Università degli Studi di Ferrara

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Chemical fingerprinting and bioactivity profile of Ayurvedic crude drugs and related preparations

Settore Scientifico Disciplinare BIO/15

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ABBREVIATIONS

2H4MB	2-hydroxy-4-methoxybenzaldehyde
2H4MBAc	2-hydroxy-4-methoxybenzoic acid
3H4MB	3-hydroxy-4-methoxybenzaldehyde
4NQO	4-nitroquinoline-1-oxide
ABTS	2,2'-azinobis (3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt
ATCC	American Type Culture Cell
BCBT	β-carotene bleaching test
DEC	Decoction
DMSO	dimethyl sulfoxide
DPPH	1,1-diphenyl-2-picrylhydrazyl
EMS	ethyl-methanesulfonate
GAE	Gallic acid equivalent
HE	Hydro-alcoholic extract
HPLC	High performance liquid chromatography
HPTLC	High performance thin layer chromatography
KI	Kovats Index
NMR	Nuclear Magnetic Resonance
R_f	Retention factor
rt	Retention time
SFE	Supercritical Fluid Extraction
WP	Whole plant

INTRODUCTION

Ayurveda, an introduction

The term "Ayu" means all aspects of life, from birth to death; the term "Veda" means knowledge. Therefore, "Ayurveda" indicates the science by which life is fully understood. Ayurveda treats man as a "whole", a combination of body, mind and soul, and designs a lifestyle able to maintain and protect health (Iannaccone, 1997).

Ayurveda is one of the oldest medicinal systems and it possesses extensive documentation of clinical experience. Traces of Ayurvedic medicine and its mythological origins are found in Indian texts dating back more than 5,000 years, but the most consistent evidence is found in the medicinal text known as *Charaka Samhita* (considered the most important work about basic Ayurvedic basic concepts), a treaty dating back to 1000 BC which describes 500 medicinal formulations in terms of preparation, dosage, and treated disorders and diseases (Iannaccone, 1997).

The principles of Ayurvedic medicine are related to the holistic conception of the human being, committed to maintaining balance with nature through medical practices that recognise the importance of natural medication in achieving this target. It is characterized by the use of plants, animals and mineral crude drugs, in simple pharmaceutical forms (e.g. powders, infusions, decoctions or tablets) but often mixed in very complex formulations (Materia Medica Ayurvedica, Ed. M.I.R., 2007). Ayurveda preparations could be composed of single drugs or mixtures of them, whose preparation and dosage depends on the disorder that has to be treated and by the psycho-somatic characteristics of the patient, categorized according to Ayurvedic principles (*doshas*) in three different types: *Vata* (air/space), *Pitta* (fire), *Kapha* (earth and water). The fact that such remedies could contain up to several mixed drugs might considerably complicate quality control and standardization.

Ayurvedic Medicine: diffusion and legislation in Italy and EU

Although the principles of this medicine are far from the roots of Hippocratic practice, Ayurveda is attracting special interest in Europe because of the different conception of health and healing system that patients achieved in modern age: the will to be involved in the curing process and not be as passive as before. It is important to be able to make the choice about which therapy is intended to be used. For this reason, the public needs a reliable current source of information regarding standards to be expected in relation to good practice ("Report of the National Working Group on the Regulation of

Complementary Therapists to the Minister for Health and Children", December 2005). The European Union has designed the traditional Ayurvedic medicinal system as non-conventional medicine, promoted formation courses, channels through which to obtain information and, overall, allowed it to be practiced by qualified doctors. Furthermore the WHO's acceptation of "health":

> "a state of complete physical, mental and social well-being
> and not merely the absence of disease or infirmity"
> (http://www.who.int/about/definition/en/print.html)

is close to Ayurvedic principles and, due to these premises, the offer of non-conventional healing centres is increasing, and consequentially the market for natural therapeutic products is moving in this direction.

In this context, scientific research has to critically evaluate the properties ascribed to the considered natural sources, through phytochemical screenings and biological assays (*in vitro, ex vivo* and *in vivo*), to determine correlations between active principles and bioactivities. Moreover, the in-depth study of Ayurvedic crude drugs could be a valuable source for the identification of new bioactive molecules, to be used as lead compounds in the development of new drugs (Guerrini & Sacchetti, 2012).

The effects of the often complex Ayurvedic formulations are widely described in the literature but the biological bases proving their effectiveness are still poorly investigated and this lack of knowledge, often associated with fragmented cultural research, makes various plants, belonging to the Ayurvedic phytotherapic culture, are still characterised, in the Italian and European commercial circuit, as "food supplements" because of the absence of a precise legal definition. In particular, herbal drugs and formulated products prepared according to the directions of the Indian Ayurvedic Pharmacopoeia are not quoted by the reports of EMEA commissions (EMEA - Committee on Herbal Medicinal Products HMPC), making them absent from the lists of drugs allowed/not allowed by the Italian Ministry of Health, but nevertheless marketed. A step in this direction, to overcome these shortcomings, was made by some European countries (Belgium, France and Italy), which are creating an updated joined list to arrive at a common framework for the use of plants and derivatives starting from the field of food supplements (Ministero della Salute, Istituto Superiore della Sanità, Prot. N. 12129, 28 marzo 2014).

GOALS

PhD goals

The main goal of this PhD research was to provide for the scarce and fragmentary knowledge about crude ayurvedic drugs and herbal derivatives. In particular, the project focused on the achievement of chemical and biological evidence about ayurvedic drugs present in preparations sold in the Italian and, in general, in the EU market. These phytotherapic realities, in fact, increasingly adopt herbal traditions belonging to distant and exotic cultures, like Ayurveda, but they implement their use considering cultural parameters unrelated to those traditions, placing them in a typical Hippocratic and allopathic context (Guerrini & Sacchetti, 2012). Furthermore, there are few studies in which there is a clear correlation between the choice of the crude drug, its formulations (e.g. decoction or hydro-alcoholic extract), its detailed chemical characterisation, the identification of the biological activities and therapeutic uses.

These statements lead to the main critical aspect: the lack of phytochemical standardization of crude drug extracts, through the choice of suitable phytomarkers that could define their quality. Therefore, starting from the firm belief that phytocomplex standardization is one of the crucial points to be able to validate their safety and effectiveness (HMPC, EMEA, 2007 and 2011), the project investigated the phytochemical composition and the biological activities of five herbal drugs preparations used in the Ayurvedic therapeutic branch called *Rasayana*: *Azadirachta indica* A. Juss (leaves), *Boerhaavia diffusa* L. (roots), *Convolvulus pluricaulis* Choisy (whole plant), *Curculigo orchioides* Gaertn. (roots) and *Hemidesmus indicus* (L.) R.Br. Ex Shultz (roots). The research focused in particular on their decoctions (DECs), cardinal extemporaneous preparations in the Ayurvedic phytotherapic tradition, alongside their hydro-alcoholic extracts (HEs), preparations belonging instead to the Western tradition that could "translate" the use of decoction of the Indian culture. Therefore the main target of the PhD research project was the standardization of these plant phytocomplexes defining some specific phytomarkers (as required by the international bodies for the approval of the use of crude drugs as medicinal plants or as a components of food supplements) and correlate the information of the chemical characterization with the biological activities, in terms of efficacy and safety (antioxidant, antigenotoxic activity and cytotoxicity against cancer cell lines), and therapeutic uses.

BOTANICAL AND PHARMACOGNOSTIC DESCRIPTION OF THE PLANT SOURCES

Azadirachta indica A. Juss

Order: Rutales

Family: Meliaceae

Genus: *Azadirachta*

Species: *Azadirachta indica* A. Juss

Parts used (crude drugs): leaves, barks, seeds, roots

Common names: neem tree, nim, margosa tree, indian lilac, margousier, neem, nimbaum, neembaum, margosa, pokok mambu, nim, neem, balnimb, nim, neem, nimba; vempu.

Description

Evergreen tree over 20 meters tall, with sympodial trunk, and rough, furrowed and greyish bark. Racemie inflorescences with large and bearing white flowers, pentamers and fragrant. Fruit is an edible yellow-green drupe. Leaves are alternate, pennate, with a margin toothed leaflets and a short petioles.

Distribution

Plant native to the Indian subcontinent, but introduced by humans in large tropical and subtropical areas of Asia and Africa, for shading of boulevards and parks, for the production of timber and for his great drought resistance and ease of cultivation (Biswas et al., 2002).

Uses

A. indica wood, particularly hard and resistant, is used in cabinet-making and for manufacturing tools. The oil obtained by the squeezing out of seeds is used as a lubricant, cosmetics, pesticide and fuel.

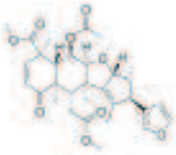
Traditional/Ethnobotanical uses: The plant species is known for numerous ethnomedicinal uses. It is used in ayurvedic medicine for many different purposes (purifying, antipruritic, antidermatite, emetic, healing, antimicrobial). In Ayurveda Neem has rasa features (taste): sweet, virya (power): refreshing, post-digestive taste: sweet; dosa: decreases Vata and Khapha, increases Pitta (Biswas et al., 2002).

Chemical constituents

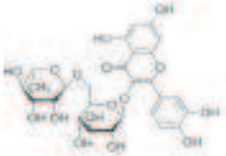
Besides the abundant polyphenols component, neem is characterized by the presence of substances belonging to the class of limonoids. These substances, mainly tetranortriterpenoids, specific to the plant species and generally called azadirachtins, are mainly characterized by a particular cyclic rearrangement. Nimbidin is the major bitter principle extracted from the neem seed oil, but other tetranortriterpenes (e.g. nimbin) have been isolated from the crude drug. Flavonoids are also present, such as rutin, quercetin, quercitrin and kaempferol; chalcones, catechins and sulphur compounds (sulphides) are correlated to the characteristic seeds smell (Atawodi et al., 2009).

azadirachtin

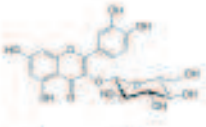
nimbidin



nimbin

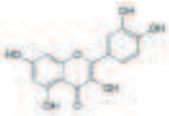


rutin

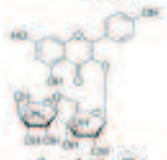


isoquercetrin

quercitrin



quecetin



kaempferol-3-O-rutinoside

Health effects known by traditional medicine and recent investigations

The use of leaves of *A. indica* is widespread in different parts of India to treat gastrointestinal disorders such as diarrhoea and cholera. The methanol extract showed significant antibacterial activity against Vibrio cholerae, etiological agent of cholera. This extract also showed antisecretory activity towards fluid secretion induced by the bacteria in mice gut and its oral administration induced antihemorrhagic activity at intestine level (Thakurta et al., 2007).

Aqueous or alcoholic flowers extracts tested in vitro showed antifilariasis activity against Setaria cervi, that consists of reversible paralysis dependent on the concentration (Mishra et al., 2005).

Antimalarial activity testing carried out on 13 different plants traditionally used, indicated *A. indica* leaves methanol extract as one of the most active preparations against *Plasmodium falciparum*, both *in vitro* (IC50<10 μg/ml, calculated as the ability to inhibit the incorporation of radio-labelled hypoxanthine by the parasite) and *in vivo*, exhibiting the

highest capacity of clearance of the parasite (89 %) among the tested plants (Gathirwa et al., 2011).

A study carried out in vivo on 30 plants traditionally used in Ayurveda for the treatment of hyperglycemia showed the capacity of *A. indica* leaf extract to significantly reduce the blood level of glucose (Kar et al., 2003). A subsequent paper also highlighted the *in vivo* inhibition of lipid peroxidation, the antioxidant blood enzymes reactivation and the levels of glutathione restoration, demonstrating its antidiabetic and antioxidant properties (Chandra et al. 2007).

Ethanol extracts of flowers, leaves and stem-bark of the variety siamensis showed a strong *in vitro* antioxidant activity in DPPH test and an inhibition of the lipid peroxidation in Chago K1 cell culture (Sithisarn et al. 2005).

Nimbin and nimbidin, tetranortriterpenoids mainly present in seed oil, exhibited an inhibition effect on the migration of macrophages in response to inflammatory stimulus also preventing phagocytosis and respiratory burst, in addition to the mitigation of degranulation in neutrophils, justifying the use of such substances for the treatment of inflammation (Kaur et al. 2004).

Regarding the potential anti-cancer activity, leaf ethanolic extract was able to induce apoptosis in prostate cancer cell lines (PC-3) in a dose-dependent manner (Kumar et al. 2006). The nimbolide, triterpene extracted from the flowers, showed antiproliferative activity against U937, HL-60, THP1, B16 cell lines at a concentration of 0.4 μM (Roy et al. 2007).

Boerhaavia diffusa L.

Order: Caryophyllales

Family: Nyctaginaceae

Genus: *Boerhaavia*

Species: *Boerhaavia diffusa* L.

Parts used (crude drug): Whole plant, but most of the Ayurvedic preparations use just the root.

Common names: B. diffusa is mostly known as Punarnava, but it has a multitude of other names depending on the language of the region in which it is used: Kahtilla, Sophaghni, Sothaghni, Varsabhu (Sanskrit); Rnaga Punarnabha (Assamense); Rakta punarnava (Bengali); Dholisaturdi, Motosatodo (Gujrati); Gadapurna, Lalpunarnava (Hindi); Sanadika, Kommeberu, Komma (Kannada); Vanjula Punarnava (Kashmiri); Chuvanna Tazhutawa (Malayalam); Ghetuli, Vasuchimuli, Satodimula, Punarnava, Khaparkhuti (Marathi); Lalapuiruni, Nalipuruni (Oriya); Itcit (Ial) Khattan(Punjabi); Mukurattai (Tamil); Atikamamidi, Erra galijeru (Telugu).

Description

Herbaceous perennial plant with strong fusiform roots, less than 1 meter tall. It has a slender and branched stem with purple hues and broadly ovate leaves with slightly round or pointed apex and round base. The flowers, white, red or pink depending on the species, are bracteolate umbels that usually contain 2-3 stamens. The cup is covered with glandular hairs. The fruit is an oval achene, sticky, monosperm, a few millimetres long and furrowed longitudinally. Thanks to their sticky nature, the fruits of the plant remain attached on the clothing of humans and legs of animals (such as small migratory birds), and it helps their dispersal from one place to another. (Rajpoot & Mishra, 2011)

Distribution

The plant is spread around the tropical, subtropical and temperate regions of the world. It is found in Australia, China, Egypt, Pakistan, Sudan, Sri Lanka, South Africa, in the US and in several countries of the Middle East. *B. diffusa* is however native to India, it grows

in all the hottest areas of the country up to an altitude of 2000 m in the Himalayan region (Awasthi et al., 2006).

Uses

In addition to the numerous therapeutic uses, the whole plant (including roots) is eaten as a vegetable in curries and soups. Roots and seeds are added to cereal, pancakes, and other foods. It also used as birds or poultry feed, and found in pastures for forage for sheep, goats and cows (Awasthi et al., 2006). Moreover, in the Indian tradition, it is a remedy against snake bites and scorpion stings, while the crushed leaves are used in disinfectant preparation to washes the eyes. The Indian common name of the plant *Punarnava* means "renewal" and indicates its purifying and regenerating use. According to Ayurvedic medicine *B. diffusa* has a refreshing, diuretic activity and is prescribed for a wide range of conditions such as alcoholism, gastrointestinal disorders, urinary tract infections, asthma, jaundice, bronchitis and anemia. *B. diffusa* root extract strengthens, tones and balances the liver (hepatotonic) (Rawat et al., 1997). It shows hepatoprotective activity (Chandan et al., 1991; Das & Agarwal, 2011). The plant is used in epilepsy, abdominal pain, dysentery, prolapsus ani, fistula ani and poison of scolopendrids (Jain & Tarafdar, 1970); in pneumonia (Saxena, 1986); jaundice (Kumar, 1992; Singh & Ali, 1992; Singh, 1993; Anis & Iqbal, 1994; Sudhakar & Chetty, 1998); anaemia, (John, 1984; Basak, 1997); as blood purifier (Tripathi et al., 1996); in enlargement of spleen (Rajwar, 1983); as stomachic, emetic, laxative, expectorant, diuretic (Jha et al., 1997); astringent, antiasthmatic, in abdominal pain cough (Das & Kant, 1988); as anti inflammatory (Kapur, 1993); in liver complaints, heart diseases (Rana et al., 1996) in dropsy, gonorrhoea, oedema, as diuretic (Singh and Aswal, 1992; Singh, 1993) in haemorrhoids (Singh et al., 1996) anaemia, calie, thoracic haemorrhage, constipation, heart disease, oedema, as antidote to rat bite poison and in rabies (Thakur et al., 1992) in urinary troubles (Maheshwari & Singh, 1984) as antiasthmatic, in anasarea, ascites (Banerjee & Banerjee, 1986) oedema, oligurea, as antidote to snake bite (Chandra et al., 1989). The fruits are used as a diuretic. The seeds are used as expectorant, carminative, tonic, anthelmintic in lumbago (Tripathi et al., 1996); jaundice and gonorrhoea (Mishra & Verma, 1995). The shoot is used in dysuria, oligurea as haematinic and uterine stimulant (Borthakur, 1996). The powder of the roots, when mixed with Mamira (*Thalictrum foliolosum*), is used to treat eye diseases such as corneal ulcer and night blindness (Awasthi et al., 2006). The people in the tribal areas are also used to accelerate the delivery (Rajpoot et al., 2011). The juice of the roots is used to

treat asthma, urinary scarcity and inflammatory disorders. The aqueous extract of the roots with a diameter of 1-3 cm has hepatoprotective (maximum effect it is recorded in the extract of the roots collected in May). The decoction of the *B. diffusa* is used in cases of cough, jaundice and in the presence of diffuse edema, caused by kidney or heart problems, for its marked diuretic effects (Agrawal et al., 2011). Flowers and seeds are used as a contraceptive (Awasthi et al., 2006). The seeds are also expectorant and carminative (Agrawal et al., 2011).

Chemical constituents

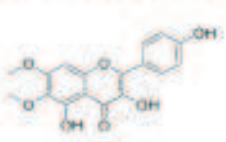
Some of the most important bioactive phytochemical constituents of *B. diffusa* plant are alkaloids, flavonoids, steroids, triterpenoids and phenolic compounds (Kadota et al., 1989; Lami et al., 1990; Jain & Khanna, 1989). It contains about 0.04% of alkaloid known as punarnavine ($C_{17}H_{22}N_2O$, mp 236-237 °C) (Surange and Pendse, 1972) and punarnavoside, an anti fibrinolytic agent. It also contains about 6% of potassium nitrate, an oily substance and ursolic acid (Kokate et al., 2005).

The green stalk of the plant has also been reported to contain boerhavin and boerhavic acid. Hentriacontane, β-sitosterol and ursolic acid along with glucose, fructose and sucrose were isolated from the root (Misra and Tiwari, 1971). A new C-methyl flavones characterized as 5, 7-dihydroxy-6-8-dimethoxy flavones was reported from root (Gupta and Ahmed, 1984) and designated as boerhavone (Ahmed and Yu, 1992). Four new compounds, boerhavisterol, boerhadiffusene, diffusarotenoid and boerhavilanastenyl benzoate and a known rotenoid, boerhavinone A were isolated from the root. Many rotanoids have been isolated from the roots of the plant (Kadota et al., 1989; Lami et al., 1990). These include a series of boeravinones: boeravinone A, boeravinone B, boeravinone C, boeravinone D, boeravinone E and boeravinone F.

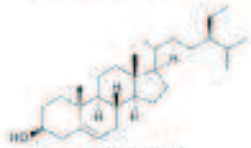
Bioactive eupalitin 3-0-β-D-galactopyranoside and eupalitin isolated from the alcoholic extract of *B. diffusa* leaves (Pandey et al., 2005) exhibited immunosuppressive properties.

A metabolite profiling and biological study was undertaken on *B. diffusa* leaves and roots and substantial differences were found between the two parts of the plant. Several compounds including terpenes, phenyl propanoids, indole compounds, norisoprenoids, among others, were identified. Organic acid analysis was also performed allowing their characterisation in this species for the first time and oxalic, ketoglutaric, pyruvic, quinic and fumaric acids were identified. Quantitative differences between two plant materials

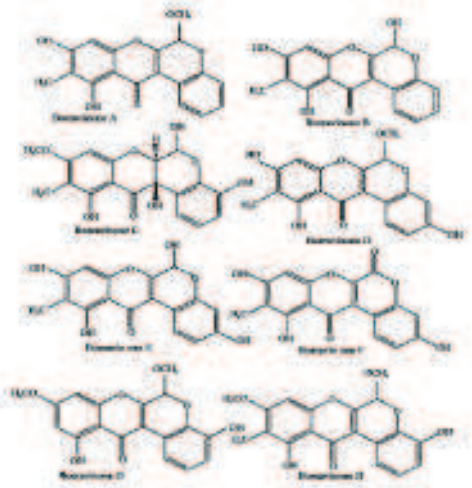
were found. Additionally, several flavonoids and one phenolic acid were also confirmed from the roots and leaves of the plant. Two known lignans, liriodendrin and syringaresinol mono-β-D-glycoside have been isolated (Lami et al., 1991). Two quinolizidine alkaloids identified as punarnavine-I and punarnavine-II were isolated from root, stem and leaves. The distribution of these alkaloids was maximum in stem and minimum in root. A relation was established between the growth process of the plant and biosynthesis of these two alkaloids. The alkaloidal content was initially low during commencement of pre-reproductive phase, gradually increased in different plant parts, becoming maximum during termination phase of reproductive stage (Nandi and Chatterjee, 1974).



eupalitin



β-sitosterol



Health effects known by traditional medicine and recent investigations

Punarnava possesses diuretic and anti-inflammatory activity and the maximum activity was observed in samples collected in the rainy season. Because of the combination of these two activities, *B. diffusa* is considered therapeutically effective for the treatment of inflammatory diseases and common clinical kidney problems such as nephrotic syndrome, edema and ascites due to early liver cirrhosis and chronic peritonitis. The plant has proven it usefulness as a growth promoter in children fed with milk fortified with drugs (Rajpoot et al., 2011).

In the form of decoction, the drug is useful in the treatment of nephrotic syndrome, and also decreases the serum level of cholesterol (Rajpoot et al., 2011).

The powder of the dried root has shown curative efficacy when administered orally for a month, for children or adults suffering from helminth infections. Patients were free from worms within five days of treatment (Awasthi et al., 2006).

The aqueous extract of roots of *B. diffusa* demonstrates analgesic and anti-inflammatory activity against edema and arthritis induced in rats. The extract if prepared with thin roots collected in May, shows hepatoprotective activities (Rawat et al., 1997).

The aqueous extract of leaves of the plant shows analgesic activity exhibiting a morphine-mimetic central action (Hiruma-Lima et al. 2000). The aqueous extract of the leaves, moreover, has proved to lower the glucose level in diabetic rats (Rajpoot et al., 2011) and is reported to be useful in the treatment of kidney (Indhumathi et al., 2011) and liver damage (Olaleye et al., 2010).

The ethanol extract of the plant showed anti-mitotic activity in *in vitro* systems (Leyon et al., 2005) and activity against cervical cancer cell line (Srivastava et al., 2011). It also showed antioxidant and anti-inflammatory properties. The ethanol extract 80% showed good adaptogenic activity: the molecules responsible for the decrease in stress may be the flavonoids due to their antioxidant capacity (Rajpoot et al., 2011). The alcoholic extract of the roots has the ability to regulate the levels of cholesterol and lipoproteins in the blood (Khan et al., 2011).

Finally, methanol extract showed spasmolytic, anticonvulsant activity (Rajpoot et al., 2011) and reduced the formation of metastases in melanoma cells B16F-10 (Leyon et al., 2005).

Convolvulus pluricaulis Choisy

Order: Solanales

Family: Convolvulaceae

Genus: *Convolvulus*

Species: *Convolvulus pluricaulis* Choisy

Parts used (crude drug): whole plant

Common names: Laghuvishnukranta, Nilasankhapuspi, Sankhapuspi, Convolvulus pluricaulis, Vaishnava, Vishnugandhi, Vishnukranthi, Vishnukranthi, Vishnukanta (Sanskrit); Shankhpushpi, Shyamakranta, Syamakranta, Vishnukanta, Shankhpushpi, Phooli, Shankaveli, Shankhpushpi, Shnkhahuli (Hindi); English speedwheel (English); Sankhali (Hurdu); Barabhutra (Bengali); Khalsa pus syi, Sa nkhapu spa, Shankhpushpi (Tibetan).

Description

C. pluricaulis is a prostrate perennial herb found on sandy or rocky ground under xerophytic conditions in northern India. Its branches are spread on the ground and can be more than 30 cm long. The flowers are blue in colour and the leaves, which are elliptic in shape, are located at alternate positions with branches or flowers (Sethiya et al., 2013).

Distribution

It is commonly found in India, especially in the state of Bihar.

Uses

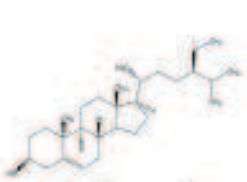
C. pluricaulis Sieb. ex Spreng. (Convolvulaceae) is a plant traditionally used in Ayurvedic medicine, it is included in the *Medhya Rasayana* section which focuses on herbal preparations aimed at delaying the ageing of the brain tissue (Singh et al., 2008). Furthermore, it is reported to possess anxiolytic, memory enhancing (Sarokte & Rao, 2014) and mood elevating effects (Verma et al., 2012). The decoction obtained with the whole plant added to milk and cumin is used to treat fever, nervous debility, and loss of memory.

The ethanolic extract of the plant reduces total serum cholesterol, triglycerides, phospholipids and nonesterified fatty-acid. In addition, it also possesses diuretic, antioxidant, antimicrobial, antidiabetic, antiulcer, hypolipidemic, antipyretic, analgesic, anti-inflammat-

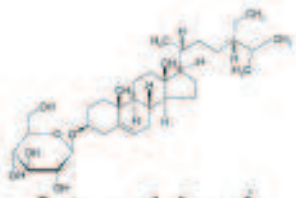
ory, hypotensive and insecticidal properties (Sharma et al., 1965; Gupta & Mudgal et al., 1974; Sairam et al., 2001).

Chemical constituents

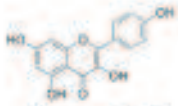
C. pluricaulis whole plant showed the presence of glycosilated compounds with phenolic aglicones, coumarins, flavonoids and alkaloids. Shankhpushpi, (the alkaloid) has been identified as active principle. β-sitosterol glycoside, hydroxy cinnamic acid, octacosanol tetracosane along with glucose, sucrose also isolated from the plant drugs (Sethiya et al., 2006).



β-sitosterol



β-sitosterol glycoside



kaempferol

scopoletin

Health effects known by traditional medicine and recent investigations

Primarily *C. pluricaulis* is used as brain tonic, and for reducing symptoms associated with anxiety, panic attacks, nervousness and insomnia (Kumar, 2006). It exhibits a reduction of anxiety and stress by controlling the production of body's stress hormones, adrenaline and cortisol. It is reported to possess anxiolytic and memory-enhancing and mood-elevating effects, and is claimed to retard brain ageing (Singh, 2008)

Alcoholic extract of *C. pluricaulis* (50 mg/kg, 250 mg/kg, 500 mg/kg, 1 g/kg of body weight) showed slight sedative but no analgesic and anticonvulsive effects in rats (Aulakh et al., 1988). Aqueous extracts of the fresh petals of *C. pluricaulis* and *E. alsinoides* were found to be almost completely fungicidal against three pathogens: *Alternaria brassicae, A. brassicola* and *Fusarium oxysporum*. While alcoholic extract of leaves and flowers inhibited the growth of the fungi *Pestalotia elasticae, Curvularia lunata* and *Fusarium moniliformae* (Aulakh et al., 1988). *C. pluricaulis* extract has been clinically investigated: at the dose of 30 ml/day, the drug exhibited anti-anxiety effects in 30 patients. Improved mental functions and relief in symptoms like nervousness, palpitation, insomnia, weakness, fatigue and dyspepsia were also observed. The immediate memory span in these patients was also increased. The patients also showed a reduction in the level of plasma cortisol and urinary catecholamines (Aulakh et al., 1988). In a clinical study, considering 25 cases of arterial hypertension, treated with the decoction of *C. pluricaulis*, a gradual fall in blood pressure along with relief in the symptoms was observed (Aulakh et al., 1988).

C. pluricaulis showed neuroprotective potential on aluminium induced neurotoxicity in rat brain (Bihaqi et al., 2009).

Alcoholic extract at 500 and 300 mg/kg dosage, produced a significant decrease in plasma glucose levels when compared with diabetic control group in alloxan induced diabetes and dexamethasone induced insulin resistance in rats (Khan & Naikwade, 2010)

Curculigo orchioides Gaertn.

Order: Asparagales

Family: Hypoxidaceae

Genus: *Curculigo*

Species: *Curculigo orchioides* Gaertn.

Parts used (crude drugs): Rhizomes are the part of the plant used in traditional Chinese medicine (Zuo et al., 2011). According to Ayurvedic medicine the root is also used.

Common names: Bhumitila (Sanskrit); Talmuli, Tailmuli (Assamense); Talmalu, Tallur (Bengali); Kalimusali (Gujrati); Syahmusali, Kalimusli (Hindi); Neltal, Neltalthigodde, Nelatale, Nelatelegadde (Kannada); Nilappenea (Malayalam); Kali musali, Bhuimaddi (Marathi); Talamuli (Oriya); Syah musali, Musali safed (Punjabi); Nilappanai (Tamil); Nel tadigadda (Telugu); Musali Siyah, Kali Musali (Urdu).

Description

A perennial herb, small (30-40 cm), stemless, with cylindrical roots, tuberous, long a few centimetres. Leaves are sessile or shortly petiolate, linear or lanceolate, 1-2 cm wide and collected in basal rosette. The flowers, appearing in summer and autumn, are bright yellow, subsessil and gathered in clusters, in which the two lower flowers are hermaphrodite, while the rest are male. The fruit is a capsule containing 1 to 4 blacks and glossy seeds (www.ayurprint.org).

Distribution

Curculigo orchioides is endemic to the mountainous areas of India and China, especially in sandy and hot areas. It is widespread in all the East Indies, northern Australia, in Japan and in Sri Lanka (Raaman et al., 2009).

Uses

C. orchioides rhizome, as well as tuberous roots, have been widely used in Indian, Pakistan and Chinese ethnomedicine to treat various diseases, including cancer, jaundice and asthma (Nagesh et al., 2009).

It showed hepatoprotective, antipyretic and immunostimulant effects. In China it is used in the treatment of kidney and sexual dysfunction, as well as for the treatment of fatigue. A mixture of *C. orchioides* with *Asparagus ascendense*, *A. racemosus*, *Chlorophytum borivilianum* and *Withania somnifera* in several pharmaceutical formulations, is used as a metabolic enhancer and aphrodisiac (Nema et al., 2010). The drug was introduced in the Charaka Samhita, used as an ingredient in cigars to relieve cough. A paste obtained from the rhizomes, goat milk or honey, applied topically to the face, has a cosmetic function illuminating the complexion (Patil et al., 2012). The Maharishi Amrit Kalash 4 and 5, Ayurvedic formulations containing *C. orchioides*, are used as a supplement to chemotherapy drugs, to reduce chemotherapy-induced vomiting, anorexia and improve the general wellness of the patients. The plant showed also estrogenic activity (Chauhan et al., 2008).

The root, according to Ayurveda, has various effects: appetite suppressants, diuretics, it is useful for the treatment of hepatic-biliary dysfunctions, metabolic and kidney disorders, haemorrhoids and joint pain, as well as antipyretic, tonic and carminative (Madhavan et al., 2007).

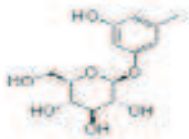
The rhizome of this plant is used topically to treat itching, skin diseases, but also systemically for bronchitis and in the treatment of leprosy and pain (analgesic). It also exhibits immunostimulant, hepatoprotective, anti-inflammatory, antioxidant and platelet regeneration capacity (Raaman et al., 2009). The rhizomes of *C. orchioides* are described in Ayurveda as Rasayana Vajikarana as aphrodisiacs and enhancers of virility; the branch Vajikarana deals, in fact, with drugs which increase the sexual power in men (Chauhan et al, 2008). The decoction of the rhizomes of the plant and the fruits of *Trachyspermum ammi* is used for the treatment of infantile syncope (Nie et al., 2013)

Chemical constituents

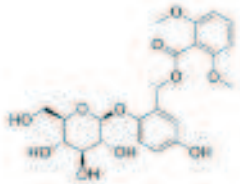
The rhizome of *C. orchioides* contains phenolic compounds, steroids, aliphatic compounds, nitrogen constituents, triterpenoids (curculigol, curculigenin A, B and C), and triterpenoidal saponins (curculigosaponins A-J) (Shrikumar et al., 2005). Three steroids, β-sitosterol, stigmasterol and yuccagenina were isolated from *C. orchioides* (Nema et al., 2010).

Among the phenolic compounds curculigoside A-C, curculigin A-D and orcinol glucoside, corchioside A and flavanone glycoside-I have been isolated and characterized (Nema etal., 2010). A number of fatty acids have been identified from the oil of the root by gas

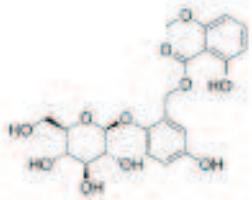
liquid chromatography. Among these there are palmitic acid, oleic acid, linoleic acid, and 2,6-dimethoxy benzoic acid. In addition to these compounds, the plant also contains glucose, mannose, xylose, glucuronic acid, resin, tannins, fat, starch and mucilages (Nema et al., 2010).



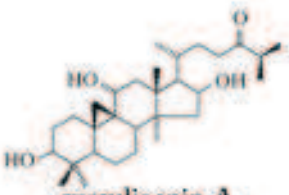
orcinol-β-D-glucoside



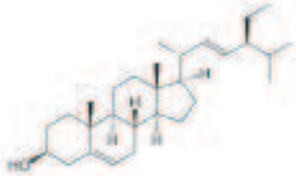
curculigoside A



curculigoside B



curculigenin A



stigmasterol

Health effects known by traditional medicine and recent investigations

Different types of plant extracts, tested *in vitro* and *in vivo* on animal models, gave preliminary indications about their effects in the prevention of osteoporosis, in increasing libido and reducing blood glucose, as hepatoprotector and bland antiseptic. *C. orchioides* also showed antispasmodic, anticancer and neuroprotective properties. In particular, curculigenin A and curculigol demonstrated hepatoprotective properties. The plant also performed regulatory effects on thyroid dysfunction in rabbits subjected to thyroidectomy (Madhavan et al., 2007).

The ethanol extract of *C. orchioides* was reported to have adaptogenic activity (improved tolerance to hypoxia and hyperthermia), and to exhibit anti-inflammatory, anticonvulsant, sedative, immunostimulant and antioxidant capacity (Venukumar et al., 2002). The alcoholic extract modified sexual behaviour, increasing mating performance in male rats (Chauhan et al., 2008). It was also effective in the treatments of hearing loss, induced by noise, in the animal model (Hong et al., 2011). In addition, the alcoholic extract of the rhizomes is promising in the treatment against skeletal diseases (Jiao et al., 2009)

Aqueous and ethanolic extracts showed significant hypoglycaemic effects in diabetic rats. The reduction of the average blood glucose level (BGL) was shown to be dose-dependent. The effects are comparable with the popular diabetes drug, glibenclamide (Madhavan et al., 2007).

The methanolic extract of rhizomes (Venukumar et al., 2002) and roots (Rathod et al., 2010) showed a good antioxidant activity. The extract obtained from the rhizomes proved also to be effective as an anti fungal (Raaman et al., 2009).

Hemidesmus indicus (L.) R.Br. Ex Shultz

Order: Gentianales

Family: Asclepiadaceae

Genus: *Hemidesmus*

Species: *Hemidesmus indicus* (L.) R.Br. Ex Shultz

Parts used (crude drugs): The most common part used is the root, but also the root bark and the dried stems are considered in some preparations.

Common names: Indian sarsaparilla (English), Anantamul (Hindi), Manipuri (Sanskrit), Nannari (Tamil).

Description

Perennial slender shrub, twining, sometimes prostrate or semi-erect. The roots are brown outside yellow-brown inside with a characteristic smell of vanilla and a sour taste. The rhizomes are woody, thick, rigid, cylindrical with a diameter between 0.5 and 2.0 cm. It presents numerous slender stalks that are swelling at the nodes. The leaves are opposite, with a small stalk and a variable shape, from oblique-oblong to linear-lanceolate; they are smooth, dark green, often with noticeable white streaks on the upper surface and, sometimes, grey-white beneath. The flowers are greenish on the outside and purple inside, gathered in axillary subsessile buds. The fruits, consisting of two follicles, are thin, about 10 cm long, cylindrical and curved. The seeds are flat, numerous, dark-grey with a white stripe.

Distribution

H. indicus is widespread in South Asia, particularly in many regions of India, Pakistan and Sri Lanka. Because of excessive harvesting in the wild is considered at risk in some parts of India.

Uses

The root is used routinely as antipyretic, antidiarrheal, diaphoretic, diuretic, for its astringent, blood purifying, tonic and refrigerants propriety. In ethnomedicine it is also used as

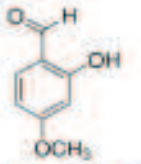
an antidote against snake bites, as a remedy for hepatic crisis, dysentery, respiratory disorders, bronchitis and asthma, skin diseases, syphilis, leprosy, leucoderma, itching, eye diseases, infantile seizures, urinary and renal disorders, loss of appetite and rheumatism. The root bark is used for dyspepsia, nutritional disorders, fever, skin diseases, ulcers, syphilis, rheumatism and whites. The stem is used as diaphoretic, diuretic, laxative, and for the treatment of anxiety. In addition, it is used against syphilis, urethritis, urinary discharge, uterine problems, leucoderma, cough and asthma (Anoop, 2008).

The Ayurvedic Pharmacopoeia reports therapeutic uses for aversion to food (aruci in Sanskrit), loss of appetite (agnimandya), diarrhoea (atisara), cough (kasa), asthma (flares), pruritus (kandu), leprosy (kustha), pyrexia (jvara) and diseases of the blood (rakta-vikara).

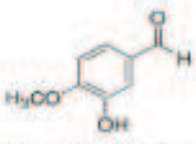
H. indicus is usually administered as decoction of root or whole plant, alone or in mixture with other plants. The simple root decoction is used to treat high fevers, skin diseases, to control menorrhagia, as antirheumatic, diuretic, anti-inflammatory, emopurificante, and against snake bite, especially *Crotalus adamenteus*. Decoction associated with *Piper longum* and *P. nigrum* is used as a tonic for physical recovery after childbirth, to improve appetite, increase blood pressure and against diarrhoea (Anoop, 2008).

Chemical components

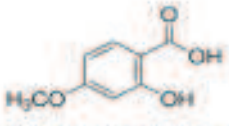
The root contains coumarino-lignoids (e.g. hemidesminin), vanillin derivatives (2-hydroxy-4-methoxybenzaldehyde, 3-hydroxy-4-methoxybenzaldehyde, 2-hydroxy-4-methoxybenzoic acid), triterpenes such as lupeol and lupeol acetate; and phytosterols like β-sitosterol, sitosterol glycosides (Aneja et al., 2008)



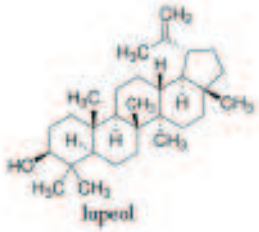
2-hydroxy-4-methoxybenzaldehyde



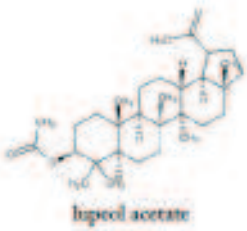
3-hydroxy-4-methoxybenzaldehyde

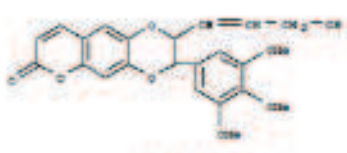


2-hydroxy-4-methoxybenzoic acid



lupeol


lupeol acetate


hemidesminin

Health effects known by traditional medicine and recent investigations

The methanol extract of the root significantly neutralizes the venom-induced lethality and the hemorrhagic activity of the venom of *Vipera russelii* in albino rats and mice (Alam et al., 1996). Furthermore, the pure compounds isolated from root extracts: lupeol acetate (Chatterjee et al., 2006) and 2-hydroxy-4-methoxybenzoic acid (Alam et al., 1998) showed antidotes adjuvant activity against the venom of *Daboia russelii* and *Naja kaouthia* snake species.

The aqueous extract (1 mg/ml) of the root possesses significant antibacterial activity when tested *in vitro* against *Staphylococcus aureus* (inhibition diameter of 14 mm), *Pseudomonas aeruginosa* (12 mm) and *Klebsiella pneumoniae* (14 mm) (Gayathri et al., 2009) and bacteriostatic activity in mice infected by *Mycobacterium leprae*, activity attributed to 2-hydroxy-4-methoxybenzaldehyde (Gupta, 1981).

The essential oil has marked antimicrobic activity towards both Gram+ and Gram- bacteria, but does not show appreciable anti fungal activity (Prasad et al., 1983).

The methanolic extract protected hepatocytes by inhibiting the lipid peroxidation induced by iron-ADP-ascorbate in liver homogenate (EC50=43.8 µg/ml) and the haemolysis induced by phenylhydrazine (EC50=9.74 µg/ml), confirming the membrane stabilization activity (Ravishankara et al., 2002).

In tests carried out in vivo on rats, the ethanolic extract of the root and the pure compound, 2-hydroxy-4-methoxybenzoic acid, showed a strong inhibitory activity towards hepatic damage by ethanol intoxication (Saravanan et al., 2007).

The aqueous extract of the root was investigated in vivo with albino mice for its antigenotoxic activity against cisplatin-induced damage. At low dosage (10-20 mg/kg) showed the genoprotective effect was high and cytotoxicity absent (Ananthi et al., 2010).

In vivo studies were carried out to evaluate the anti-nociceptive activity of the ethanolic extract of the root using three different methods of induction of nociception (acetic acid, hot plate and formalin) in order to evaluate its effects, respectively, at the peripheral level, at central level and the two combined. The extract was administered prior the induction of pain and in all three methods was observed dose-dependent anti-nociceptive activity (Verma et al., 2005).

The hydro-alcoholic extract of the root showed significant antiulcer activity in a dose dependent manner and reduces the formation of gastric and duodenal lesions induced in animal models (Anoop et al., 2003).

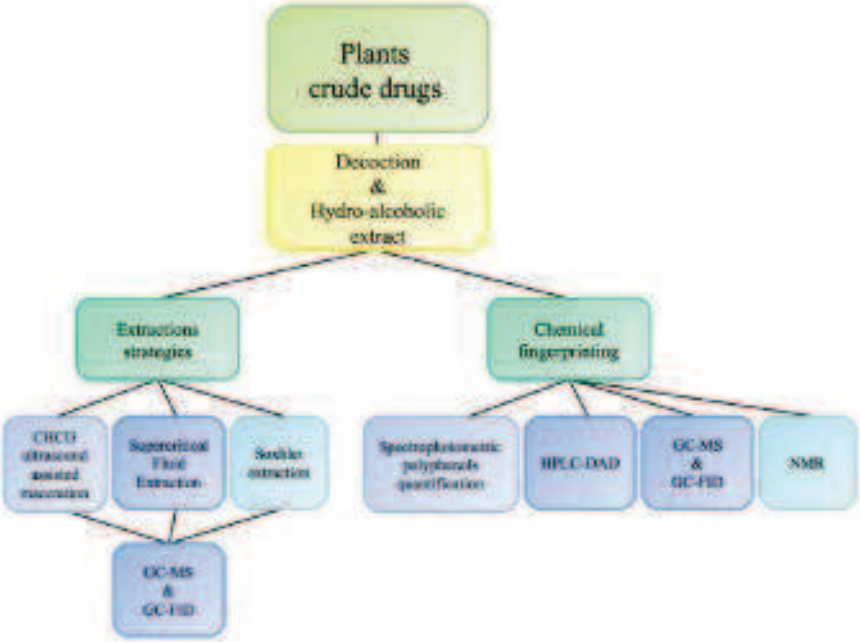
With regards to the anti carcinogenic activity, a dose-dependent cytotoxic activity was observed by testing the decoction *H. indicus*, *Nigella sativa* and *Smilax glabra* against human HepG2 cell lines (Thabrew et al., 2005).

The cytotoxic potential of the root methanolic extract was evaluated against Ehrlich Ascites tumor. *In vitro*, the extract showed a significant cytotoxic activity, while *in vivo* reduced the weight of the tumoral mass in a dose dependent manner (Zarei et al., 2012).

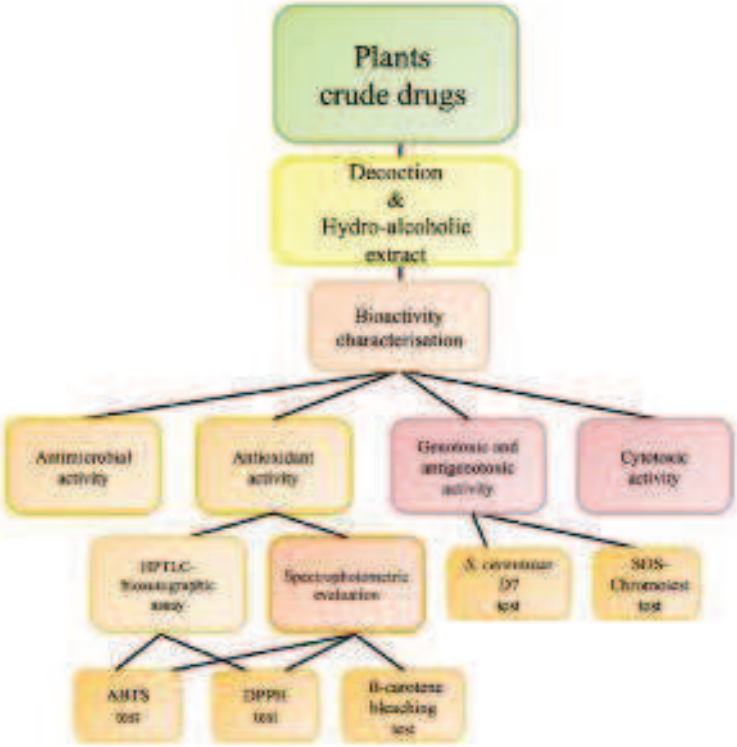
MATERIALS and METHODS

Flow chart schemes of the research

Phytochemical fingerprinting



Biological activities evaluation



PLANT MATERIAL

Leaves of *Azadirachta indica* (Batch No. 0158, Mfg Date 10/12/2010), roots of *Boerhaavia diffusa* (Batch No. 5188, Mfg Date 15/02/2011), whole plant of *Convolvulus pluricaulis* (Batch No. 7028, Mgf Date 25/10/2011), roots *Curculigo orchioides* (Batch No. 2045, Mgf Date 19/08/2011), and roots of *Hemidesmus indicus* (Batch No. 3904, Mgf Date 24/08/2011) were collected from Ram Bagh (Rajasthan, India), authenticated by Dr. MR Uniyal, Maharishi Ayurveda Product Ltd., Noida, India; and imported by MAP Italia. After harvesting, the roots were cleaned and cut into small pieces before being dried. All samples were then ground to a fine powder and kept at -20°C until used for the extractions.

A. indica

B. diffusa

C. pluricaulis

C. orchioides

H. indicus

Plants decoction and hydro-alcoholic extract preparation

Decoction

Decoctions (DECs) were prepared in triplicate according to the procedures reported in the Ayurvedic Pharmacopoeia of India: 10 g of dried grinded crude drug were mixed with 300 mL of water and put on a hotplate. After a period of gentle boiling the volume reduced to 75 mL. DECs were then filtered, lyophilized and stored at -20 °C. Immediately before the use, the samples were resuspended in water. The process is described by the images below.

Hydro-alcoholic extract

Hydro-alcoholic extracts (HEs) preparation followed the procedure reported in the European Pharmacopoeia (Ed. 8.0, 2011): 50 g of ground root were mixed with 450mL of 30% ethanol–water solution. Mixtures were left for 21 days and were stirred constantly. Extracts were then filtered, lyophilised and stored at -20 °C. All the extractions were performed in triplicate. Immediately before the use, the samples were resuspended in 30% ethanol–water solution.

PHYTOCHEMICAL FINGERPRINTING

EXTRACTIONS METHODS

Supercritical fluid extraction (SFE), chloroform and soxhlet extractions were carried out on DECs and HEs in order to obtaining the best chemical characterisation of the preparations, with particular attention to low-abundance compounds which are often deemed responsible for biological activity (Guerrini and Sacchetti, 2012). Every extract was then investigated by gas chromatography.

Supercritical fluid extraction

SFE was performed using an Applied Separations extractor (Allentown, PA, USA) model Spe-ed$_{TM}$ SFE Prime. The CO_2 used was 4.5 purity grade (99.995 %) supplied by SOL. S.p.a.. A 10 mL extraction vessel was then loaded with about 1.5 g of DEC, or 0.5 g of HE, mixed with an equal amount of Spe-ed$_{TM}$ Matrix. After the sample compression a plug of wool (Spe-ed$_{TM}$ Wool) was place on top and the empty space was filled with matrix. The extraction CO_2 flow rate was maintained at an average level of 3.5 L/min, and the process was performed using different combination of pressure and temperature, for 15 min of static, followed by 45 min of dynamic extraction at an average flow rate of 3.5 l/ml. The total duration of the process was 1 hour. Each extract, performed in triplicate, were collected and analysed by GC-MS.

$CHCl_3$ extraction

An aliquot of 0.5 g of lyophilised samples (DECs or HEs) were placed in a glass test tube with 5 ml of $CHCl_3$. The suspension was placed in ultrasonic bath for 20 minutes. After this extraction process the sample was centrifuged for 5 minutes at 3000 rpm to separate the solvent from the not dissolved lyophilised material. The supernatant was collected and replaced with the same volume of fresh $CHCl_3$ and the process was repeated 4 more times. Five consecutive extractions were made on the starting botanical, for a total volume of 25 ml that was then filtered through a PTFE syringe filter with a porosity of 0.45 microns. Finally, the $CHCl_3$ was evaporated until the samples were dried.

Soxhlet extractions

Soxhlet extraction was performed in order to extract nonvolatile and semivolatile organic compounds from dried formulations samples (DECs or HEs). The following method was applied: an aliquot of 1 g of solid samples were place in the soxhlet apparatus under reflux conditions with 40 ml of a 1:1 mixture of $CHCl_3$ and EtOH (heating mantle set to 200 °C) for 6 hours. Subsequently, 10 ml of ddH_2O were added to the extraction mixture in a separation fennel, and after shaken the two phases were separately collected and dried.

SEPARATION OF THE SUGAR FRACTION

Since the carbohydrate (polysaccharides) portion of some preparations (in particular *Hemidesmus indicus*) interfered with the chemical characterisation of the secondary metabolites, a separation strategy using a gravimetric column fill up of Amberlite XAD-2 resin (Sigma–Aldrich) was adopted.

The sample of *H. indicus* DEC and HE were solubilised using the concentrations calculate from the yield of their respective formulation processes: 34.07 mg/ml for DEC and 24.41 mg/ml for HE. The solutions were then filtered, the filtrate passed through an Amberlite XAD-2 column (Sigma–Aldrich), previously activated, and washed with 150 ml of ddH_2O. The sugar fraction were then eluted, collected, freeze-dried and stored at -20 °C before being analysed. The column was then washed with 150 ml of methanol. The solvent was collected, dried in a Rotavapor and stored in the dark at -20 °C before the HPLC analyses.

Spectrophotometric assays for the determination of the total polyphenol content of DECs and HEs

Polyphenols, flavonoids and procyanidins were monitored spectrophotometically following the approaches described below.

An aliquot of DECs or HEs was dissolved in water or 30 % ethanol solution and used for polyphenols determination. Folin–Ciocalteau method for the evaluation of total polyphenols fraction was adopted, as reported in Bruni et al. (2006). Each extract (0.1 ml) has been diluted with water (7.9 ml), and added to a 0.5 ml Folin-Ciocalteu reagent. After 2 min, 1.5 ml of saturated Na_2CO_3 solution (ca. 20%) was added. The solutions were gently shaken at room temperature in the dark. The reaction was measured after 2 hr at 765 nm,

with reference to a control. Gallic acid, at different concentrations ranging from 0 to 5.0 mg/ml, was used as standard to perform the calibration curve solution. Results of total phenolic for DECs and HEs were expressed as milligram gallic acid equivalents (GAE) per gram of samples (mg GAE/g).

The total flavonoids in the formulations were checked in accordance to Lamaison & Carnat (1991): 1 mg of each dried extract has been dissolved in methanol [1 ml] and added to 1 ml of $AlCl_3 \bullet 6H_2O$ solution (2%). The solutions were gently shaken at room temperature, in the dark and then analysed. The reaction was measured after 10 min at 394 nm, in comparison with a control (hyperoside). The calibration curve was prepared with a solution of hyperoside (ranging 0 - 60 µg/ml). Results of total flavonoids content of the preparations were expressed as milligram of hyperoside equivalents (HE) per gram of samples (mg HE/g).

The total procyanidins in the extracts have been checked as reported by Porter (1986): 1 mg of each dried extract has been dissolved in methanol [1 ml] and added to 6 ml of n-butanol/chloridric acid solution (95:5), and 0.2 ml of 2% $NH_4Fe(SO_4)_2 \bullet 12\ H_2O$ in HCl 2M solution. The solutions were gently shaken at 95° C for 40 min in the dark, then analysed. The reaction was measured, at room temperature, at 550 nm in comparison to blank. Cyanidin chloride solutions (ranging 0 - 60 µg/ml) were used to perform a calibration curve. Results of total procyanidins evaluations in DECs and HEs were expressed as milligram of cyaniding chloride equivalents (CCIE) per gram of samples (mg CCIE/g).

Chromatographic approach to a joined evaluation of phytochemical and biological evidences: high performance thin layer chromatography (HPTLC) separation and bioautographic assays for the evaluation of antioxidant activity

Samples of DECs and HEs were resuspended in the same solvent used for their formulation (water for DECs and 30 % ethanol for HEs) at the concentrations calculated after lyophilisation (Tab. 1). Pure compounds, instead, were solubilised in methanol at the concentration of 1 mg/ml. Five microlitres of each sample were applied in triplicate to silica gel 60 F_{254}-precoated high performance thin layer chromatographic plates (Merck, Germany) as 10 mm wide bands with automatic sampler Linomat V (CAMAG), programmed through winCATS Planar Chromatography Manager software, 1.4.7 version (CAMAG). The formulations were flanked by the pure compounds identified inside then

from previous research found in literature. The plates were eluted with a double solvent system (Wagner et al., 2001), the first from the point of deposition to R_f 0.5, the second from the deposition point to reach R_f 1. The first elution step consist of a mixture of ethyl acetate-formic acid-glacial acetic acid-water (100:11:11:26), the second step were a solution of toluene-ethyl acetate-acetic acid (100:90:10). After solvent evaporation, one chromatogram was sprayed with a solution of NP/PEG (Natural products/polyethylene glycol reagent) to separate the compounds present in the phytocomplexes and, in particular, to highlight flavonoids and flavonols. The second and the third chromatograms were respectively derivatised with a solution of ABTS (2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid), 0.011 g/10 ml of ddH_2O with 100 µl of $K_2S_2O_8$ 70 mM) and DPPH (2,2-diphenyl-1-picrylhydrazyl, 20 mg/100 ml of methanol) to identify antioxidant active compounds. The eluted plate was then observed under UV light at a wavelength of 365 nm, with a TLC Visualizer (CAMAG), to verify the separation grade, and in the visible light to highlight the antioxidant activity of the phytocomplexes fractions.

The same technique was used to achieve a semi-quantitative evaluation of the glycosilated flavonol rutin inside *A. indica* DEC and HE. On the same HPTLC plate, aliquots of DEC, HE, and calibration solutions of rutin in three different concentrations were applied in double. The silica plate, after NP/PEG development using the method described above, was then photographed with the TLC Visualizer (CAMAG). A rutin calibration curve was built using the VideoScan 1.02 software (CAMAG), and the concentration of the flavonol was evaluated inside the formulations.

Deposited Formulation	Conc. (mg/ml)
A. indica DEC	41,62
A. indica HE	28,66
B. diffusa DEC	23,47
B. diffusa HE	14,38
C. pluricaulis DEC	22,30
C. pluricaulis HE	14,82
C. orchioides DEC	11,25
C. orchioides HE	27,74
H. indicus DEC	34,07
H indicus HE	24,41

Table 1. Decoctions and hydro-alcoholic macerations yields.

High Performance Liquid Chromatography (HPLC) analyses of DECs and HEs

DECs and HEs were subjected to RP-HPLC analysis to identify and quantify their main phytomarkers. The reference compounds (all obtained from Sigma Aldrich, Milano) were used as external standards to set up and calculate appropriate calibration curves. The analyses were performed using a Jasco modular HPLC (Tokyo, Japan, model PU 2089) coupled to a diode array apparatus (MD 2010 Plus) linked to an injection valve with a 20 µl sampler loop (injection volume: 40 µl). The columns used were a Tracer Extrasil ODS2 (5πm, 25x0,46 cm) or an Eclipse-PLUS-C18 (5πm, 25x0,46 cm) with a flow rate of 1.0 mL/min. Mobile phases and gradients were chosen depending on the phytocomplex or the compound to analyse (Tab. 2-5).

Following chromatogram recording, samples peaks were identified by comparing their UV spectra and retention time with those from the pure standards. Dedicated Borwin software (Borwin ver. 1.22, JMBS Developments, Grenoble, France) was used to calculate peak area by integration.

Standard solution and calibration procedure.

Individual stock solutions of each formulations phytomarkers were prepared in methanol. The calibration curves of the considered compounds were prepared within a different range (Tab. 2-5) Each calibration solution was injected into HPLC in triplicate. The calibration graphs were provided by the regression analysis of peak area of the analytes versus the related concentrations. The analysis of the phytocomplexes was performed under the same experimental conditions. The obtained calibration graphs allowed the determination of the concentration of the phytomarkers inside the formulations.

	rutin
Gradient	0 min75%A - 25%B 20 min 60%A - 40%B 25 min 50%A - 50%B 30 min 0%A - 100%B 35 min 100%A - 0%B 45 min 75% A - 25%B
Solvent system	A: H_2O:Ac. formic acid (99.5:0.5) B: MeOH:CH_3CN:Formic Acid (49.75:49.75:0.5)
Calibration curve conc. range	The flavonoids quantification was conducted by constructing a rutin calibration curve (concentration range from 20 µg/ml to 500 µg/ml) and by applying the internal standardization method, calculating the response factors obtained by injecting a sample of commercial standards of the each flavonoids all at the same concentration (24 µg/ml).
r^2	0,9986
LOD	2.1 µg/ml
LOQ	6.3 µg/ml

Response factor	rutin: 1	isoquercetin: 0.69	kampferol-3-O-rutinoside: 0.85	kampferol-3-O-glucoside: 0.53	quercitrin: 0.53	quercetin: 0.76

Table 2.

	boeravinone B	**eupalitin**	**vanillin**	**ferulic acid**
Gradient	0 min 50%A - 50%B 25 min 0%A - 100%B 300 min 0%A - 100%B 45 min 50%A - 50%B	0 min 50%A - 50%B 25 min 0%A - 100%B 300 min 0%A - 100%B 45 min 50%A - 50%B	0 min 75%A - 25%B 20 min 60%A - 40%B 25 min 50%A - 50%B 30 min 0%A - 100%B 35 min 0%A - 100%B 45 min 75%A - 25%B	0 min 75%A - 25%B 20 min 60%A - 40%B 25 min 50%A - 50%B 30 min 0%A - 100%B 35 min 0%A - 100%B 45 min 75%A - 25%B,
Solvent system (A & B)	A: H_2O:Ac. formic acid (99.5:0.5) B: MeOH:CH_3CN:Formic Acid (49.75:49.75:0.5)	A: H_2O:Ac. formic acid (99.5:0.5) B: MeOH:CH_3CN:Formic Acid (49.75:49.75:0.5)	A: H_2O:Ac. formic acid (99.5:0.5) B: MeOH:CH_3CN:Formic Acid (49.75:49.75:0.5)	A: H_2O:Ac. formic acid (99.5:0.5) B: MeOH:CH_3CN:Formic Acid (49.75:49.75:0.5)
Calibration curve conc. range	5 standard solutions in a range from 1.25 µg/ml to 10 µg/ml	5 standard solutions in a range from 1.25 µg/ml to 25 µg/ml	5 standard solutions in a range from 1.25 µg/ml to 25 µg/ml	5 standard solutions in a range from 1.25 µg/ml to 25 µg/ml
r^2	0,9949	0,9951	0,9952	0,9975
LOD	0.136 µg/ml	0.400 µg/ml	0.064 µg/ml	0.103 µg/ml
LOQ	0.412 µg/ml	1.212 µg/ml	0.195 µg/ml	0.311 µg/ml

Table 3.

	curculigoside A	**orcinol-β-D-glucoside**
Gradient	0 min 75%A - 25%B 20 min 60%A - 40%B 25 min 50%A - 50%B 30 min 0%A - 100%B 35 min 0%A - 100%B 45 min 75%A - 25%B	0 min 90%A - 10%B 30 min 50%A - 50%B 35 min 0%A - 100%B 40 min 0%A - 100%B 45 min 90%A - 10%B
Solvent system	A: H_2O:Ac. formic acid (99.5:0.5) B: MeOH:CH_3CN:Formic Acid (49.75:49.75:0,5)	A: H_2O:Ac. formic acid (99.5:0.5) B: MeOH:CH_3CN:Formic Acid (49.75:49.75:0,5)
Calibration curve conc. range	5 standard solutions in a range from 5 µg/ml to 10 µg/ml	5 standard solutions in a range from 5 µg/ml to 450 µg/ml
r^2	0,9994	0,9982
LOD	1.032 µg/ml	4.210 µg/ml
LOQ	3.127 µg/ml	12,758 µg/ml

Table 4.

	2H4MB	**3H4MB**	**2H4MBAc**
Gradient	0 min 75%A - 25%B 20 min 60%A - 40%B 25 min 50%A - 50%B 30 min 0%A - 100%B 35 min 100%A - 0%B 45 min 75% A - 25%B	0 min 75%A - 25%B 20 min 60%A - 40%B 25 min 50%A - 50%B 30 min 0%A - 100%B 35 min 100%A - 0%B 45 min 75% A - 25%B	0 min 75%A - 25%B 20 min 60%A - 40%B 25 min 50%A - 50%B 30 min 0%A - 100%B 35 min 100%A - 0%B 45 min 75% A - 25%B
Solvent system	A: H_2O:Ac. formic acid (99.5:0.5) B: MeOH:CH_3CN:Formic Acid (49.75:49.75:0,5)	A: H_2O:Ac. formic acid (99.5:0.5) B: MeOH:CH_3CN:Formic Acid (49.75:49.75:0,5)	A: H_2O:Ac. formic acid (99.5:0.5) B: MeOH:CH_3CN:Formic Acid (49.75:49.75:0,5)
Calibration curve conc. range	Standard solutions in a range from 0.5 µg/ml to 450 µg/ml	Standard solutions in a range from 0.5 µg/ml to 450 µg/ml	Standard solutions in a range from 0.5 µg/ml to 450 µg/ml

r^2	0,9993	0,9982	0,9957
LOD	0.56 µg/ml	0.74 µg/ml	1.28 µg/ml
LOQ	0.93 µg/ml	2.05 µg/ml	1.97 µg/ml

Table 5.

Gas chromatographic coupled with a flame ionisation detector (GC-FID) analyses

DECs and HEs gas chromatographic analyses were performed using a Thermo-Quest GC-Trace gas-chromatograph equipped with a flame ionisation detector (FID) and a Varian FactorFour VF-5ms poly-5%-phenyl-95%-dimethyl-siloxane bonded phase column (i.d., 0.25 mm; length, 30 m; film thickness, 0.25 µm). Operating conditions were as follows: injector temperature 300 °C; FID temperature 300 °C, Carrier (Helium) flow rate 1.2 ml/min and split ratio 1:50. The chromatographic analyses of chloroform and supercritical fluids extracts of DECs and HEs were performed by applying the following temperature program: 75 °C to 300 °C with a gradient of 5 °C/min, then in isotherm for 10 min. The total duration of the chromatogram acquisition was 55 min. One microliter of calibration solutions of the considered pure compounds was injected, in triplicate, to prepare calibration curves. Correlation factor R^2 in the chosen linearity range, limit of detection (LOD) and limit of quantification (LOQ) were calculated for each calibation curve. The analysis of the phytocomplexes was performed under the same experimental conditions to determine the concentration of the phytomarkers inside the phytocomplexes.

Gas chromatographic coupled with a mass spectrometry detector (GC-FID) analyses

DECs and HEs were also analysed by a Varian GC-3800 gas chromatograph equipped with a Varian MS-4000 mass spectrometer using electron impact and hooked to NIST library. The constituents of the considered formulations were identified by comparing their GC retention times, KI and the MS fragmentation pattern with those of pure compounds and by matching the MS fragmentation patterns and retention indices with the above mentioned mass spectra library. A Varian FactorFour VF-5ms poly-5% phenyl-95%-dimethyl-siloxane bonded phase column (i.d., 0.25 mm; length, 30 m; film thickness, 0.25 µm) was used. Operating GC conditions were as follows: injector temperature 300 °C; carrier (Helium) flow rate 1.2 ml/min and split ratio 1:50. Oven

temperature changed depending on the analyses method.

The mass spectrometer conditions applied were: ionization voltage, 70 eV; emission current, 10 μAmp; scan rate, 1 scan/sec; mass range, 20-900 Da; ionic trap temperature, 150 °C, transfer line temperature, 300 °C. In order to determine the Kovats index of the components, a mixture of alkanes (C8–C32) (Sigma-Aldrich, Italy) was injected in the GC–MS and analysed using the same conditions described above.

Nuclear Magnetic Resonance

To better define the phytochemical fingerprint, ^{1}H-NMR spectroscopic analysis have been employed. The ^{1}H-NMR spectra were recorded on a Varian Gemini-400 spectrometer operating at 399.97 MHz and at a temperature of 303 K. Every extract was dissolved in proper deuterated solvents (D_2O, CD_3OD or $CDCl_3$) into a 5 mm NMR tube, and the solvent signal was used for spectral calibration. ^{1}H spectra were run using a standard pulse sequence "s2pul", with 45.0 degrees pulse, 3.00 s acquisition time, 8 repetitions, 4000 Hz spectral width, and 0.33 Hz FID resolution. Characteristic resonances of ^{1}H-NMR spectra for standards used were detected according to literature data, and by comparison with chemical shifts observed for single constituent solutions.

BIOACTIVITY

Antioxidant activity

The prevention of oxidative stress is critical because of its strong link to cellular ageing and gene mutations, factors that could expose tissues to an increased risk of oncogenesis (Stoll et al., 2013). Based on the indications of Ayurveda with respect to these plants source (*Rasayana*) the study of the antioxidant activity was a priority. Because of their chemical complexity, the antioxidant evaluation of herbal-derived preparations could lead to scattered results, according to the method adopted. Therefore, the recourse to a pool of methods rather than to a single assay is highly advisable (Rossi et al., 2011).The 2,2'-azinobis (3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt radical (ABTS) assay, the 1,1-diphenyl-2-picrylhydrazyl (DPPH) test and β-carotene bleaching test (BCBT) were performed, in order to evaluate the radical scavenging activity, the capacity of the phytocomplexes to block the radical propagation, and to determine the activity of both hydrophilic and lipophilic species, ensuring a better comparison of the results and covering a wider range of possible applications. In particular, ABTS and DPPH test were performed by spectrophotometric methods and (HP)TLC-bioautographic assay (Rossi et al., Guerrini & Sacchetti, 2014). Using the latter test, the most active fractions of the preparations were pinpointed after (HP)TLC plates elution (See HPTLC paragraph in the section "Material and methods").

$ABTS^{\bullet+}$ assay

The method (Miller et al., 1993; Scartezzini et al., 2006) involves the use of ABTS converted in its radical cation by the addition of sodium persulfate 70 mM. 1 mL of radicalized ABTS was added to 25 mL of EtOH and the absorbance was measured at 734 nm to obtain 0.70 ± 0.02. The instrument blanc was obtained mixing 900 µl of ABTS solution with 100 µl of EtOH and mesuring the absorbance at 734 nm after 1 min with a spectrophotometer (ThermoSpectronic Helios γ, Cambridge, UK). The same procedure was performed with each sample and the antioxidant activity was expressed as inhibition percentage ($Ip^{ABTS}\%$) and calculated using the following expression:

$$Ip^{ABTS}\% = 100(1 - AbsABTS_{SAMPLE} / AbsABTS_{BLANC})$$

A 1 mg/ml solution of trolox (Sigma–Aldrich) was used as positive control. The assays were performed in triplicate for each sample. Subsequently, a regression curve was created

correlating the $ABTS^{\cdot+}$ inhibition percentage with the sample concentration and the IC_{50} value was calculated.

$DPPH^{\cdot}$ assay

Free radical-scavenging activity of DECs and HEs was performed according to the procedure of Wang et al. (1998). An aliquot of 100 µl of DECs and HEs was resuspended in the appropriate solvent and added to a 10^{-4} M methanolic solution of 1,1-diphenyl-2-picrylhydrazyl (DPPH, Sigma–Aldrich) to obtain assay sample solutions in the appropriate range of concentrations. The assay solutions were then placed in an orbital shaker (Universal Table Shaker 709; 200 rpm) at room temperature. After 30 min of incubation, the absorbance of each sample was measured at 517 nm with a spectrophotometer (ThermoSpectronic Helios c, Cambridge, UK). A 1 mg/ml solution of trolox (Sigma–Aldrich) was used as positive control. Antioxidant activity was expressed as $DPPH^{\cdot}$ inhibition percentage ($Ip^{DPPH}\%$) and calculated using the following expression:

$$Ip^{DPPH}\% = 100(1 - AbsDPPH_1 / AbsDPPH_2)$$

where, $AbsDPPH_1$ is the absorbance of the DPPH solution that reacted with phytocomplex sample; and $AbsDPPH_2$ is the absorbance value of the DPPH solution with methanol only (negative control). The assays were performed in triplicate for each sample. Subsequently, a regression curve was created correlating the $DPPH^{\cdot}$ inhibition percentage with the sample concentration and the IC_{50} value was calculated.

BCBT

Antioxidant activity of DECs and HEs was also determined through the ` -carotene bleaching test (Taga, Miller, & Pratt, 1984). An aliquot of 10 mg of ` -carotene (type I synthetic, Sigma–Aldrich) was dissolved in 10 ml of $CHCl_3$ with 20 µl of linoleic acid (Sigma–Aldrich) and 200 µl of Tween 40 as emulsifier. $CHCl_3$ was removed using a rotary evaporator (B¼chi 461 Switzerland) at 40 °C, and distilled water (50 ml), previously O_2-saturated for 30 min, was added to obtain an emulsion. Samples of DECs or HEs (0.2 ml), in a range of concentration, were then added to the emulsion and incubated at 50 °C for one hour. Absorbance was monitored with a spectrophotometer (ThermoSpectronic Helios c, Cambridge, UK) before incubation, and after 60 min at 470 nm wavelength. To set up the spectrophotometer, a solution composed by 20 µl of linoleic acid, 200 µl of Tween 40 and 50 ml of distilled water (without tested sample) was employed. A 1 mg/ml methanolic solution of trolox (Sigma–Aldrich) was used as positive

control. Negative controls were set up with appropriate aliquots of methanol. The antioxidant activity (AA) expressed as inhibition percentage of the ' -carotene oxidation, was calculated as follows:

$$AA = 100(DRc - DR)/DRc$$

where, DRc [=ln(a/b)/60] is control percentage degradation; Drs [=ln(a/b)/60] is sample percentage degradation; a = absorbance before incubation; b = absorbance after 60 min of incubation. For each sample, assays were performed in triplicate.

Antimicrobial activity

The antimicrobial activity was performed using three Gram-positive bacteria: *Enterococcus faecalis* (ATCC 29212); *Staphylococcus aureus* (ATCC 29213); *Staphylococcus epidermidis* (ATCC 14990); three Gram-negative: *Escherichia coli* (ATCC 4350); *Klebsiella oxytoca* (ATCC 29516); *Pseudomonas aeruginosa* and one yeast: *Candida albicans*

For bacteria and yeast a 96-well microplate was used.

The preparations were tested starting from the concentrations calculated from the yield of their respective formulation processes followed by a series of two fold dilutions for seven concentrations (Foutsa et al., 2013). The experiments were flanked by a negative control (solvent only without plant sample). In all wells was added a bacterial suspension to obtain a final concentration of 1 x 10^7 CFU/mL (or 1 x 10^6 CFU/mL for *C. albicans*) and a final volume of 200 µL/well. The microplate was incubated 8 h (24 h for *C. albicans*) at 37 °C in agitation at 110 rpm then was added 40 µL (of a solution of 20 mg/mL) of the dye 2,3,5-Triphenyl-tetrazolium chloride (Sigma-Aldrich) and was measured the absorbance at 415 nm before and after 1 h of the addition of the dye to test microbial viability.

Genotoxic and antigenotoxic properties

The evaluation of the genotoxic properties was preparatory for the antiogenotoxic assay. It was performed through *Saccharomyces cerevisiae* D7 test and SOS-Chromotest, and as expected the preparations did not exhibit any mutagenic activity, but the main target of this biological tests was to highlight the possible mutagen-protective capacity of the tested botanical.

Saccharomyces cerevisiae D7 test

Cytotoxicity and mutagenic pre-test was performed on preparations, employing yeast cells (D7 diploid strain of *S. cerevisiae* ATCC 201137). Complete liquid (YEP), solid (YEPD), and selective media were prepared according to literature (Zimmermann, Rasenberger, 1975; Rossi et al., 2011; Muzzoli & Sacchetti, 2001). Cells from a culture with low spontaneous gene conversion and reverse point mutation frequencies were grown in a liquid medium at 28-30°C, until they reached the stationary growth phase. The yeast cells were pelleted and re-suspended in a volume of 0.1 M sterile potassium phosphate buffer, pH 7.4, to obtain the final mixture of 2 x10^8 cell/ml. The test solutions (4 ml) were composed of 1 ml of cell suspension, potassium phosphate buffer, and sample of phytocomplexes. The negative control was assessed with ddH_2O for DEC and a 30 % EtOH solution for HE, while a positive control was set up with Ethyl Methane Sulphonate (EMS) (0.01 mg/plate). The mixture was incubated under shaking for 2 h at 37°C. Then the cells were plated in complete and selective media to ascertain survival, *trp*- (convertants) and *ilv*- (revertants). The plates were then incubated at 29 ± 1°C and, after 5 days, the grown colonies were counted to determine the gene conversion at *trp* locus (*trp* convertants) and point mutation at *ilv* locus (*ilv* revertants) frequencies on the basis of the colonies ratio numbered on selective and complete media. In light of the results achieved with the above-described method, the inhibitory effects (i.e. mutagen-protective activities) of each preparations was evaluated on gene conversion and reverse point mutation against direct acting mutagen Ethyl Methane Sulphonate (EMS). Starting from a mixture of 2 x 10^8 cell/ml, as described above, experiments were processed in the same way adopted for toxicity and mutagenic evaluation, with the presence of EMS (0.01 mg/plate) included in the test solution. Colony-forming Units (CFU) were assessed after the plates were incubated at 29 ± 1°C and, after 5 days and compared with that of control where no test samples were added. Every genoactivities for each samples were evaluated by visual estimation (colony counting) and integrated by statistical analysis.

SOS-Chromotest

Genotoxicity and antigenotoxicity assays were performed in accordance with Quillardet and Hofnung (1985). An aliquot of 500 µl of a bacterial culture of *E. coli* PQ37 was added to 5 ml of fresh La medium (LB medium plus 20 µg/ml ampicillin) and left to grow overnight and shaken constantly at 37°C. One ml of culture was added to 5 ml of fresh

La medium and was grown at 37°C for 3.5 h. At this point the bacterial concentration was $2x10^8$ UFC/ml; the solution had an optical density of λ=0,6±0,02.

This solution was diluted 1:10 with fresh LB medium and 0,6 ml were distributed into test tubes containing 20 µl of 4-nitroquinoline 1-oxide (4NQO), the genotoxic agent, or 20 µl of a solution of tested material (DECs, HEs, or pure compounds) in several concentrations. For the evaluation of the antigenotoxic activity the same assay was performed, but with the addiction of 20 µl a of 2.5 µg/ml 4NQO solution in each well used to test the phytocomplexes. After 2 h of incubation at 37°C started the evaluation of the genotoxic/antigenotoxic activity (β-galactosidase) and the cell viability (alkaline phosphatase). The genotoxic and antigenotoxic assay (evaluation of the β-galactosidase quantity) was performed using 0.3 ml of the last obtained bacterial solution added to 2.7 ml of B buffer. After a period of incubation of 10 minutes at 37°C, 0.6 ml of a 0.4% solution of o-nitrophenyl-d-galactopyranoside (ONPG) was added. After anther 60 minutes of incubation, the addition of 2 ml of Na_2CO_3 1M solution stopped the reaction. The colour of the mixture was read with a spectrophotometer (ThermoSpectronic Helios γ, Cambridge, UK) at wave length of 420 nm.

At the same time the viability assay (evaluation of the alkaline phosphatase quantity) was performed using 0.3 ml of bacterial solution added to 2.7 ml of P buffer. In this case, after a period of incubation of 10 minutes at 37°C, 0.6 ml of a 0.4% solution of p-nitrophenyl phosphate (PNPP) was added. After anther 60 minutes of incubation, the addition of 1 ml of HCl 2.5M stopped the reaction and cause the colour disappearance. Five minutes later, the addition of 1 ml of tris(hydroxymethyl)aminomethane 2M changed the pH restoring the colour. The mixture was read with the spectrophotometer at wave length of 420 nm.

Cytotoxic properties

The evaluation of the cytotoxic capacity of DECs, HEs, fractions and pure molecules was performed through MTT and resazurin assays. These test aimed to establish the viability of *in vitro* cancer cell lines exposed to different concentrations of the above mentioned substances compared to untreated cells.

MTT assay

Cell lines and culture conditions: Human colorectal carcinoma (CaCo-2), human breast

cancer (MCF-7) and human lung carcinoma (A549)were purchased by Istituto Zooprofilattico Sperimentale della Lombardia e dell'Emilia-Romagna, Brescia, Italy. CaCo-2 and MCF-7 cells were maintained respectively in DMEM containing 4,5 g/L and 1 g/L glucose; A549 cells were grown in Ham's F12 medium. Cell lines were cultured in medium supplemented with 10% fetal bovine serum (FBS), 100 U/mL penicillin/streptomycin, and 2 mM L-glutamine. CaCo-2, MCF-7 and A549 cell lines, were routinely grown in 75 cm^2 flasks in a humidified 5% CO_2-95% air atmosphere at 37°C until 80% confluence.

Cell viability assay: cell viability was determined by MTT colorimetric assay (Mosmann, 1983) as reflected by the activity of succinate dehydrogenase. Cells were seeded at the density of 2 x 10^4 cells/well on a 96-well plate. After 24 h, cells were exposed to different concentrations of DECs and HEs preparations in a final volume of 200 µl of culture medium. Control culture was exposed to only vehicle (medium containing 2% FBS). After 24h of incubation, 20 µl of MTT (5mg/ml in phosphate-buffered saline, PBS) were added in each well and the plates were incubated for 4 h at 37°C. The medium was removed and replaced with 100 µl dimethyl sulphoxide to dissolve the formazan crystals. The extent of MTT reduction was measured spectrophotometrically at 570 nm using a microplate reader (680 XR, BIO-RAD).

Resazurin assay

Resazurin reduction assay was performed to assess the cytotoxicity of plant preparations, fractions and identified pure molecules towards one sensitive (CCRF-CEM) and its equivalent resistant leukaemia cell line (CEM-ADR5000). This bioactivity evaluation was performed in the laboratories of Doctor Professor Thomas Efferth in the Institute of Pharmacy and Biochemistry, University of Mainz (Germany), during two periods: 01 November 2013 - 15 December 2013; and 01 July 2014 - 30 September 2014.

The assay is based on the capability of viable cells to reduce the indicator dye resazurin in the highly fluorescent resorufin, while non-viable cells rapidly lose this metabolic capacity without producing fluorescent signal. The assay was performed in accordance with the method described in Kuete et al. (2013). 100 µl of a cell solution containing 2 x 10^4 cells were seeded in each well of a 96-wells-plate. Another aliquot of 100 µl containing the sample to analyse in various concentrations (from 1000 to 0.5 µg/mL for extract and from 100 to 0.005 µg/mL for pure molecules) was then added to obtain a total volume of

200 µl per well. After 72 h, 20 µl of a 0.01 % w/v resazurin solution in ddH_2O were added to each well and the plates were incubated at 37 °C for 4 h. Fluorescence was measured on an Infinite M2000Pro™ plate reader (Tecan, Germany) using an excitation wave length of 544 nm and an emission wave length of 590 nm. Each assay was repeated three times, with six replicates each. The viability was evaluated based on a comparison with untreated cells. IC_{50} values represent sample's concentrations required to inhibit 50% of cell proliferation and were calculated from a calibration curve.

Statistical analysis

Data are reported as mean ± standard error of the mean and "n" was the number of independent experiments performed in triplicate. The statistical analyses were calculated using one-way analysis of variance (ANOVA), followed by Dunnett's Test. The results were considered significant with $p < 0.05$ compared to untreated cells.

RESULTS and DISCUSSION

AZADIRACHTA INDICA

Chemical characterisation

Decoction (DEC) and hydro-alcoholic extract (HE) were formulated following the Ayurvedic indication and the those of the European Pharmacopoeia, starting from 10 g and 50 g of *Azadirachta indica* dried crude drug respectively. The procedures gave the following yields: 3.12±0.18 g (31.2%) for DEC and 10.46±0.06 g (20.92%) for HE.

The chemical characterisation started with the total phenolic quantification through spectrophotometric assay using Folin-Ciocalteau reagent. Curiously, the results obtained by the analyses of the traditional formulations were comparable (Tab. 6) even if the preparation procedures involves different methods and solvents.

	DEC	HE
Polyphenols (mg GAE/g)	9.99 ± 1,93	7.29 ± 0.71
Flavonoids (mg HE/g)	2.22 ± 0,13	1.97 ± 0.12
Proanthocyanidins (mg CCIE/g)	< 0.3	< 0.3

Table 6. Yield of total phenolic quantification of *A. indica* preparations.

Literature reports similar results for the evaluation of total phenolic content of an aqueous extract obtained from the leaves, but data showed a significant discrepancy about the flavonoids quantification (Ghimeray et al., 2009). Furthermore, the results reported in literature about the total polyphenol and flavonoids quantification of an 80% ethanolic extracts of *Azadirachta indica* leaves revealed a direct correlation between the alcohol percentage in the extraction mixture and the total phenolic extraction efficacy.

The ^{1}H-NMR analyses of DEC and HE of *A. indica* (Fig. 1) highlighted the presence of a substantial sugar fraction.

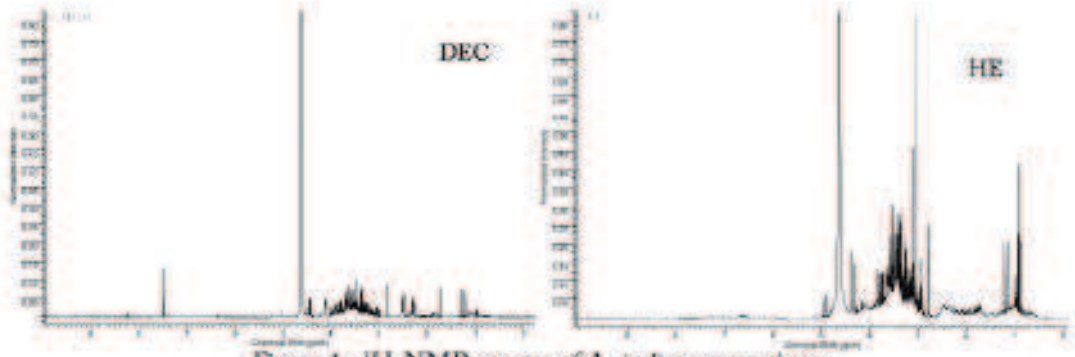


Figure 1. ^{1}H-NMR spectra of A. *indica* preparations.

Subsequently, a RP-HPLC-DAD analyses led to the identification of some flavonoids: rutin, isoquercitrin, quercitrin, kaempferol-3-O-glucoside, kaempferol-3-O-rutinoside in DEC and the same molecules plus quercetin in HE (Fig. 2).

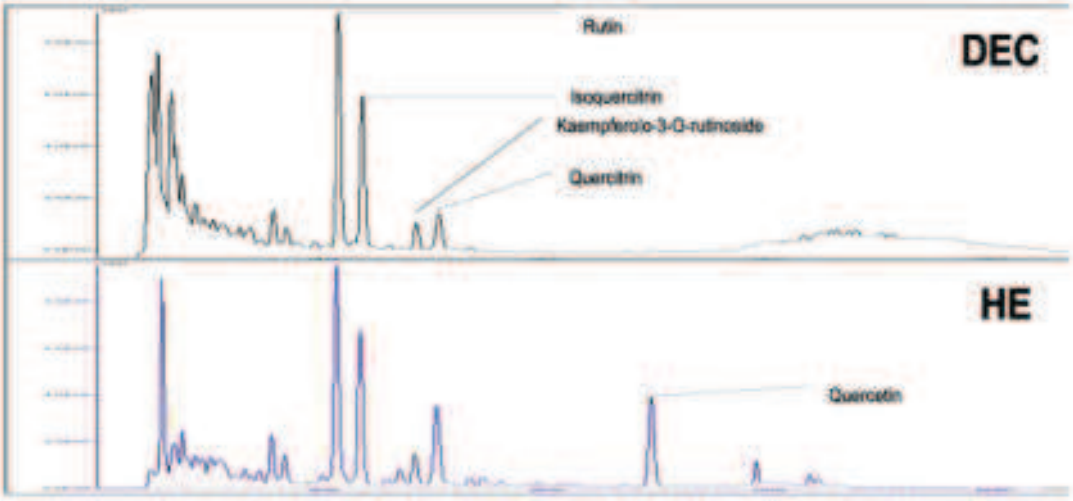


Figure 2. RP-HPLC-DAD chromatograms of *A. indica* preparations.

The identified compounds were then quantified in both preparations (Tab. 7) using the same chromatographic technique to build a calibration curve to evaluate the flavonoids concentration using the internal normalisation method.

	DEC (mg/ml)	HE (mg/ml)
rutin	0.330±0.003	0.453±0.014
isoquercitrin	0.140±0.007	0.233±0.012
kaempferol-3-O-rutinoside	0.041±0.002	0.053±0.002
quercitrin / kaempferol-3-O-glucoside	0.047±0.002	0.100±0.004
quercetin	n.d.	0.047±0.004

Table 7. Flavonoids quantification in *A. indica* preparations (n.d.= not detectable).

In general hydro-alcoholic maceration showed a better extraction capacity in terms of yield and quality of flavonoids than the aqueous extract (DEC), in contrast with the spectrophotometric evaluation data, but confirming the proposed hypothesis. Rutin was also chosen to conduct a quantification using a plane chromatographic support (HPTLC) coupled to a CAMAG UV Visualiser (Fig. 3).

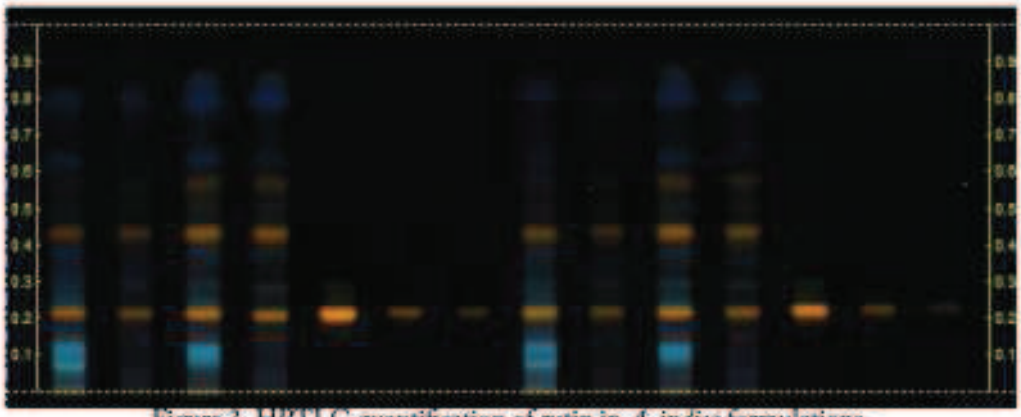


Figure 3. HPTLC quantification of rutin in *A. indica* formulations.

The results showed a slight deviation of the values obtained by HPTLC-VISUALIZER from those obtained by RP-HPLC-DAD, most evident in the hydro-alcoholic extract. Data relating the validation methods, evaluation of LOD, LOQ, precision and accuracy, indicate RP-HPLC-DAD quantification method as the most accurate by the 3-12 % (Tab. 8).

	HPLC-DAD	HPTLC-VISUALIZER
R^2	0,9986	0,9985
LOD (mg/ml)	0,0020	0,0110
LOQ (mg/ml)	0,0063	0,0340
RIPETIBILITA' (R.S.D.%)	1,60%	0,42%
ACCURACY	94-103%	91-115%
	YIELDS	
DEC (mg/ml)	0.330±0.003	0.360±0.011
HE (mg/ml)	0.453±0.014	0.587±0.009

Table 8. Comparison HPLC-DAD - HPTLC-VISUALIZER for the quantitative determination of rutin in *A. indica* DEC and HE.

However, it should be stressed that these data are preliminary and still in progress, starting from the optimization of HPTLC-VISUALIZER quantification method.

In order to better characterize the lipophilic fractions of the considered preparations, three different polarity extractions were performed, by ultrasound assisted maceration in chloroform, soxhlet and supercritical fluid extraction. Using these techniques it was pos-

sible to highlight substances which, although present, were hardly identifiable and quantifiable with the chromatographic methods previously applied (HPTLC, HPLC), because of their low concentration in the formulations. Given the difficult solubilisation of the samples, the extraction process was repeated until depletion of the original phytocomplex. The GC-MS-FID analyses of the phytocomplexes described above allowed the identification and the quantification of β-sitosterol, campesterol and stigmasterol in the apolar fraction of DEC (Fig. 4) and just β-sitosterol in HE (Fig. 5).

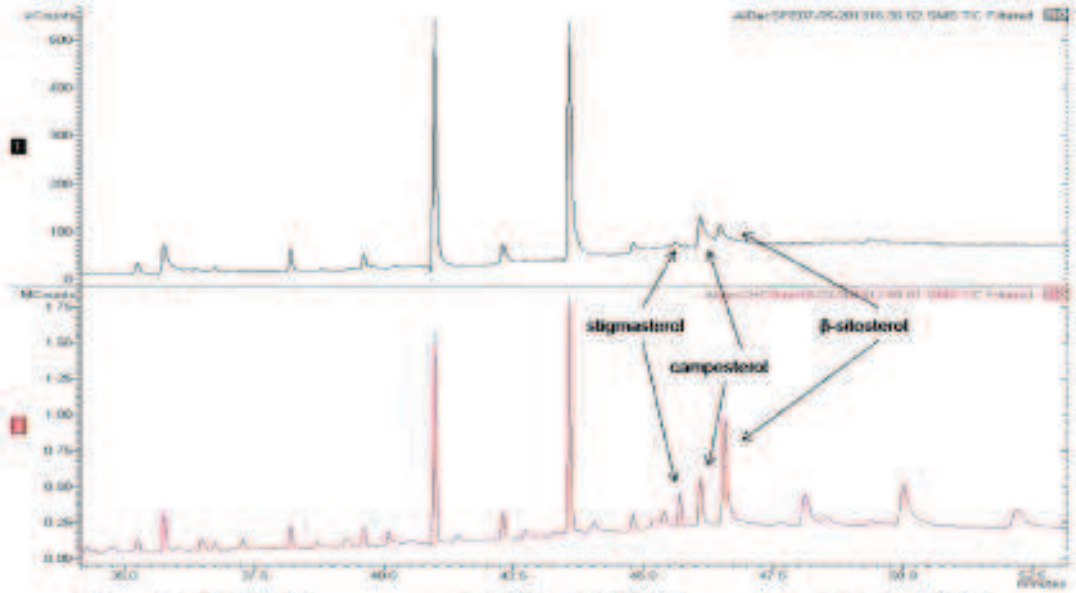


Figure 4. GC-MS chromatograms of SFE and CHCl3 extraction of *A. indica* DEC.

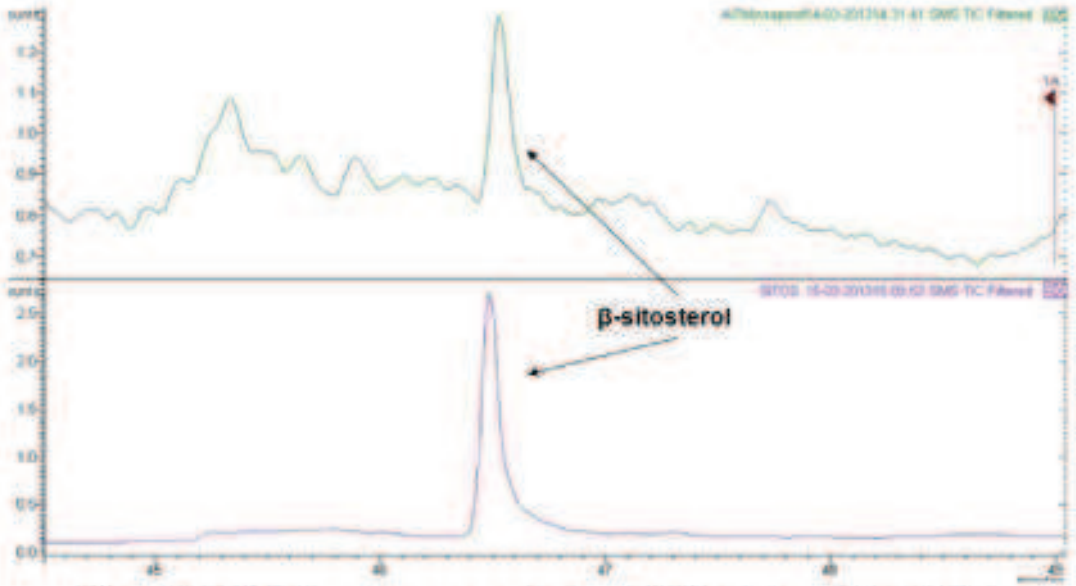


Figure 5. GC-MS chromatograms magnification of SFE extraction of *A. indica* HE.

The data were coherent with literature (Siddiqui et al. 1986; Nguyen et al. 2004). The GC-FID analyses, performed to achieve the quantification of DEC compounds, showed a higher presence of β-sitosterol in the chloroform extract compared to the SFE or soxhlet samples, while the evaluated quantities of stigmasterol appeared to be comparable. Chloroform extract of HE exhibit a quantity of β-sitosterol below the LOQ value, while SFE extract, performed on the same preparation, showed a concentration slightly higher. Moreover, the β-sitosterol concentration in HE resulted to be higher compared to DEC (Tab. 9).

	DEC $CHCl_3$ extraction (μg/ml)	DEC SFE extraction (μg/ml)	HE $CHCl_3$ extraction (μg/ml)	HE SFE extraction (μg/ml)
β-sitosterol	5.447±0.441	3.739±0.339	/	7.145±0.437
stigmasterol	0.759±0.068	0.640±0.038	/	/

Table 9. Quantitation of β-sitosterol and stigmasterol in *A. indica* formulations extracts.

In conclusion, the extraction of β-sitosterol was more efficient with chloroform in DEC, while SFE was the most performing extraction techniques for the same compound in HE. This contrasting results could be due to chemically different nature of the phytocomplexes in which different chemical interactions among β-sitosterol and other compounds could have arose and interfered with the extraction efficiency.

The GC-MS analyses of the soxhlet extraction of DEC, confirmed the presence of β-sitosterol and highlighted the presence of further molecules, as, in order of retention time, benzoic acid, vanillin, dihydroactinidiolide and phytol. The same analyses performed on HE exhibited the presence of 4-terpineol, a monoterpene alcohol, and dihydroactinidiolide. The identification of these substances, with proved biological activities, can be an opportunity to explain the multiple beneficial effects attributed to the medicinal plant.

Biological activities

Antioxidant activity

The evaluation of the antioxidant capacity of *A. indica* DEC and HE performed by ABTS and DPPH test led to concordant results showing the higher activity of hydro-alcoholic extract than that of decoction. Rutin, isoquercitrin and quercitrin, exhibited a high antioxidant capacity highlighting, through ABTS and DPPH tests, IC_{50} values lower than those of positive control (Tab. 10).

IC50 (μg/ml)	ABTS	DPPH	BCBT
HE	22.64 ± 0.17	125.11 ± 6.29	542.75 ± 2.28
DEC	42.80 ± 0.22	211.96 ± 11.34	148.28 ± 1.84
Quercitrin	1.34 ± 0.07	6.11 ± 0.33	n.d.
Isoquercitrin	1.21 ± 0.06	5.31 ± 0.28	n.d.
Rutin	3.51 ± 0.02	7.76 ± 0.49	n.d.
TROLOX	4.01 ± 0.21	13.61 ± 0.71	0.35 ± 0.01

Table 10. Antioxidant capacity of *A. indica* preparation and pure compounds (n.d. = not detectable).

In particular, isoquercitrin appears to be the molecule with the highest antioxidant capacity exhibiting an IC_{50} of 1.21±0.06 μg/ml and 5.31±0.20 μg/ml in the ABTS and DPPH test respectively. BCBT was performed to evaluate the capacity of the phytocomplexes to block the radical propagation starting from the oxidation of linoleic acid and it showed an opposite trend compared to the radical scavenging activity results obtained by the ABTS and DPPH test. BCBT reports a higher antioxidant capacity for the Ayurvedic traditional formulation compared to that of the hydro-alcoholic extract, and it did not show any activity for the pure flavonoids compounds. Literature regarding comparable DPPH assay data obtained testing a 70 % EtOH hydro-alcoholic extract (IC_{50}=105.68 μg/ml; Manikandan et al., 2008), showed a slightly higher antioxidant activity than the experimental results. In light of the increased phenolic extraction capacity of the 70 % solution compared to the 30 % solution, the literature results are coherent with the experimental evidences.

Considering DEC, literature reports a DPPH test IC_{50} value of 31.41 μg/ml (Shitisarn et al., 2006), discordant from the experimental results, most probably because of the extraction was performed on *A. siamensis* variety instead on *A. indica*. The activity of the tested flavonoids (rutin, quercitrin and isoquercitrin) is well known and confirmed by

several studies (Jianxiong et al., 2007; Sankhadip et al., 2013; Silva et al., 2009).

Both HPTLC-bioautographic test using ABTS and DPPH (Fig. 6) underlined the contribution to the antioxidant activity of flavonoids, in full agreement with what already discussed.

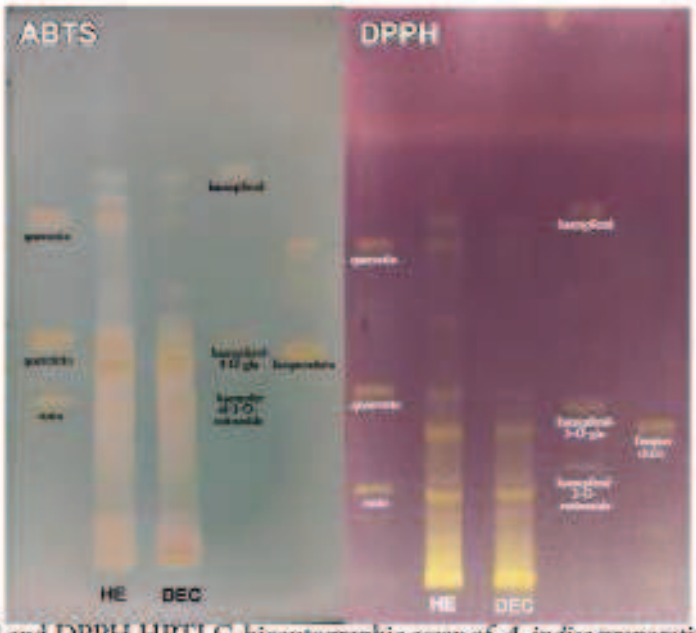


Figure 6. ABTS and DPPH HPTLC-bioautographic assay of *A. indica* preparations, for the evaluation of the antioxidant activity.

Antimicrobial activity

The considered preparations showed a mild antibacterial activity (data not shown) with particular reference to the Gram positive bacteria *S. aureus*. Related literature confirms the experimental data (Jahan et al., 2012). The assays performed with other microorganisms did not highlight any other noteworthy antimicrobial activity (data not shown).

Genotoxic and antigenotoxic activity

To monitor the safety and efficacy of the phytocomplexes, two different tests were performed in order to highlight distinct types of mutations using Test D7 with *Saccharomyces cerevisiae* D7 strain and SOS-Chromotest with *Escherichia coli* PQ37 strain.

D7 test provided information about phytocomplexes cytotoxicity (indicated as percentage of survivors) and genotoxicity (expressed as gene conversion and point mutation). Neither DEC nor HE showed cytotoxic activity at all concentrations tested, as the percentage of survivors (number of colonies grown on complete medium A) is comparable with the control. In addition, data showed that neither DEC nor HE possess genotoxic activity

because the values of gene conversion and point mutation are within the physiological parameters of the negative control (respectively 0.4-0.8 x 10^{-5} for gene conversion and 0.1-0.5 x 10^{-6} for point mutation) in contrast to those obtained with EMS (ethyl-methanesulfonate), used as positive control. The evaluation of the antigenotoxic potential against a known mutagen (EMS) did not exhibit noteworthy results (Tab. 11) for any of the two preparations. In both tests there was a sharp increase in the number of yeast colony in the plates with selective medium (lacking tryptophan and isoleucine to highlight respectively gene conversion and point mutation) treated with EMS, proving that neither DEC nor HE counteract its mutagenic activity.

Genotoxicity DEC

(mg/plate)	Survival%	Conversants x10⁷ UFC	Revertants x10⁷ UFC
H_2O	100.00 ± 3.01	0.54 ± 0.10	0.19 ± 0.08
0.33	100.65 ± 0.97	0.53 ± 0.23	0.38 ± 0.29
0.65	99.91 ± 1.49	0.53 ± 0.06	0.24 ± 0.09
1.30	99.18 ± 2.24	0.58 ± 0.32	0.34 ± 0.27
2.60	100.49 ± 1.85	0.72 ± 0.32	0.24 ± 0.09
5.20	100.97 ± 1.57	0.77 ± 0.49	0.38 ± 0.08
10.41	101.46 ± 2.06	0.62 ± 0.10	0.29 ± 0.02
20.83	98.06 ± 3.02	0.58 ± 0.14	0.35 ± 0.09
41.62	101.94 ± 1.26	0.76 ± 0.13	0.43 ± 0.26
EMS 0.2%	94.17 ± 3.02	6.13 ± 1.35	38.76 ± 6.08

Antigenotoxicity DEC

(mg/plate)	Survival%	Conversants x10⁷ UFC	Revertants x10⁷ UFC
H_2O	100.00 ± 4.32	0.45 ± 0.17	0.26 ± 0.14
0.33	99.97 ± 2.41	13.90 ± 0.90	77.85 ± 5.97
0.65	97.55 ± 2.74	12.94 ± 3.10	81.35 ± 4.97
1.30	99.11 ± 2.62	14.04 ± 0.19	86.52 ± 5.72
2.60	97.08 ± 2.18	13.76 ± 1.71	82.48 ± 18.03
5.20	102.98 ± 3.30	12.47 ± 2.40	81.37 ± 18.50
10.41	100.00 ± 3.08	13.21 ± 1.09	79.86 ± 4.02
20.83	100.86 ± 2.77	13.45 ± 1.22	86.48 ± 2.57
41.62	100.43 ± 1.71	13.59 ± 0.73	82.36 ± 6.99
EMS 0.2%	39.42 ± 3.42	13.74 ± 0.95	80.75 ± 7.56

Genotoxicity HE

(mg/plate)	Survival%	Conversants x10⁷ UFC	Revertants x10⁷ UFC
0.0015	100.00 ± 16.56	0.45 ± 0.27	0.35 ± 0.15
0.11	94.95 ± 2.40	0.54 ± 0.29	0.43 ± 0.22
0.22	100.00 ± 2.31	0.73 ± 0.26	0.39 ± 0.06
0.45	99.10 ± 4.22	0.52 ± 0.23	0.36 ± 0.19
0.90	91.92 ± 3.50	0.58 ± 0.19	0.33 ± 0.14
1.79	100.00 ± 4.37	0.51 ± 0.36	0.20 ± 0.11
3.58	91.92 ± 6.96	0.68 ± 0.35	0.36 ± 0.14
7.17	88.38 ± 3.25	0.59 ± 0.08	0.37 ± 0.16
14.33	91.41 ± 5.94	0.52 ± 0.24	0.23 ± 0.12
EMS 0.2%	95.40 ± 2.67	15.24 ± 1.89	100.86 ± 14.46

Antigenotoxicity HE

(mg/plate)	Survival%	Conversants x10⁷ UFC	Revertants x10⁷ UFC
0.0015	100.00 ± 8.56	0.50 ± 0.20	0.27 ± 0.21
0.11	99.20 ± 1.72	13.95 ± 0.42	71.09 ± 7.30
0.22	97.30 ± 2.23	14.03 ± 0.71	77.04 ± 9.04
0.45	99.20 ± 2.50	13.92 ± 2.15	82.19 ± 14.91
0.90	98.65 ± 1.21	15.64 ± 0.88	78.72 ± 1.71
1.79	98.65 ± 1.21	11.46 ± 0.37	72.15 ± 3.95
3.58	99.20 ± 0.95	12.86 ± 1.26	71.91 ± 10.39
7.17	98.65 ± 2.54	13.01 ± 1.33	80.29 ± 7.08
14.33	97.30 ± 1.85	12.41 ± 2.29	71.11 ± 6.73
EMS 0.2%	93.69 ± 2.37	13.16 ± 1.25	95.19 ± 15.88

Tab 11. D7 test: Genotoxic and antigenotoxic activity of *A. indica* DEC and HE

Considering the SOS-Chromotest, DEC showed cytotoxicity at the highest three concentrations tested, probably because of the antibacterial effect towards *E. coli* (Susmitha et al., 2013), however evidenced by the antimicrobial assay. The non-cytotoxic concentrations showed a slight antigenotoxic action: about 20 % inhibition towards the activation of the SOS system caused by the mutagen 4NQO (Fig. 7). HE showed cytotoxicity at the first two concentrations tested but no antigenotoxic capacity. In general, the assays confirmed the absence of genotoxicity but also the lack of antigenotoxic potential towards 4NQO.

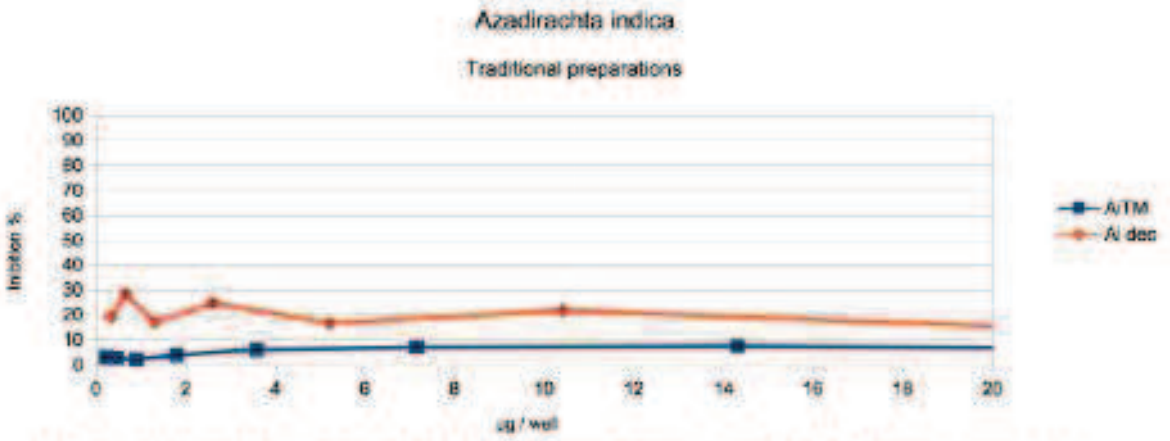


Figure 7. Antigenotoxic capacity of *A. indica* preparations test with SOS-Chromotest agains the known genotoxic agent 4NQO. Data were presented as the mean ± SD (*n*=3).

Three flavonoids were tested for their antigenotoxic activity as pure compounds: rutin, isoquercitrin and quercetin (Fig. 8). Quercetin was the only compound showing antigenotoxic capacity confirming similar results given in literature (Mersch-Sundermann et al., 1994).

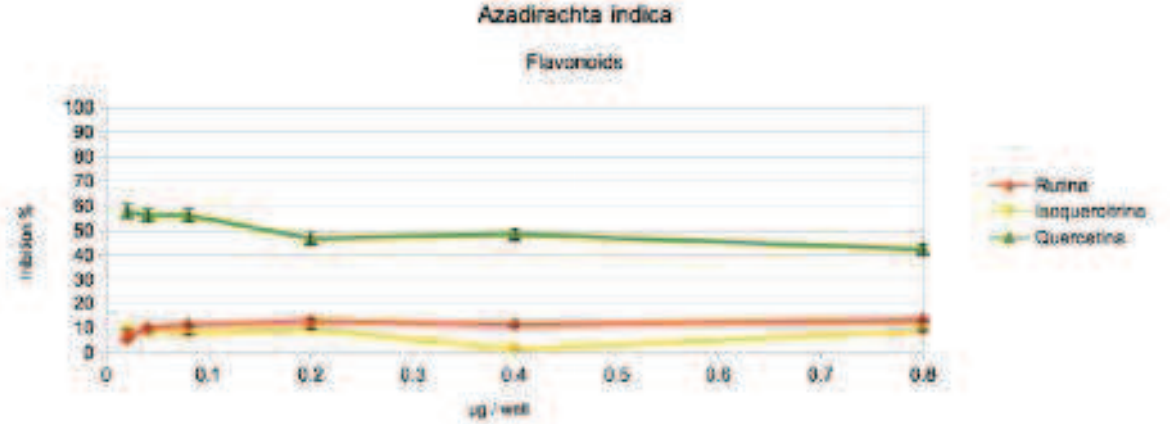


Figure 8. Antigenotoxic capacity of rutin, isoquercetrin and quercetrin tested with SOS-Chromotest agains the known genotoxic agent 4NQO. Data were presented as the mean ± SD (*n*=3).

Antiproliferative activity

The evaluation of the antiproliferative activity of the herbal preparations, DEC and HE, started from the *in vitro* test against heterogeneous human epithelial colorectal adenocarcinoma cells (CaCo2). The cells were exposed to an increasing concentrations of HE (10, 50, 100 µg/ml) and DEC (10, 50, 100, 500, 1000 µg/ml), and they were flanked by a negative control, characterised by cells cultured in medium containing only vehicle

(DMSO 0.1 %). After 24 hours of treatment, both HE and DEC (Fig. 9) did not changed the index of cell viability at any concentrations tested compared to the control.

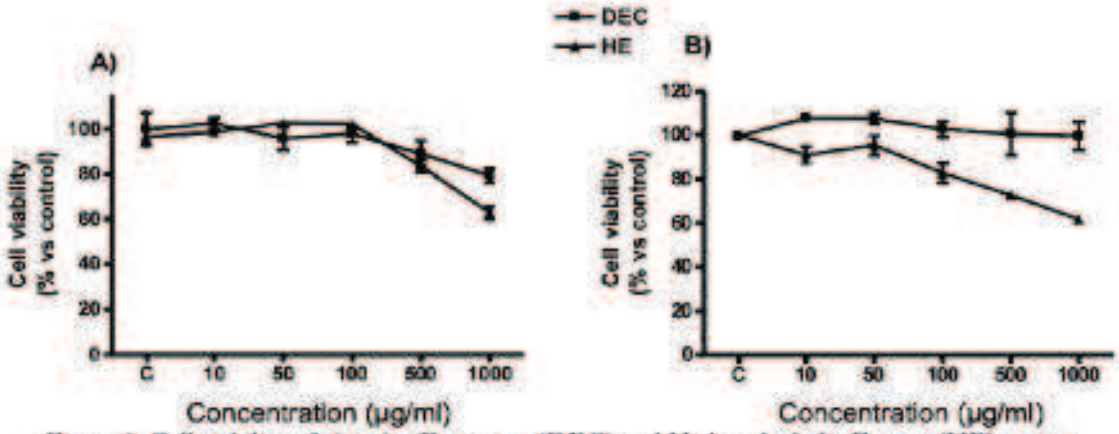


Figure 9. Cell viability of *A. indica* Decoction (DEC) and Hydro-alcoholic Extract (HE) against A549 (A) and CaCo-2 (B) cell lines after 24 h. Data were presented as the mean ± SD (n=3).

Continuing the evaluation of the cytotoxic activity, adenocarcinomic human alveolar basal epithelial cells (A549), breast cancer cells (MCF7), human colon adenocarcinoma cell (LoVo) and human liver carcinoma cell line (HepG2) were considered. *A. indica* formulations did not exhibit any noteworthy activity against the first three cell lines (A549, MCF7 and LoVo) at the highest concentration tested, but it reached IC_{50} values in the tests involving HepG2 cells, showing an interesting selectivity against this cell line. In this case, DEC exhibited a higher antiproliferative activity than HE, showing an IC_{50} of 217.70±0.431 µg/mL, value far from those stated by the American National Cancer Institute (Suffness & Pezzuto, 1991). The investigation of the cytotoxic capacity of the herbal preparation in the context of drug resistance problems, involving a number of mechanisms often poorly decoded associated to the over-production of P-glycoproteins due to the increased expression of the multidrug resistance locus, resulted particularly interesting. In order to highlight eventual effects of DEC and HE against this phenomenon, the antiproliferative activity was tested against one drug-sensitive leukaemia cell lines (CCRF-CEM) and compared with its multi-drug-resistant version (CEM/ADR5000), that overexpress the P-glycoprotein. HE showed a higher bioactivity compared to DEC against both cell lines (Tab. 12), unlike the results obtained against HepG2, but still far from the value stated by the American National Cancer Institute, and even higher than those of doxorubicin (113.17±8.29 µg/mL, Kuete et al., 2014).

	DEC IC_{50} (µg/mL)	HE IC_{50} (µg/mL)
CCRF-CEM	298.84±12.99	137.61±19.86
CEM/ADR5000	>1000	337.79±43.75

Table 12. Cytotoxic IC_{50} values of *A. indica* preparation against CCRF-CEM and CEM/ADR5000 cell lines. Data were presented as the mean ± SD (*n*=3).

Furthermore, DEC did not reach the 50 % proliferation inhibition against CEM/ADR5000 at the maximum concentration tested.

BOERHAAVIA DIFFUSA

Chemical characterisation

The ^{1}H-NMR analyses of DEC and HE of *B. diffusa* (Fig. 10) highlighted the presence of a substantial sugar fraction and a phenolic component, and subsequently the preparations were investigated through spectrophotometric and chromatographic techniques. This strategy brought to the identification and quantification of some phenolic compounds of the classes of benzaldehydes, benzoic acids, cinnamic acid and phenilpropanoids, typically considered interesting as potential active compounds and never identified in the considered herbal preparations of this species.

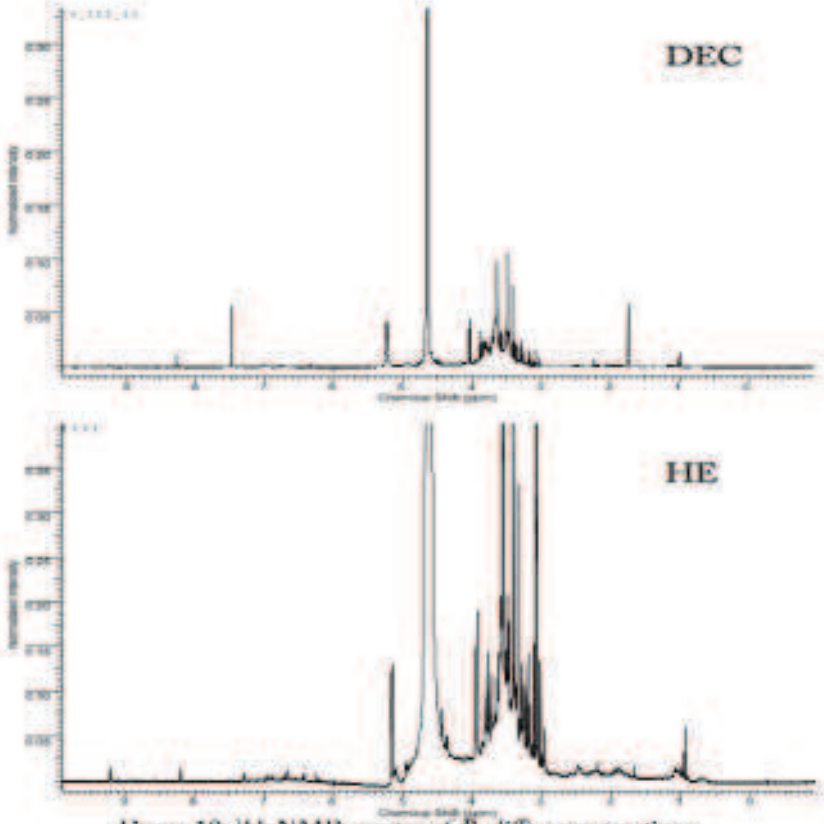


Figure 10. ^{1}H-NMR spectra of *B. diffusa* preparations.

The yields of the formulation processes were 1.760±0.172 g (17.60%) for DEC and 4.461±0.218 g (8.92%) for HE.

The spectrophotometric quantification of the total phenolic content of DEC and HE, was carried out using Folin-Ciocalteau reagent and the results are reported in Tab. 13.

	DEC	HE
Polyphenols (mg GAE/g)	4.60 ± 0.25	9.71 ± 1.11
Flavonoids (mg HE/g)	2.18 ± 0.11	1.14 ± 0.04
Proanthocyanidins (mg CCIE/g)	0.56 ± 0.02	< 0.3

Table 13. Yield of total phenolic quantification of *B. diffusa* preparations.

The aqueous extract (DEC) exhibited a lower capacity of polyphenol extraction than HE, but the trend was inverted for flavonoids, since the quantification was double of the amount compared to those detected in the hydro-alcoholic extract. Literature reports very few information about the *B. diffusa* DEC and HE phenolic content, relying on preparation protocols different from those used in Ayurveda, which makes data hardly comparable (Hua-Bin et al., 2007).

The RP-HPLC-DAD characterization of the phenolic fraction of *B. diffusa* preparations, performed with a fully validated method, showed two different chemical profiles (Fig. 11).

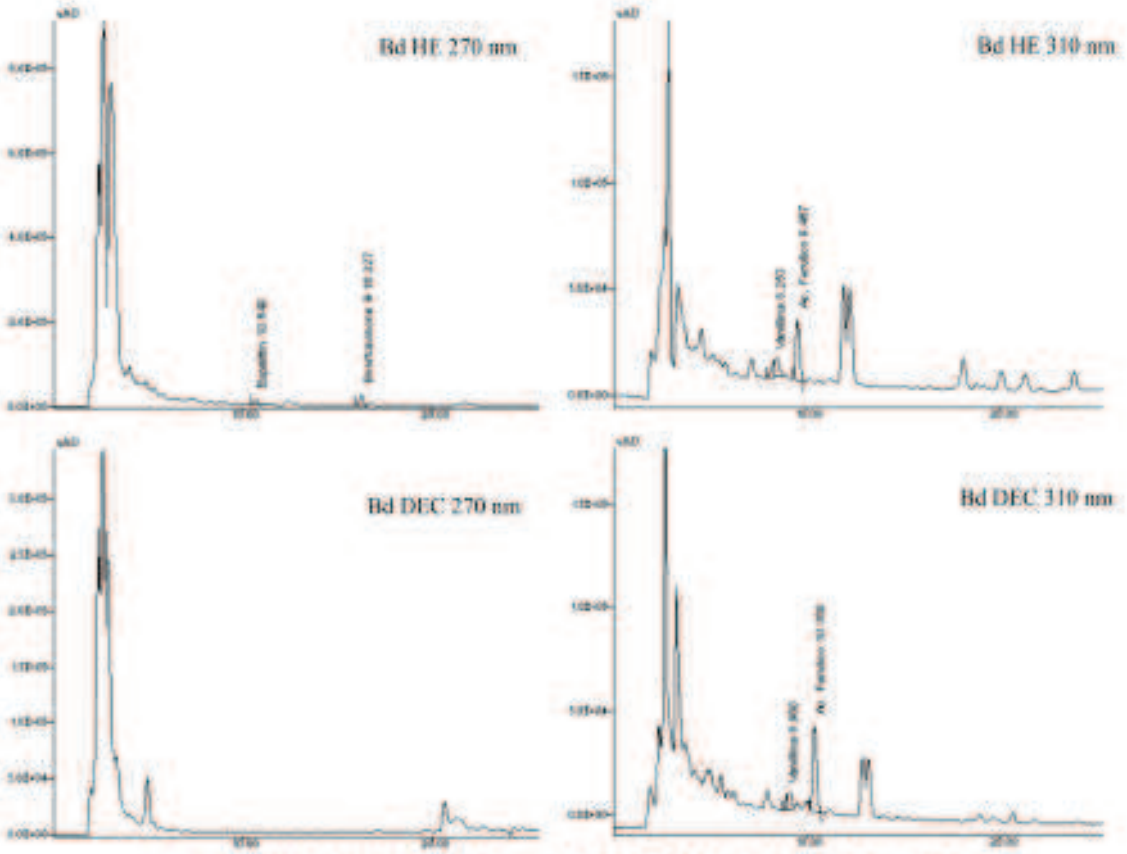


Figure 11. RP-HPLC-DAD chemical profiles of *B. diffusa* DEC and HE.

Vanillin and ferulic acid were the most abundant compounds in DEC, while HE, in addition to these molecules, was also characterised by the presence of boeravinone B and eupalitin, which were instead not detectable in DEC (Tab. 14).

	DEC (µg/ml)	HE (µg/ml)
boeravinone B	n.d.	1.944±0.020
eupalitin	n.d.	2.284±0.055
ferulic acid	6.803±0.022	4.644±0.288
vanillin	1.833±1.387	3.447±0.408

Table 14. Pure compounds quantification in *B. diffusa* preparations. (n.d. = not detectable)

The quantitative comparison between formulations highlighted a higher concentration of vanillin in HE compared to DEC (46.82 %), while ferulic acid had an opposite trend (-31.97%), likely as a consequence of its better solubility in hot water than in alcoholic solution (Merck index, 12th Ed, 1996).

With the aim of obtaining the best chemical characterisation of the preparations, with particular attention to low-abundance compounds which are often deemed responsible for biological activity (Guerrini and Sacchetti, 2012), both preparations were investigated through gas-chromatographic analyses after $CHCl_3$, supercritical CO_2, and soxhlet extraction. The evidences, emerged from GC-MS qualitative analysis of the chloroform extraction, indicated the presence of β-sitosterol, stigmasterol, 3,5-dimethoxy-4-hydroxybenzaldehyde, 3,5-dimethoxy-4-hydroxy-cinnamaldehyde, 4-hydroxy-2-methoxy-cinnamaldehyde in both DEC (Fig. 12) and HE (Fig. 13).

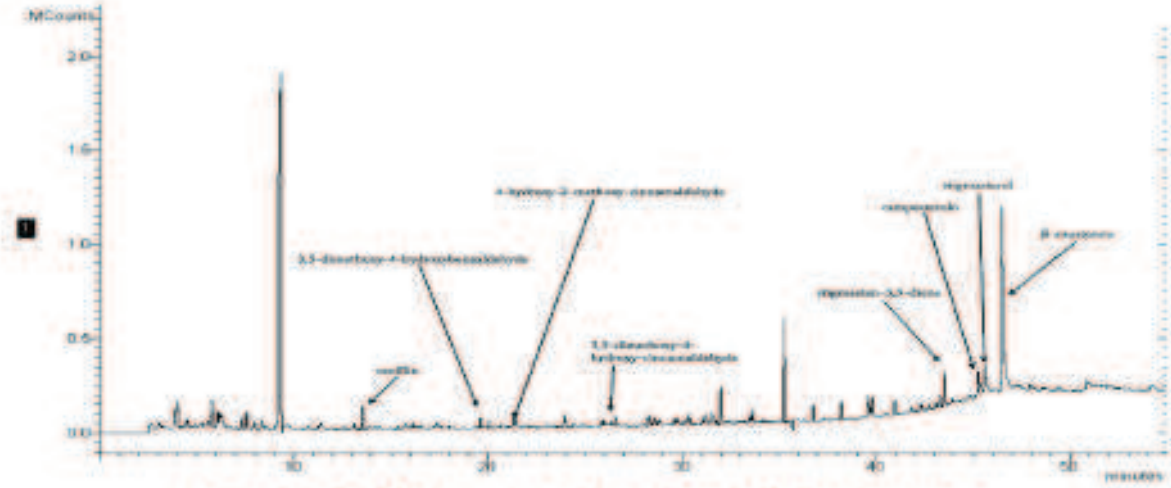


Figure 12. GC-MS chromatograms of *B. diffusa* DEC $CHCl_3$ extraction.

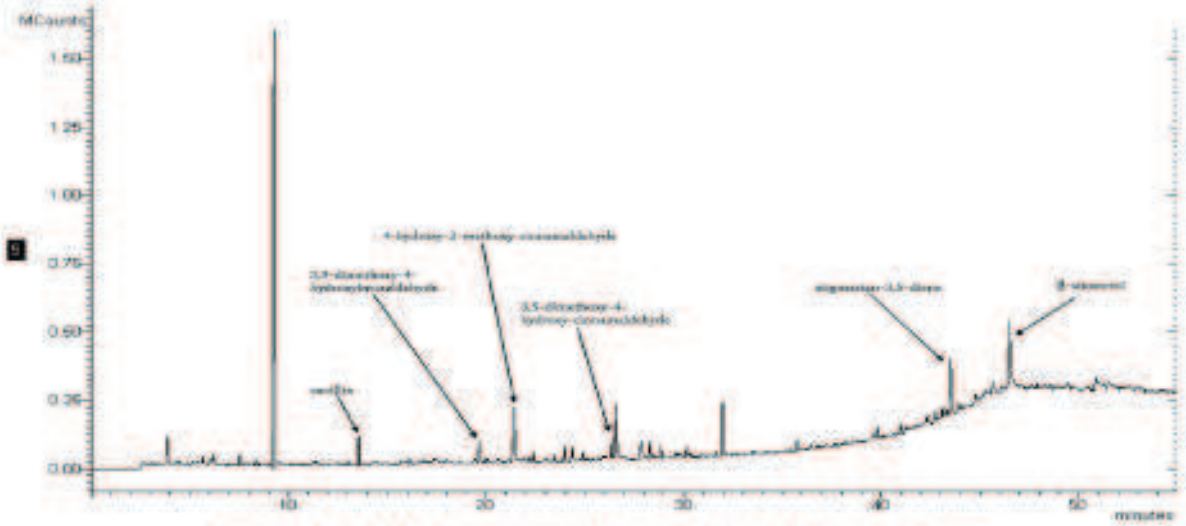


Figure 13. GC-MS chromatograms of *B. diffusa* HE $CHCl_3$ extraction.

Furthermore, the Ayurvedic traditional preparation of *B. diffusa* (DEC) exhibit the presence of campesterol, phytosterol not detected in the hydro-alcoholic extract (HE).
The supercritical fluids extraction (SFE) confirmed the presence of the compounds already detected in the $CHCl_3$ extraction, but the process was not as affective as the previous one. In particular, for β-sitosterol the concentration ratio between the extract obtained with supercritical CO_2 and those obtained by $CHCl_3$ maceration was approximately 1:2 (Tab. 15).

	DEC $CHCl_3$ extraction (μg/ml)	DEC SFE extraction (μg/ml)	HE $CHCl_3$ extraction (μg/ml)	HE SFE extraction (μg/ml)
β-sitosterol	2.500±0.342	1.063±0.006	0.411±0.189	0.192±0.042

Table 15. Quantitation of β-sitosterol in *B. diffusa* formulations extracts

The chromatographic analyses performed on the phytocomplex obtained after DEC extraction by soxhlet apparatus confirmed the presence of vanillin, β-sitosterol and it showed the presence of two new identified molecules: the 4-hydroxy-3,5-dimethoxybenzaldehyde and the stigmastan-3,5-diene.

Biological activities

Antioxidant activity

Because of their chemical complexity, the antioxidant evaluation of herbal-derived preparations could lead to scattered results, according to the method adopted. Therefore, the recourse to a pool of methods rather than a single assay is highly advisable (Rossi et al., 2011). ABTS test, DPPH assay and β-carotene bleaching test (BCBT) were performed in order to evaluate the radical scavenging activity, the capacity of the phytocomplexes to block the radical propagation, and to determine the activity of both hydrophilic and lipophilic species, ensuring a global vision of the antioxidant capacity and covering a wider range of possible applications. In particular, ABTS and DPPH test were performed by spectrophotometric and (HP)TLC-bioautographic assay. Using the latter test, the preparations most active fractions were pinpointed after (HP)TLC plates elution (Rossi et al., 2011; Guerrini & Sacchetti, 2014).

The ABTS spectrophotometric assay of the whole preparations and pure compounds was more performing compared to DPPH and BCBT data (Tab. 16).

IC_{50} (ng/ml)	ABTS	DPPH	BCBT
DEC	150.794±6.241	n.d.	n.d.
HE	33.272±3.406	258.409±11.735	226.179±9.211
vanillin	6.058±0.943	n.d.	n.d.
boeravinone B	4.814±0.011	n.d.	n.d.
eupalitin	4.402±0.002	15.749±0.120	0.077±0.003
ferulic acid	1.081±0.043	9.477±0.229	n.d.
Trolox	2.040±0.101	4.090±0.203	0.059±0.002

Table 16. Antioxidant capacity of *B. diffusa* preparation and pure compounds (n.d. = not detectable).

The only exception noticed was eupalitin, which showed its highest antioxidant capacity in the BCBT, highlighting the different outcomes that may emerge from different mechanism of action. Regarding *B. diffusa* DEC, its antioxidant activity was evaluable just by ABTS test, resulting 77.93% weaker than the antioxidant capacity of HE, and it was neg-

ligible in the other assays. The hydro-alcoholic extract, instead, exhibited a weak capability in every performed test, showing its highest antioxidant capacity in the ABTS assay.
In order to determine the chemical classes involved in the antioxidant activity and to verify the contribution of the identified compounds in the *B. diffusa* preparations, DPPH and ABTS test was performed also by (HP)TLC-bioautographic assay (Fig. 14).

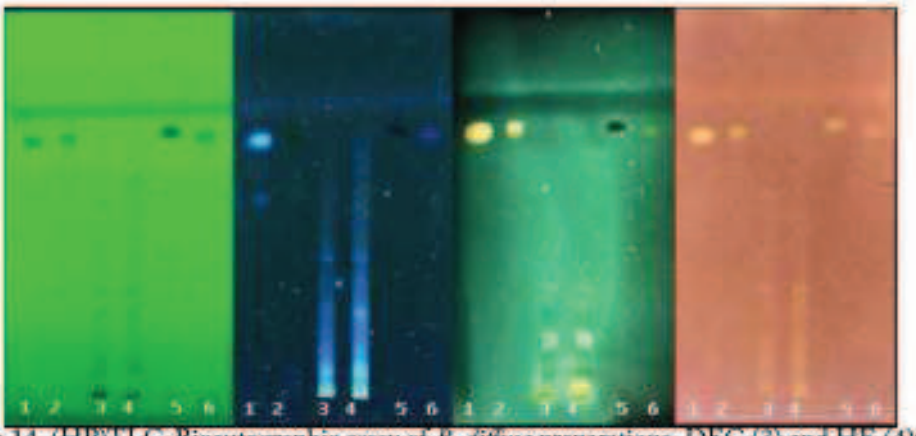


Figure 14. (HP)TLC-Bioautographic assay of *B. diffusa* preparations, DEC (3) and HE (4); and pure molecules, ferulic acid (1, Rf: 0.88), eupalitin (2, Rf: 0.91), boeravinone B (5, Rf: 0.94) and vanillin (6, Rf: 0.91). The first and the second sectors are visualised at 254 and 366 nm, while the third and the fourth are derivatised with ABTS and DPPH respectively.

The experiments confirmed that ABTS offers a better affinity than DPPH for both preparations. The eluted (HP)TLC plate displayed the absence of radical scavenging reactions at the Rf corresponding to the most characterising molecules identified. Further spectrophotometric analyses, carried out to understand the motivation that lead to these results, revealed that among the identified compounds the only one showing activity in all the performed tests was eupalitin, with a preference towards BCTC where it showed its best IC_{50} (98.25% lower than ABTS test result), most probably as a consequence of its lipophilic properties. In the ABTS test ferulic acid showed a radical scavenging activity higher than positive control (+47.01%), while in the DPPH test the antioxidant activity was lower (-56.84%) than Trolox. Ferulic acid did not show any antioxidant capacity in both (HP)TLC-bioautographic assays, and, in light of evidence otherwise obtained by spectrophotometric analyses, we could suppose that these negative results could be related to its low concentration on the silica gel plate, hundred fold lower than that used to reach significant reactivity. Vanillin and boeravinone B exhibited an antioxidant potential just towards the ABTS test, with IC_{50} values 66.33% and 57.62% higher than positive control. The moderate activity of these three molecules in the BCBT (46.87% for ferulic acid, 30.27% for vanillin and 26.62% for boeravinone B) could be explained by the "polar para-

dox" (Porter, 1993; Frankel et al., 1994), since apolar molecules exhibit stronger antioxidant capacity in emulsions because they concentrate on the lipid-air surface, thus ensuring an higher protection of the emulsion itself. On the other hand, polar antioxidants, remaining in the aqueous phase, are diluted and therefore less effective in protecting the lipid (Koleva et al., 2002). The literature reported IC_{50} values for *B. diffusa* ethanolic extract , different from those previous described, but similar evidences have been pointed out about the radical scavenging activity of the pure molecules identified (Khalid et al., 2011). In the DPPH test, data comparison highlighted just a 0.4% difference between experimental ferulic acid IC_{50} value and related data reported in literature (Terpinc et al., 2010).

We must conclude that, at least for the evaluated plants and notwithstanding what otherwise reported, an hydro-alcoholic maceration obtained following Eur. Ph. specifications outperforms the orthodox ayurvedic decoctions in terms of antioxidant performance and overall polyphenolic content (Li et al., 2007).

Antimicrobial activity

The preliminary results highlighted a lack of antibacterial activity against the Gram + (*S. aureus*) and Gram- (*E. coli*) strains considered. The tests extended to other bacterial and fungi strains to better verify the antimicrobial activity on human and plant pathogens did not report any considerable results.

Genotoxic and antigenotoxic activity

Safety of preparations was investigated through two established tests: D7 test, using *Saccharomyces cerevisiae* D7 strain; SOS-Chromotest, using *Escherichia coli* PQ37 strain. Both analyses gave indication of the citotoxicity of the preparation/compound considered (survivor %, expressed as number of grown colonies on complete medium, in D7; % of activation of alkaline phosphatase in SOS-Chromotest), but they differed in the information about their genotoxic potential: D7 test evaluate genotoxicity and antigenotoxicity expressed as capacity of the phytocomplexes to generate, or protect from the generation, of gene conversion and point mutation; while SOS-Chromotest evaluate the activation of the bacterial SOS system as measure of genetic damage (Muzzoli & Sacchetti, 2001).

D7 test experimental data showed that neither DEC nor HE exhibit cytotoxic activity at all concentrations tested, and that they did not have genotoxic activity because the values

of gene conversion and point mutation remained within the physiological parameters (respectively 0.4-0,8 x 10^{-5} for gene conversion and 0.1-0.5 x 10^{-6} for the point mutation) in contrast to those obtained with positive control (EMS). The evaluation of the antigenotoxic activity against a known mutagen (EMS) did not give any appreciable results for both preparations. Both of them generated a sharp increase in the number of survivors in the plates with selective medium treated with EMS, which shows that neither DEC nor HE counteract its mutagenic activity (data not shown).

The SOS-Chromotest was performed in order to have further evidences of the lack of cytotoxicity, genotoxicity highlighted by the previous test. DEC and HE did not display any citotoxicity or DNA damage with reference to the parameters of the test. The only exception was given by boeravinone B, which showed a limited cytotoxicity at a concentration about sixty-folds higher than the amount present in the hydro-alcoholic extract.

To evaluate antigenotoxicity (Tab. 17), DEC and HE were checked in a range of concentration starting from the maximum extractions yield, obtained respectively from 10 and 50 g of crude drug, following a two fold dilution scheme from 1:2 to 1:32. Pure molecules were tested following the same dilution pattern, in order to include in the tested range the concentrations actually available in the preparations. The antigenotoxic capacity was tested against a 2.5 µg/mL solution of 4-Nitroquinoline N-oxide (4NQO), which cause an induction of the SOS system equal to the double of the test performed on the sole 1% DMSO-saline solution.

Preparations			Pure compounds		
	Dose (µg/ml)	Inhibition of genotoxicity (%)		Dose (µg/ml)	Inhibition of genotoxicity (%)
Bd DEC	733.44	1.89	ferulic acid	200.00	25.66
	1466.88	3.40		400.00	26.79
	2933.75	5.28		800.00	27.92
	5867.50	6.04		1600.00	32.08
	11735.00	6.79		3200.00	33.21
	23470.00	9.81		6400.00	35.09
Bd HE	449.38	1.10	vanillin	200.00	19.06
	898.75	1.89		400.00	26.79
	1797.50	6.04		800.00	28.68
	3595.00	6.79		1600.00	30.57
	7190.00	7.55		3200.00	34.34
	14380.00	7.55		6400.00	36.60
			eupalitin	187.50	7.92
				375.00	14.34
				750.00	14.72
				1500.00	16.23
				3000.00	17.74
				6000.00	22.26
			boeravinone B	265.63	16.60
				531.25	24.53
				1062.50	n.d.
				2125.00	n.d.
				4250.00	n.d.
				8500.00	n.d.

Table 17. Effect of *B. diffusa* preparations and pure molecules on genotoxicity induced by 4NQO (2.5 µg/mL) in the SOS-Chromotest (n.d. = not detectable).

Both *B. diffusa* preparations showed a weak antigenotoxic activity, slightly higher for DEC than HE (9.81% and 7.55% respectively). The highest antigenotoxic capacity, with dose-response correlation, was showed by ferulic acid (35.09% at the concentration of 10 µg/ml); and vanillin (36.60% at the concentration of 10 µg/ml), values in accordance with related literature (Ohta et al., 1986). In general, the experimental evidences pointed out an higher activity for DEC in comparison to HE showing an opposite trend with respect to the antioxidant capacity.

Antiproliferative activity

Decoction (DEC) and hydro-alcoholic extract (HE) were then tested against the cell lines: MCF7, A549, CaCo2, HepG2 and LoVo. Both phytocomplexes did not reach the IC_{50} threasold when tested against A549, CaCo2 (Fig. 15), MCF7 and LoVo, but HE showed a better activity than DEC against CaCo2 cell line. DEC showed a weak anti-proliferative potential against HpeG2 cell line with an IC_{50} value of 203.60±0.58 µg/ml, far from the value stated by the American National Cancer Institute (Suffness & Pezzuto, 1991).

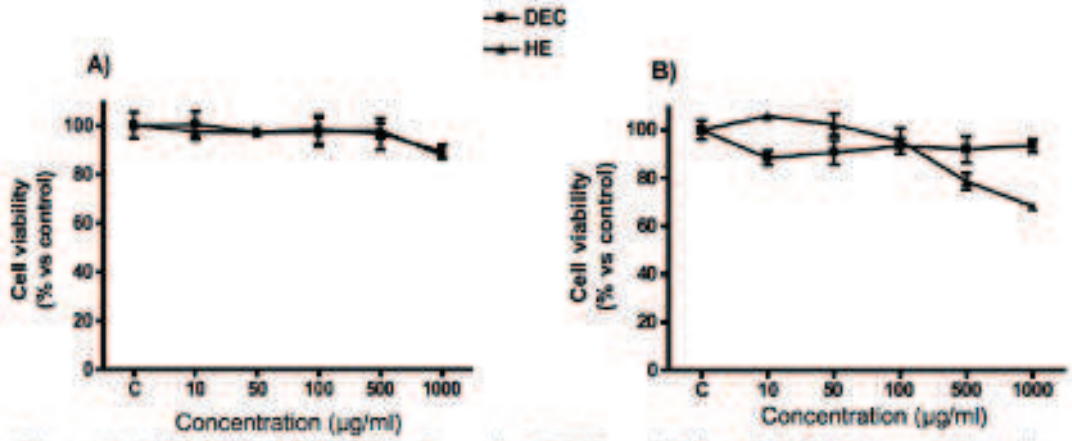


Figure 15. Cell viability of *B. diffusa* Decoction (DEC) and Hydro-alcoholic Extract (HE) against A549 (A) and CaCo-2 (B) cell lines after 24 h. Data were presented as the mean ± SD (n=3).

The antiproliferative activity was also tested against two leukaemia cell lines, one drug-sensitive (CCRF-CEM) and one multi-drug-resistant (CEM/ADR5000). Each extract reached IC_{50} inhibition value (Tab. 18) against both considered cell lines.

Plant extracts and pure molecules	CCRF-CEM cell line IC_{50} (µg/ml)	CEM/ADR5000 cell line IC_{50} (µg/ml)
Boerhaavia diffusa – DEC	428.07 ± 4.01	1119.87 ± 45.31
Boerhaavia diffusa – HE	320.98 ± 17.40	849.33 ± 6.43
eupalitin	13.12 ± 0.76	23.34 ± 0.33
boeravinone B	>30	>30
vanillin	>30	>30
ferulic acid	>30	>30

Table 18. Plant extracts and pure molecules IC_{50} values against CCRF-CEM and CEM/ADR5000

The results of the cytotoxicity evaluation followed the same trend of the results of the antioxidant activity, showing higher activity for the hydro-alcoholic extractions compared to decoctions, but, in this case too far away from the value stated by the American National Cancer Institute. Among the molecules pannier considered (ferulic acid, vanillin, boeravinone B and eupalitin), the only compound that showed an IC_{50} value of cytotoxic activity was eupalitin. It exhibit IC_{50} values of 13.12±0.76 µg/mL against CCRF-CEM cell line and 23.34±0.33 µg/mL against CEM/ADR5000. Though P-glycoprotein-overexpressing CEM/ADR5000 cells showed 1.78-fold degree of cross-resistance compared to its drug-sensitive counterpart, they were more sensitive to this compound than towards doxorubicin (113.17±8.29 µg/mL; Kuete et al., 2014).

CONVOLVULUS PLURICAULIS

Chemical characterisation

Literature references about *C. pluricaulis* lack of scientific sources regarding the precise chemical characterization of the whole plant as crude drug. To fill this gap, it was decided to proceed with the analysis of the preparations decoction (DEC) and hydro-alcoholic extract (HE), as planned in the whole PhD research project, and in parallel perform supercritical CO_2, $CHCl_3$ and soxhlet extractions on the whole plant crude drug (WP).
The ^{1}H-NMR analyses of DEC and HE of *C. pluricaulis* (Fig. 16) highlighted a presence of a substantial sugar fraction and exhibited some polyphenols signals.

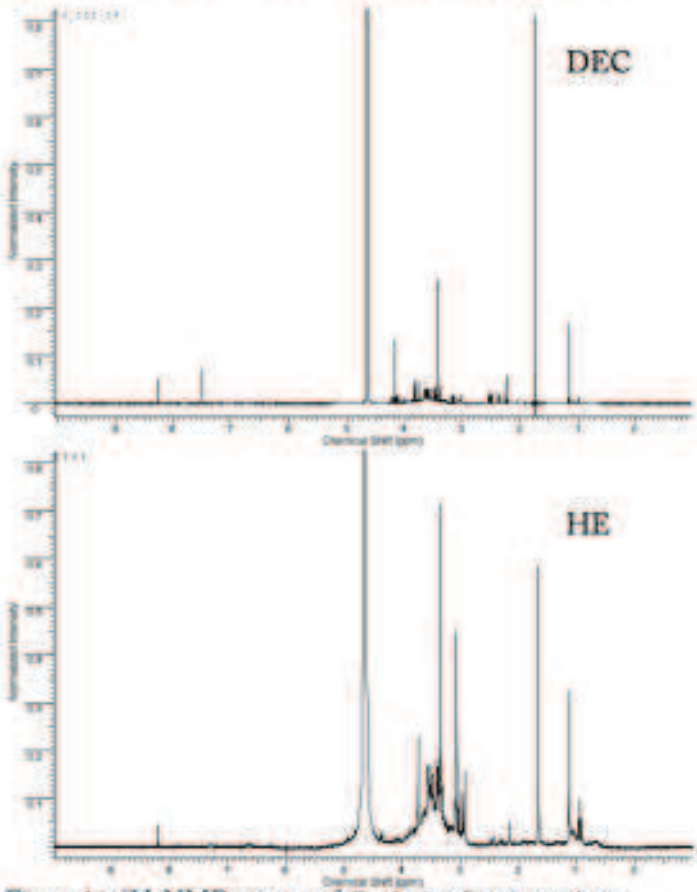


Figure 16. ^{1}H-NMR spectra of *C. pluricaulis* preparations.

Yields of decoction and maceration processes are reposted in Tab. 19.

	g	Yields	%
DEC	18.40±0.15		16.73±0.14
HE	4.74±0.01		9.49±0.02

Table 19. Yields of *C. pluricaulis* DEC and HE processes.

To conform to the method used so far, and since the preparations are characterised by extractions performed on the whole plant, the chemical characterization began with the quantitative assessment of polyphenols, flavonoids, and proanthocyanidins present in DEC and HE, by spectrophotometric method (Tab. 20).

	DEC	HE
Polyphenols (mg GAE/g)	6.05±0.28	6.38±0.89
Flavonoids (mg HE/g)	4.87±0.32	1.18±0.06
Proanthocyanidins (mg CCIE/g)	0.48±0.01	< 0.3

Table 20. Yield of total phenolic quantification of *C. pluricaulis* preparations.

The two phytocomplexes showed a similar total phenolic content (5.2% greater for HE than DEC), but a substantial different amount of flavonoids (99.9% higher in DEC compared to HE) and proanthocyanidins.

Decoction (DEC), traditional Ayurvedic preparation, was the first phytocomplex to be examined. The RP-HPLC-DAD analysis produced chromatograms characterized by multiple peaks, of which the most evident was identified as caftaric acid (Fig. 17). Others two, less intense than the previous one were found to be caffeic acid and p-coumaric acid. Other peaks with gradually lower intensity have been identified as: iso-ferulic acid and tr-ferulic acid.

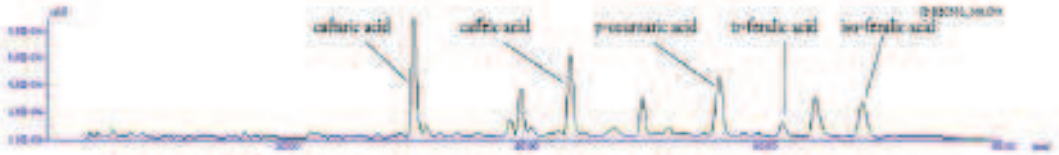


Figure 17. RP-HPLC-DAD chemical profiles of *C. pluricaulis* DEC at 310nm.

DEC was then fractionated through a series of extractions with different polarity solvent system (soxhlet apparatus, $CHCl_3$ maceration in ultrasound and supercritical CO_2) with the aim of obtaining the best chemical characterisation. This extractions scheme emphasize the presence of those compounds in lesser abundance, but which are often deemed responsible for the biological activity, as single compounds or in synergy (Guerrini and Sacchetti, 2012). The three phytocomplexes obtained (from DEC and HE), were analysed by GC-MS. The following figure (Fig. 18) shows the results of DEC chemical characterization.

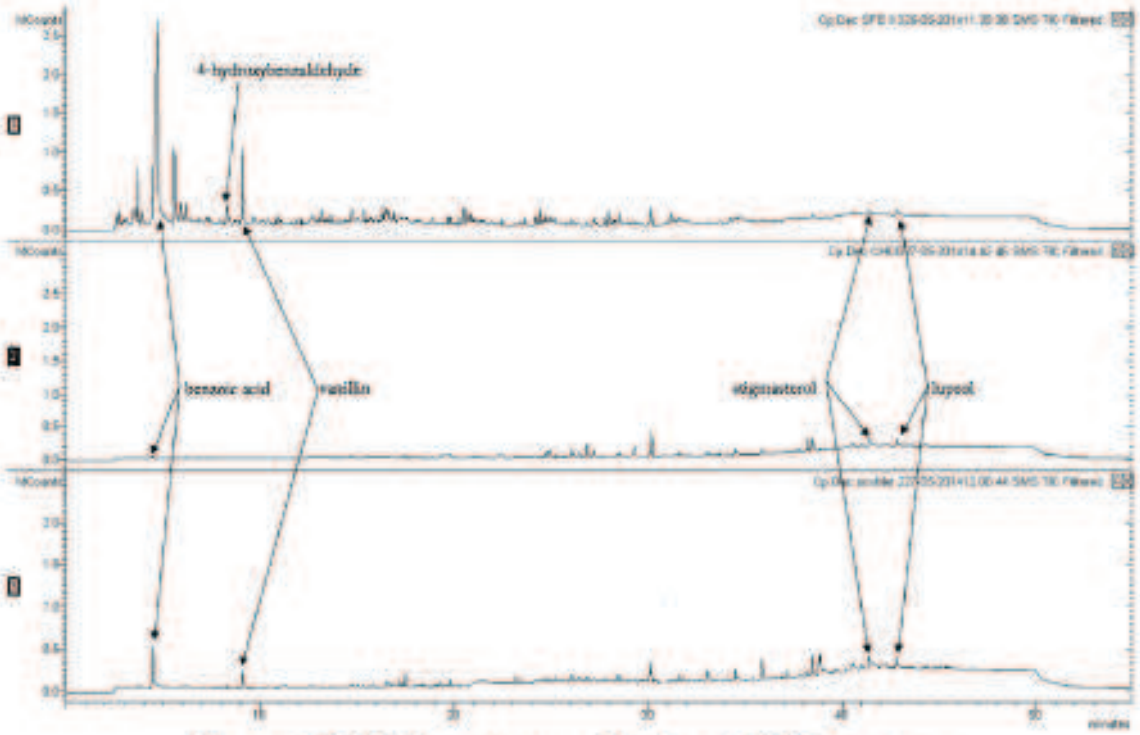


Figure 18. GC-MS chromatograms of *C. pluricaulis* DEC extractions.

The GC-MS chromatograms highlighted the presence of different types of compounds from benzoic acid, 4-hydroxybenzaldehyde and vanillin, to stigmasterol (phytosterol) and lupeol (triterpenoid), but other molecules are in the identification process.

The first experimental evidences related to the supercritical CO_2 extractions indicate that higher temperatures (80 °C) affect the extraction enriching the phytocomplex of phytosterols (data also confirmed by literature: Zhao & Zhang, 2013) and other different molecules (e.g. lupeol), while at lower temperatures (60 °C) the phytocomplexes is enriched of molecules with simple structure like vanillin or its isomers.

At a later stage, hydro-alcoholic extract (HE) was considered and, as expected, it showed a different phytochemical profile from DEC. The RP-HPLC-DAD analysis (Fig. 19) aimed to the recognition of flavonoids and phenolic acids led to the identification of the same compounds already seen in DEC.

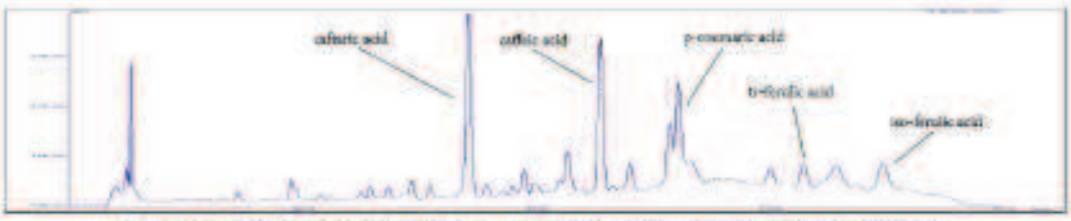


Figure 19. RP-HPLC-DAD chemical profiles of *C. pluricaulis* HE at 310nm.

The soxhlet apparatus extractive strategy used for the characterization of this phytocomplex highlighted the absence of phytosterols, shifting the focus to lower retention times (form 10 to 20 min) in which benzoic acid, eugenol, vanillin and a cinnamic acid derivative were detected (Fig. 20). Further investigation to identify compounds detected between 30 and 35 min are still in progress.

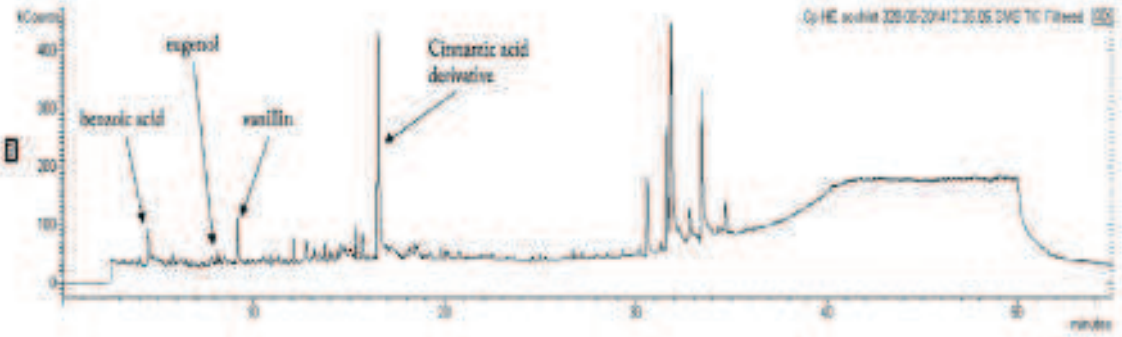


Figure 20. GC-MS chromatograms of *C. pluricaulis* HE soxhlet extraction.

C. pluricaulis preparations were the last crude drug to be characterized, therefore GC-MS analyses of HE extractions with $CHCl_3$ and supercritical CO_2 are still in progress, but the lack of phytosterols resulted evident.

As it can be seen from the comparison of Fig. 17 and 19, the RP-HPLC-DAD analysis showed a preponderance of phenolic acids in DEC and HE. Further evidences from GC-MS showed the absence of the phytosterolic component in the latter phytocomplex instead present in DEC. In both preparations is evident the presence of vanillin.

Identification and quantification of the phenolic acids in DEC and HE by gas chromatography (Tab. 21) was performed following a full validated method (Caligiani et al., 2013) in collaboration with the University of Parma (Italy).

Compound (mg/kg of preparation)	Decoction (DEC)	Hydro-alcoholic Extract HE)
2-furancarboxylic acid	56.6±2.8	1.1±0.04
benzoic acid	572.6±27.3	122.0±5.9
valeric acid	0.6±0.04	0.9±0.05
benzeneacetic acid	46.2±2.1	46.1±2.5
succinic acid	80.2±3.7	61.1±3.1
fumaric acid	21.1±1.1	67.2±3.5

salicylate	444.5±20.3	1298.7±72.3
4-hydroxybenzaldehyde	78.6±3.6	136.4±5.8
resorcinol	16.1±0.7	11.1±0.6
3-hydroxy butanoic acid	168.2±8.6	530.7±24.3
5-methyl resorcinol	4.8±0.1	n.d.
eugenol	4.1±0.3	6.6±0.4
salicylic acid	1.9±0.1	64.1±3.2
vanillin	101.5±4.9	284.9±14.25
3-hydroxybenzoic acid	107.7±6.3	73.4±3.8
tirosol	23.8±1.1	53.1±2.4
4-hydroxybenzoic acid	226.3±11.3	756.5±38.7
4-hydroxybenzeneacetic acid	13.7±0.5	40.1±1.9
3.5-dimethoxy-4-hydroxybenzaldehyde	27.1±1.2	46.8±2.2
vanillic acid	162.2±7.7	693.1±35.6
azelaic acid	157.5±8.9	649.3±32.5
3.4-dihydroxybenzoic acid	130.5±6.4	400.3±19.2
p-cumaric acid	578.7±27.3	1113.8±54.6
isoferulic acid	394.8±18.7	617.9±29.5
tr-ferulic acid	111.1±4.8	139.1±5.4
caffeic acid	276.2±12.1	103.5±5.2
sinapinic acid	11.6±0.4	11.9±0.4

Table 21. Quantification of phenolic acids in *C. pluricaulis* DEC and HE by gas chromatography (n.d. = not detectable).

Data shown in Tab. 21, confirm the identification carried out with HPLC and indicated the presence of further phenolic acid.

As already mentioned, the whole plant crude drug (WP), considered in parallel to the two preparations, was taken into account for this plant. The preliminary results of the different polarity extractions are reported in Fig. 21.

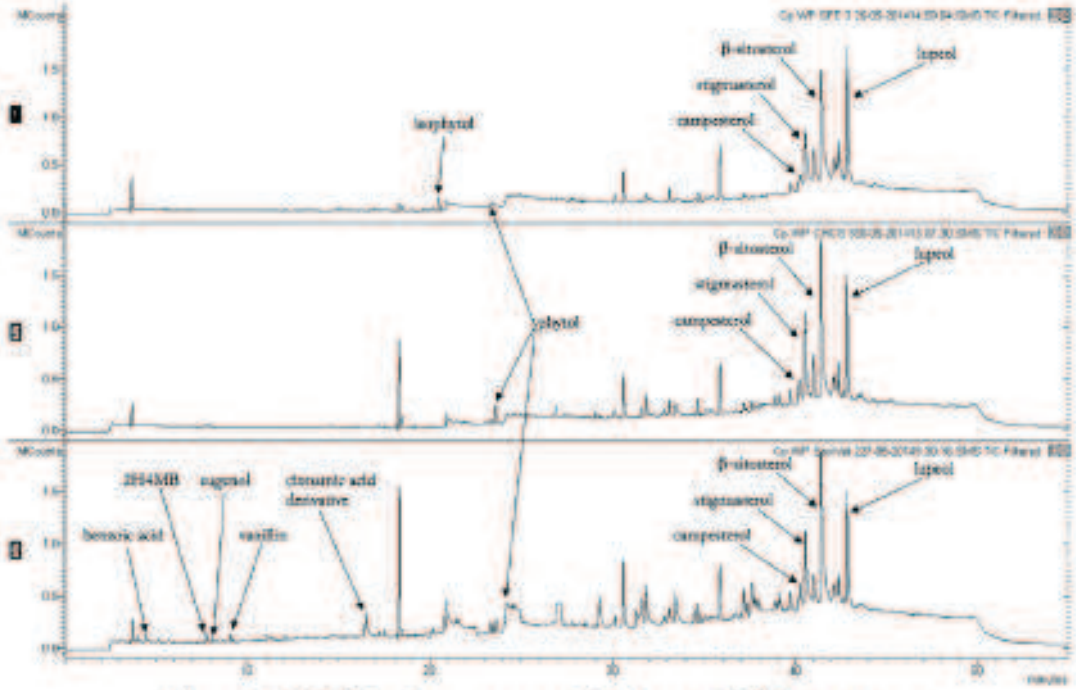


Figure 21. GC-MS chromatograms of *C. pluricaulis* WP extractions.

The chromatograms of the botanicals derived from extractions at different polarity of the whole plant crude drug, instead of on a traditional preparation, exhibited the highest molecular variety. In particular, the main constituents of these phytocomplexes were phytosterols (campesterol, stigmasterol and β-sitosterol) and terpenoids (e.g. lupeol). Among them, two acyclic diterpene alcohol precursors of vitamin E, phytol and isophytol (identified just in the supercritical CO_2 extract), not present in the preparations considered so far (DEC and HE). These molecules are already known in the pharmaceutical field thanks to their antiinflammatory and antirheumatic activity (Sudha et al., 2013). Furthermore, another derivative of Vit. E is still in identification phase.

Most of the molecules reported by this thesis are are newly identified in this drug (e.g. phenolic acids, vanillin, stigmasterol and campesterol, lupeol and vit. E precursors). Literature reports a general reference to phytosterols and flavonoids (Sethiya et al., 2009). The presence of other molecules reported in literature (alkaloids) has not yet been verified.

Biological activities

Antioxidant activity

The evaluation of the antioxidant activity of DEC and HE through spectrophotometric methods showed the higher sensitivity of ABTS test compared to DPPH assay and β-carotene bleaching test (BCBT) towards the considered botanicals (Tab. 22).

IC50 (mg/ml)	ABTS	DPPH	BCBT
DEC	0.170 ± 0.010	0.380 ± 0.020	0.165 ± 0.002
HE	0.050 ± 0.002	n.d.	0.259 ± 0.002
Trolox	0.005 ± 0.0002	0.016 ± 0.001	0.035 ± 0.001

Table 22. Antioxidant capacity of *C. pluricaulis* preparation (n.d. = not detectable).

DEC showed a comparable activity in the ABTS assay and BCBT, and a lower antioxidant capacity in the DPPH test (44.74 %). Regarding the ABTS assay, the highest activity was showed by HE (+70.59 % higher than DEC), but its IC_{50} value differs by a factor 10 from those of the positive control.

The opposite trend was shown in BCBT, where DEC showed a better antioxidant capacity compared to HE (+36.29 %). The bioactivity of DEC might be linked to the presence of phenolic acids (caftatic, caffeic, p-coumaric, tr-ferulic and iso-ferulic acid), molecules whose antioxidant activity is already well-known in literature (Apostolou et al., 2013; Kelebek et al., 2013; Fernandez-Pachon et al., 2006; Wang et al., 2010; Yi et al., 2011). HPTLC-bioautographic assays performed with ABTS and DPPH (Fig. 22), confirming the results of the spectrophotometric analyses, allowed to the identification of some molecules/fractions involved in the radical scavenging activity. These compounds activity was evident in the $ABTS^{\bullet+}$ test at rf 0.25, 0.3 and 0.75 for DEC; and rf 0.3, 0.44, 0.69 and 0.75 for HE, but the recognition process in still in progress.

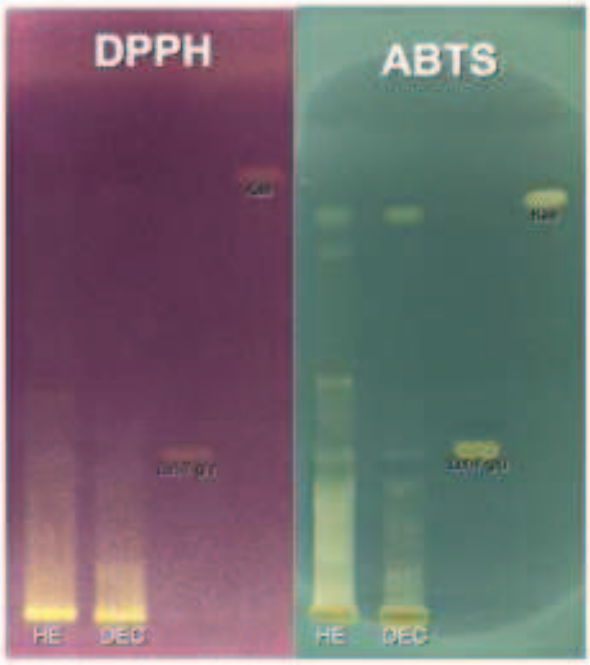


Figure 22. (HP)TLC-Bioautographic assay of *C. pluricaulis* preparations DEC and HE, with kaempferol (kae) and luteolin-7-O-glucoside (lut-7-glu).

Antimicrobial activity

The evaluation of the antimicrobial capacity of *C. pluricaulis* preparations was performed on four phytopathogens (*A. tumefaciensi, A. vitis, C. michiganensis, P. syringae*) and 5 human pathogens (*S. aureus, L. grayi, E. coli, P. aeruginosa* and *C. albicans*).

The tests performed on human pathogens did not showed any antimicrobial activity, in contrast to those carried out on the plant pathogens, in which DEC showed to be active against *P. syringae* and *C. michiganensis*, with the possibility of calculating the respective IC_{50} values (Tab. 23)

	IC_{50} (mg/ml)
Pseudomonas syringae	18.37
Clavibacter michiganensis	2.93

Table 23. IC_{50} values of *C. pluricaulis* DEC antimicrobial activity.

Genotoxic and antigenotoxic activity

Cytotoxic and genotoxic/antigenotoxic properties of the whole preparations have been assessed by *Saccharomyces cerevisiae* D7 strain test and SOS-Chromotest. The first test confirmed the safety of the phytocomplexes but the substantial ineffectiveness as antigenotoxic agent against EMS (data not shown). DEC and HE were then tested with SOS-Chromotest in a concentration range of progressive dilutions starting from 1:2 up to 1:32 (v/v) to assess cytotoxicity and genotoxicity. The tests did not highlight any cytotoxic or genotoxic potential at any concentrations tested, confirming the safety of the formulations. The evaluation of the antigenotoxic capacity (Tab. 24) was carried out against a 2.5 µg/ml solution of 4-nitroquinoline N-oxide (4NQO). HE did not show any noteworthy activity, while DEC, even if it was not active enough to reach an IC_{50} value, inhibited the bacterial SOS system activation of the 30.10±1.92 %, exhibiting a weak antigenotoxic potential without a dose-effect correlation.

Preparations		
	Dose (µg/ml)	Inhibition of genotoxicity (%)
DEC	6.97	30.10
	13.94	27.23
	27.88	27.23
	55.75	18.83
	111.50	18.83
	223.00	16.54
HE	2.32	5.36
	4.63	6.25
	9.26	7.59
	18.53	7.14
	37.05	8.48
	74.10	4.91
	148.20	1.34

Table 24. Effect of *C. pluricaulis* preparations on genotoxicity induced by 4NQO (2.5 µg/mL) in the SOS-Chromotest.

Antiproliferative activity

C. pluricaulis formulations were tested against A549, CaCo-2 (Fig. 23), MCF7, LoVo and HepG2 cell lines. Cells were exposed for 24 hours to different concentrations of DEC and HE flanked by negative controls: 10 % FBS medium for DEC and 0.1 % DMSO medium for HE. Both considered formulation did not show any activity against the first four cell lines at the highest concentration tested. Considering HepG2 cell line,

DEC, in contrast with HE, exhibited a weak inhibition of proliferation, making possible to calculate its IC_{50} value (111.50±0.12 µg/ml). In light of this experimental growth inhibition data, and comparing them with IC_{50} values that plant extract should exhibit to be considered active by the American National Cancer Institute (<30 µg/ml), it could be concluded that the data obtained are not significant.

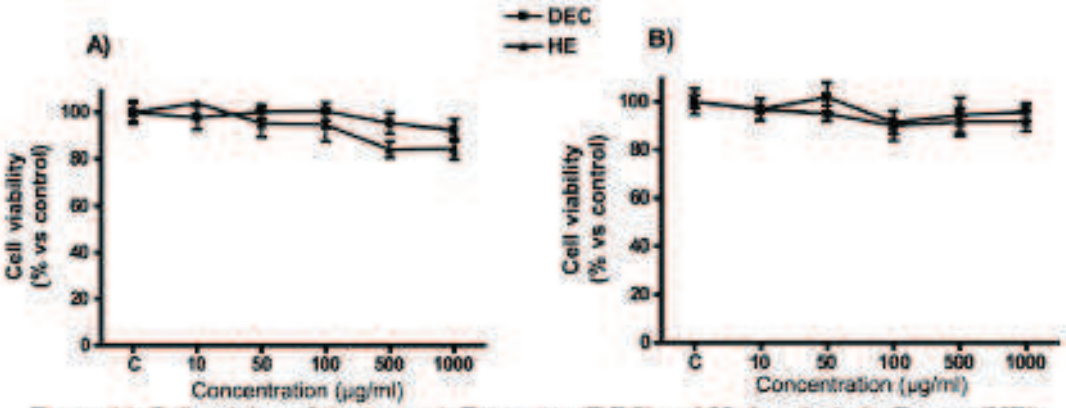


Figure 23. Cell viability of *C. pluricaulis* Decoction (DEC) and Hydro-alcoholic Extract (HE) against A549 (A) and CaCo-2 (B) cell lines after 24 h. Data were presented as the mean ± SD (n=3).

To proceed the investigation of *C. pluricaulis* cytotoxic activity, DEC, HE, and some of their extracts obtained with different techniques (maceration in $CHCl_3$, soxhlet apparatus and SFE), were tested against two leukaemia cell lines, drug-sensitive (CCRF-CEM), and multi-drug-resistant (CEM/ADR5000). The aim of this further investigation was to understand which compound or fraction was most responsible for this bioactivity and to highlight eventual cross-resistance phenomenon. These analyses were performed in Doctor Professor T. Efferth facilities at the Institute of Pharmacy and Biochemistry of the University of Mainz (Germany), thanks to the international mobility grant for young unstructured researchers of the University of Ferrara supported by 5 x 1000 funds of the year 2011.

A screening of 25 samples (15 phytocomplexes and 10 pure compounds) was preliminarily performed to a single concentration (100 µg/mL for phytocomplexes and 100 µM for molecules) on the sole CCRF-CEM cell line. These tests showed at least 50 % inhibition of cell proliferation for 11 samples: 8 extracts and 3 pure molecules in them identified. In relation to these results, every sample was tested against both cell lines (CCRF-CEM and CEM-ADR5000) in a series of 10 dilutions. The phytocomplexes and the pure molecules showed a dose-dependent reduction of CCRF-CEM cell line viability

with IC_{50} values higher than doxorubicin (Tab. 25).

	CCRF-CEM IC_{50}	CEM/ADR5000 IC_{50}
Preparations and extracts (μg/ml)		
DEC	190.88±2.90	>1000
DEC SFE	78.07±3.77	>1000
DEC $CHCl_3$ extract	16.18±0.79	36.90±1.91
DEC soxhlet extract (apolar)	63.93±2.33	76.62±0.40
HE	592.78±27.90	>1000
HE SFE	71.19±2.97	>1000
HE CHCl3 extract	39.74±2.05	>1000
HE soxhlet extract (apolar)	20.19±1.18	68.92±0.17
Pure compounds (μM)		
caffeic acid	56.69±1.96	51.58±6.99
kaempferol	27.48±0.69	29.31±1.45
doxorubicin	0.20 ± 0.06	195.12 ± 14.30

Table 25. Cell viability of *C. pluricaulis* DEC, HE, various polarity extracts and pure compounds against CCRF-CEM and CEM/ADR5000 cell lines after 72 h. Data were presented as the mean ± SD (n=3)

The phytocomplexes obtained by DEC maceration in $CHCl_3$ and the apolar fraction of the HE soxhlet extraction showed IC_{50} values (respectively 16.18±0.79 μg/mL and 20.19±1.18 μg/mL) in line with the value stated by the American National Cancer Institute (Suffness & Pezzuto, 1991). They also reached the 50 % cytotoxicity in the test performed against CEM-ADR5000 with IC_{50} values lower than doxorubicin but respectively about 2 and 3 fold bigger than experimental data obtained in the tests against CCRF-CEM, showing cross-resistance phenomena.

Among ten tested pure compounds just two (caffeic acid and kaempferol) revealed to be active against CCRF-CEM and CEM/ADR5000 cell lines. Kaempferol and caffeic acid exhibited higher IC_{50} values compared to those of the American National Cancer Institute for both considered cell lines, but their respectively values against CCRF-CEM and CEM/ADR5000 were similar, indicating the absence of cross-resistance phenomena.

CURCULIGO ORCHIOIDES

Chemical characterisation

Decoction (DEC) and hydro-alcoholic maceration (HE) procedures were performed following the indications of the Ayurvedic Pharmacopoeia of India and the European Pharmacopoeia (European Pharmacopoeia Ed. 7.0, 2011). Traditional Ayurvedic decoctions presented a total extraction yield of 8.44±2.51%, while the hydro-alcoholic extraction yields was 17.75±0.08%. The chemical characterization of DEC and HE of *C. orchioides* by ^{1}H-NMR showed a fingerprinting profile characterized mainly by a predominant carbohydrate fraction (Fig. 24).

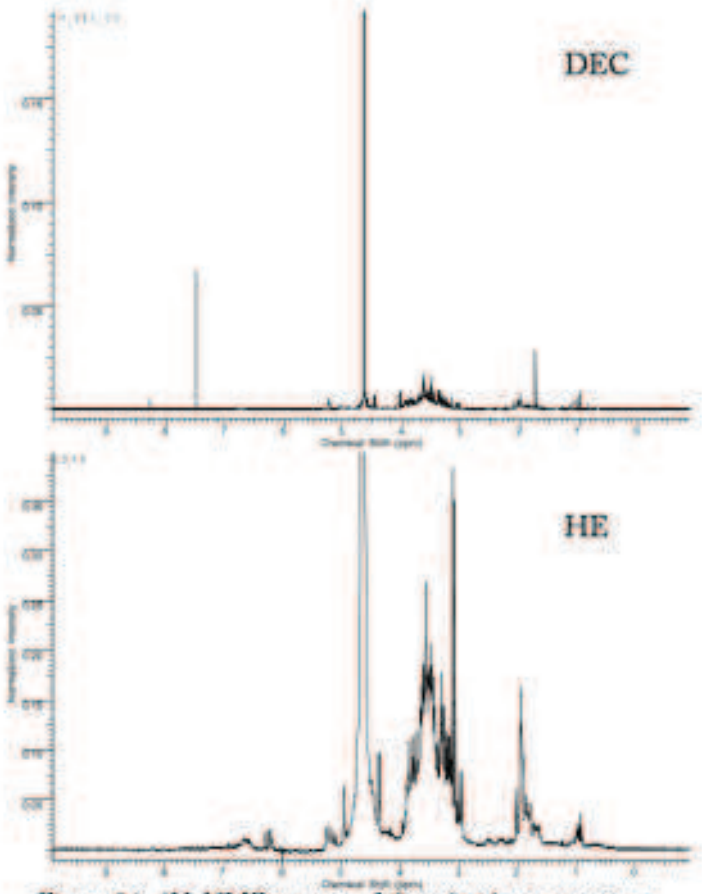


Figure 24. ^{1}H-NMR spectra of *C. orchioides* preparations.

The quantitative spectrophotometric analyses of the phenolic fractions using Folin-Ciocalteu reagent revealed an higher content of polyphenols (63,89 %) for HE than that of DEC (Tab. 26).

	DEC	HE
Polyphenols (mg GAE/g)	6.28 ± 0.31	17.39 ± 2.08
Flavonoids (mg HE/g)	5.41 ± 0.37	1.35 ± 0.04
Proanthocyanidins (mg CCIE/g)	0.56 ± 0.03	< 0.3

Table 26. Yield of total phenolic quantification of *C. orchioides* preparations.

Regarding the phenolic content of DEC, very few reports are available and they rely on preparation protocols different from the Ayurvedic one, which makes data hardly comparable (Hua-Bin et al., 2007). A similar scenario concerns hydro-alcoholic macerations. In particular, the amount of starting plant material, the ratio of plant material to solvent, the ratio of water to alcohol mixture and the extraction time are considerably different from the official Eur. Ph. protocol here adopted. Such variability, moreover, highlights a drawback in experimental design that must be noticed, as may lead to scattered and unreliable results in the literature both from the phytochemical and the bioactivity standpoint. In particular, *C. orchioides* hydro-alcoholic extract showed a value 65.40 % lower than experimental data about HE (Hua-Bin et al., 2007; Gacche & Dhole, 2006).

Subsequently, through chromatographic techniques (RP-HPLC-DAD and GC-MS-FID), started the qualitative and quantitative evaluation of the chemical profile of the two preparations. The RP-HPLC-DAD characterization of the phenolic fraction of *C. orchioides* preparations (Fig. 25-26), performed with fully validated methods, highlighted the presence of curculigoside A and orcinol-β-D-glucoside as main compounds in both DEC and HE (Tab. 27), and both molecules resulted to be more abundant in HE than in DEC: +82.29% for curculigoside A and +68.57% for orcinol-β-D-glucoside. Furthermore, orcinol-β-D-glucoside is more abundant than curculigoside A in both preparation, with a relative abundance of +90.14 % in DEC and +82.49 % in HE.

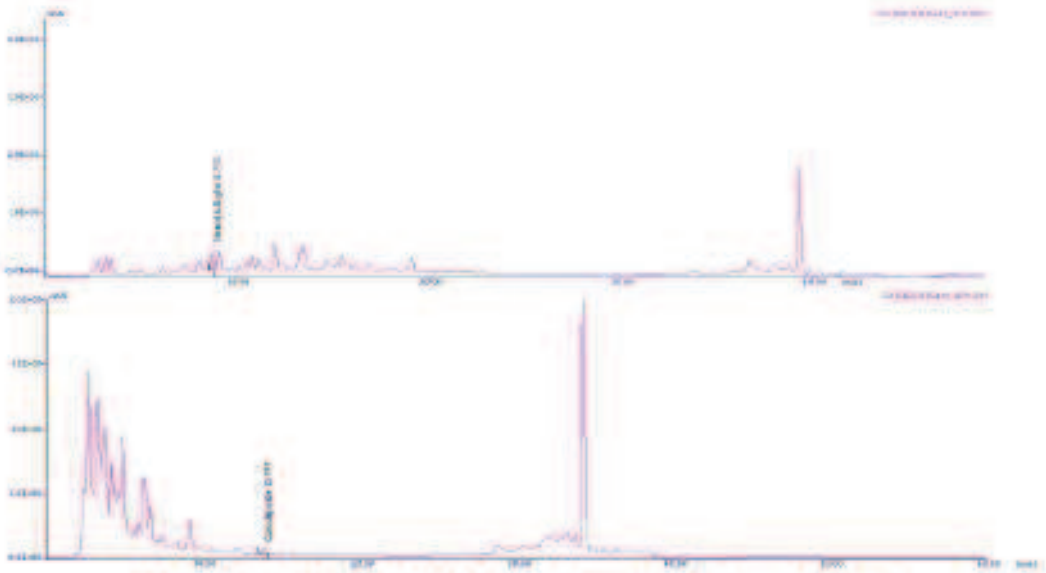


Figure 25. RP-HPLC-DAD chromatograms of *C. orchioides* DEC.

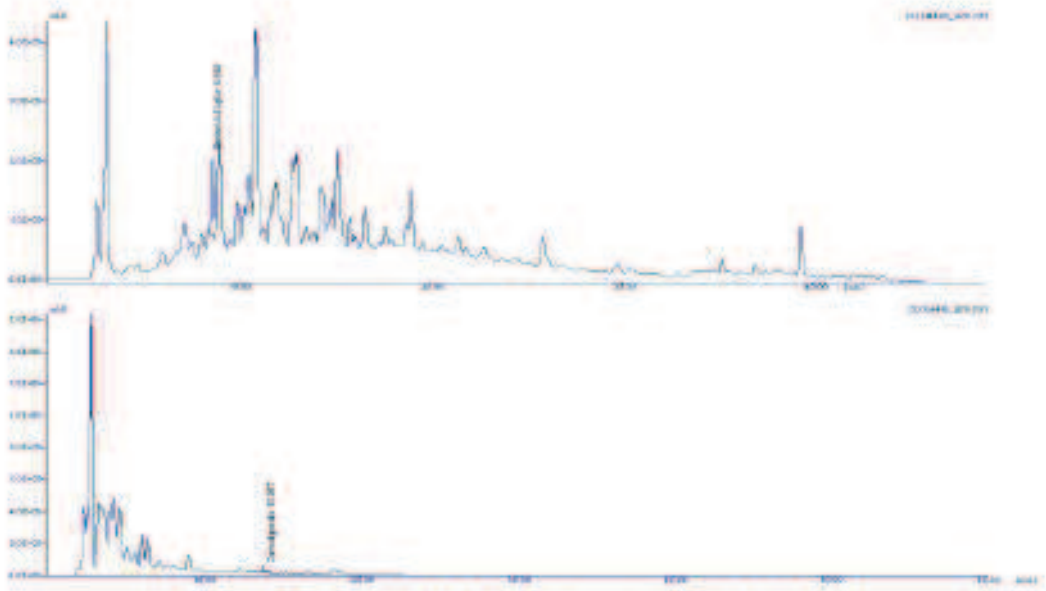


Figure 26. RP-HPLC-DAD chromatograms of *C. orchioides* HE.

	DEC (µg/ml)	HE (µg/ml)
orcinol-β-D-glucoside	72.93 ± 0.43	232.04 ± 8.49
curculigoside A	7.19 ±1.43	40.62 ± 6.57

Table 27. Pure compounds quantification in *C. orchioides* preparations.

Three different extraction techniques were performed in parallel to facilitate the chemical characterization of the compounds with apolar character present in phytocomplexes: extraction in chloroform with ultrasounds, in soxhlet apparatus and with supercritical CO_2. GC-MS spectra of DEC $CHCl_3$ extraction (Fig. 27), compared to the NIST library or to the pure compound spectra, and after the Kovats Index calculation, allowed the identification of: 4-hydroxybenzaldehyde, orcinol, vanillin, stigmastan-3,5-diene, β-sitosterol, stigmasterol.

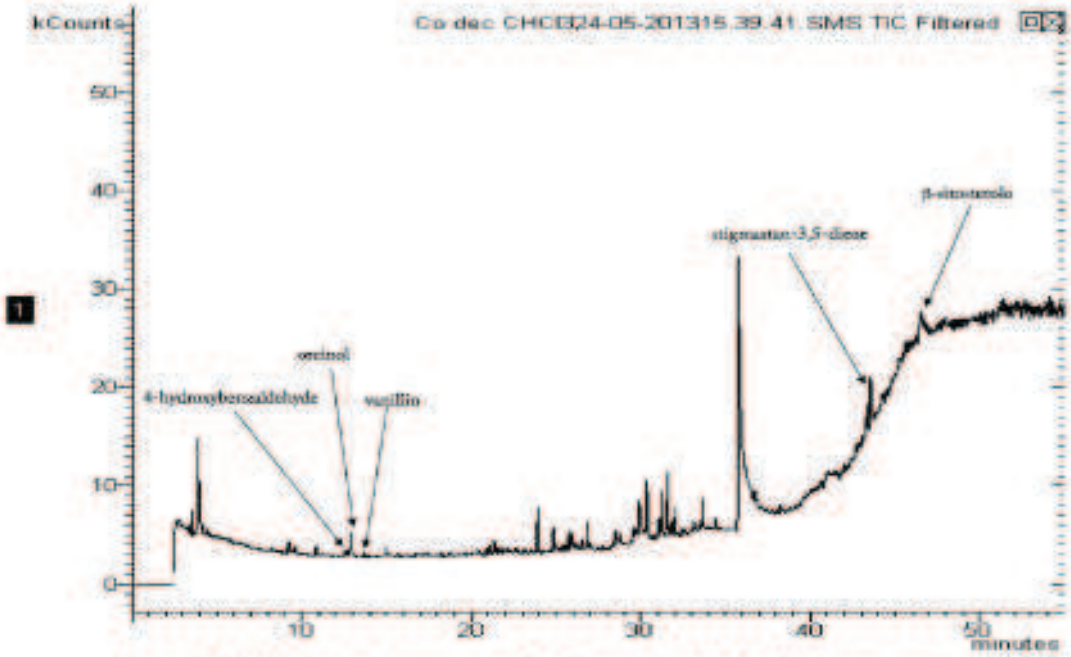


Figure 27. GC-MS chromatograms of *C. orchioides* DEC $CHCl_3$ extraction.

Similarly the phytocomplex obtained by supercritical CO_2 extraction (Fig. 28) showed the presence of: 4-hydroxybenzaldehyde, methylcinnamate, vanillin, stigmastan-3,5-diene, β-sitosterol and stigmasterol.

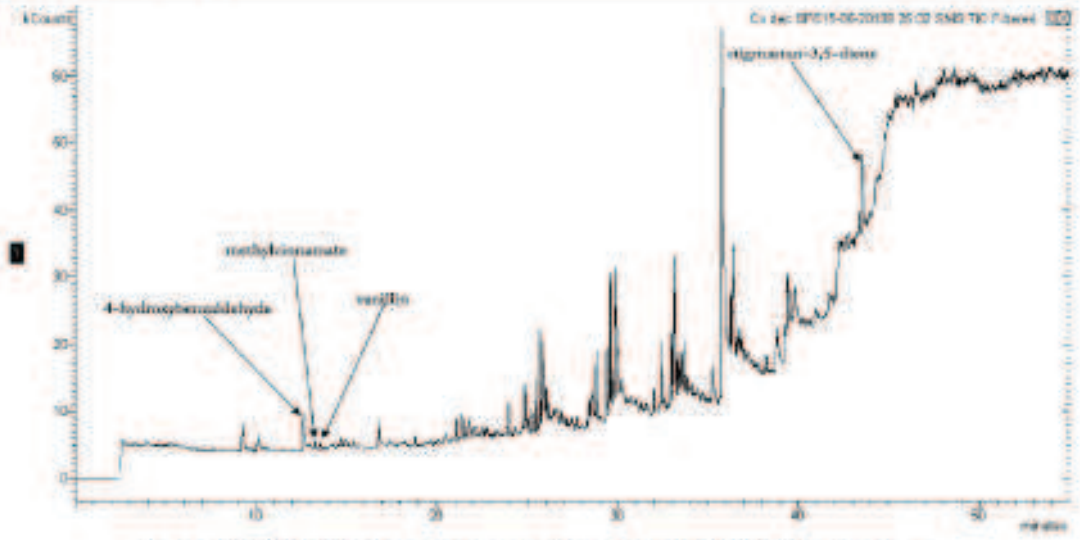


Figure 28. GC-MS chromatograms of *C. orchioides* DEC SFE extraction.

Some differences are notable between the chromatographic profiles of the two phytocomplexes obtained with different methods. In particular, the supercritical fluid extraction resulted richer in peaks than the chromatogram of the previous extraction in the area between 25 and 40 min (still under process of identification), moreover this technique is more effective in the extraction of aldehydes.

Considering HE, the GC-MS analyses highlight the presence of further molecules compared to the Ayurvedic preparation (DEC). In the phytocomplex obtained after the $CHCl_3$ extraction, (Fig. 29) the following compounds were identified: eugenol, orcinol, methylcinnamate, cinnamylacetate, 4-hydroxybenzoic acid and stigmastan-3,5-diene;

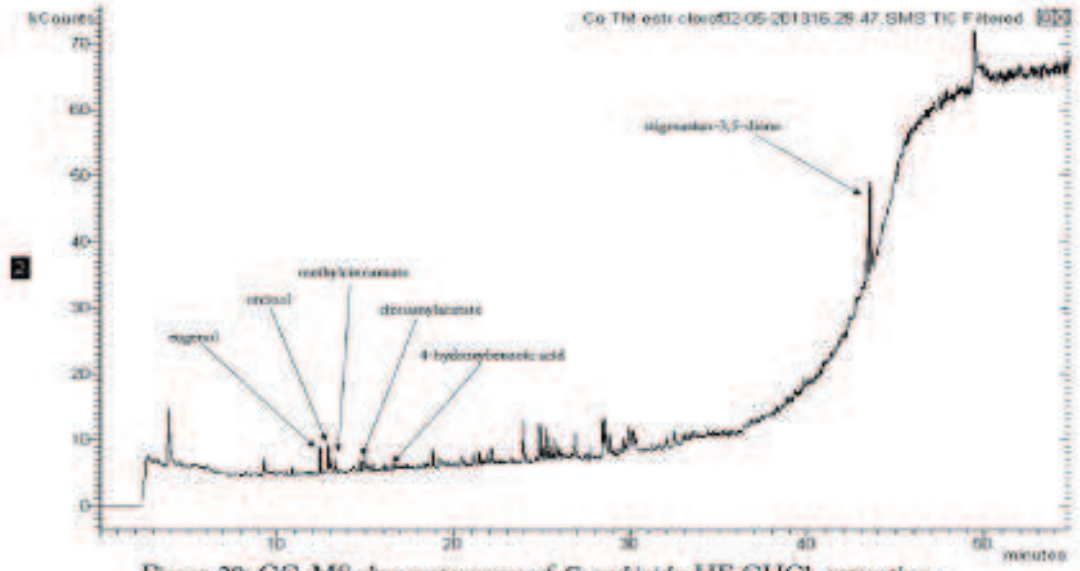


Figure 29. GC-MS chromatograms of *C. orchioides* HE $CHCl_3$ extraction.

The CO_2 supercritical extract of HE (Fig. 30) exhibit the presence of: 4-hydroxybenzaldehyde, orcinol, 4-hydroxybenzoic acid, etil p-hydroxycinnamate and stigmastan-3,5-diene.

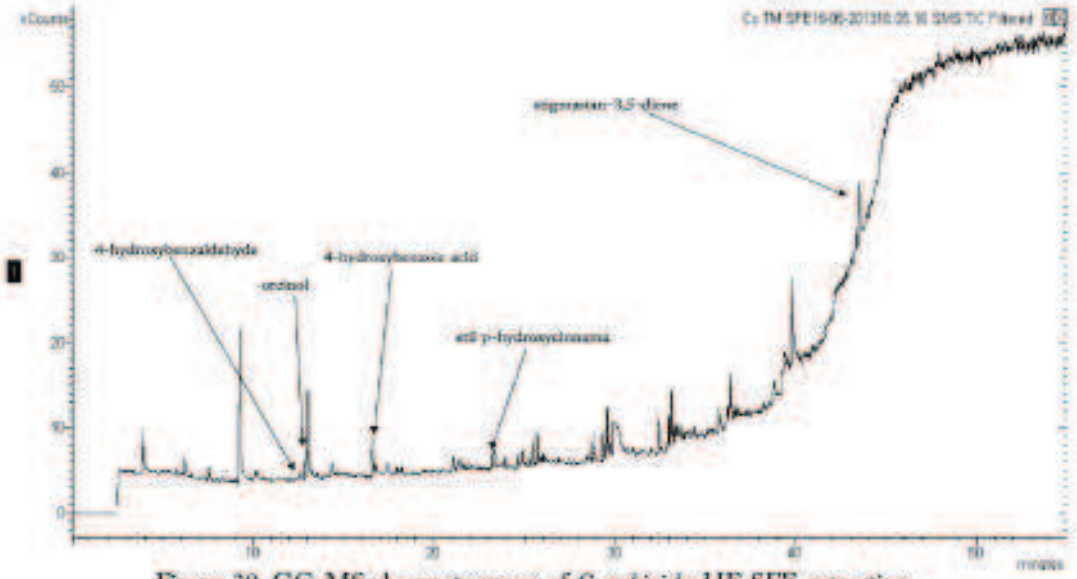


Figure 30. GC-MS chromatograms of *C. orchioides* HE SFE extraction.

In qualitative terms, comparing the chromatograms obtained, it can be pointed out the rich phytochemical profile provided by both extractive methods and the slightly different extraction specificity of the formulations strategies. In fact, while orcinol was identified in both phytocomplexes, the chloroform extract showed the presence of eugenol, methylcinnammate and cinnamylacetate not identified in the supercritical one; vice versa SFE extract was characterized by the presence of 4-hydroxybenzaldehyde and 4-ethyl-hydroxycinnamate missing in the $CHCl_3$ one.

Biological activities

Antioxidant activity

Curculigo orchioides hydro-alcoholic preparations was 91.47 % more active than decoction in the ABTS test and showed a wide range of antioxidant capacity, being active as radical scavenger and interfering at the same time with radical propagation (Tab. 29).

IC50 (µg/ml)	ABTS	DPPH	BCBT
DEC	46.895±1.643	217.354±0.310	n.d.
HE	4.435±0.090	35.777±0.624	226.627±10.154
curculigoside A	4.752±0.197	n.d.	n.d.
orcinol-β-d-glucoside	4.900±0.011	n.d.	n.d.
TROLOX	2.040±0.101	4.090±0.203	0.059±0.002

Table 29. Antioxidant capacity of *C. orchioides* preparation and pure compounds (n.d. = not detectable).

The Ayurvedic traditional preparation (DEC) exhibited a lower activity in the DPPH assay too when compared to HE (that showed IC_{50} value comparable to the trolox, positive control) and reached just the 45.75% of inhibition in BCBT at the highest concentration tested (11.25 mg/ml). Such behaviour reflects the higher phenolic content of *C. orchioides* preparations: HE with higher polyphenolic concentration showed the best antioxidant capacity. Considering the identified compounds, curculigoside A and orcinol-β-D-glucoside, they exhibited an IC_{50} values in the ABTS test approximately two-fold higher than positive control (57.07% for curculigoside A and 58.37% for orcinol-β-D-glucoside), while they did not reach the 50% inhibition at the highest concentration tested in the DPPH test and in the BCBT. Considering the concentration of the pure compounds in HE, and comparing their results in the ABTS test with those of the whole preparation, a possible contribution of curculigoside A and orcinol-β-D-glucoside in the antioxidant activity of HE could be suggested. On the other hand, DPPH tests and BCBT did not reveal the same trend, leaving possibilities for further investigations with other methods. (HP)TLC-bioautographic assay highlighted, for both *C. orchioides* preparations, a variety of molecules with antioxidant activity (Fig. 31). Most of these compounds are in the bottom part of the silica plate, the part of the TLC in which the most polar molecules could

be found, therefore it could hypothesize that antioxidant activity was united to this fraction, but the identification process is still in progress.

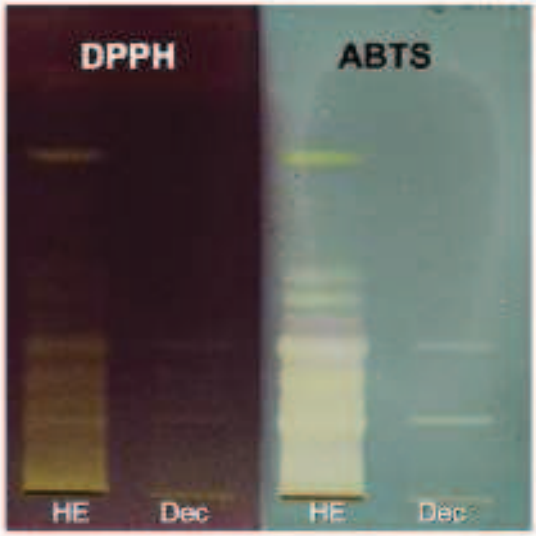


Figure 31. ABTS and DPPH HPTLC-bioautographic assay of *C. orchioides* preparations.

We must conclude that, at least for the evaluated phytocomplexes and notwithstanding what otherwise reported, an hydro-alcoholic maceration obtained following Eur. Ph. specifications outperforms the orthodox ayurvedic decoctions in terms of antioxidant performance and overall polyphenolic content (Li et al., 2007).

	Inhib % (DPPH test)
curculigoside A	4.99
orcinol-β-D-glucoside	1.08

Table 30. Inhibition percentage of curculigoside A and orcinol-β-D-glucoside in DPPH test.

	Inhib. % (BCBT)
C. orchioides DEC	45.75
curculigoside A	23.14
orcinol-β-D-glucoside	14.70

Table 31. Inhibition percentage of *C. orchioides* DEC, curculigoside A and orcinol-β-D-glucoside in BCBT.

Although the phytocomplexes are not directly comparable, Rathod et al. (2010) evaluated the antioxidant activity of a methanolic extract of *C. orchioides* roots using DPPH test, reporting an IC_{50} value of 29.97 mg/ml, that seem in line with the

experimental data obtained with the hydroalcoholic extract (35.78 mg/ml).
Regarding the pure compounds considered, the data obtained so far are innovative, because of the lack of scientific publications about it.

Antimicrobial activity

The results of the antimicrobial test performed with *C. orchioides* DEC and HE highlighted a lack of antibacterial activity on the Gram + (S. aureus) and Gram- (E. coli) strains considered. Further tests against other bacterial and fungi strains to better verify the antimicrobial activity on human and plant pathogens did not highlight any activity.

Genotoxic and antigenotoxic activity

Safety of preparations was investigated through two established tests using *Saccharomyces cerevisiae* D7 strain (D7 test) and *Escherichia coli* PQ37 strain (SOS-Chromotest). Both analyses give indication of the citotoxicity of the preparation/compound (survivor %, expressed as number of grown colonies on complete medium, in D7 test; percentage of activation of alkaline phosphatase in SOS-Chromotest), but they differ in the information about their genotoxic potential: D7 test evaluate genotoxicity and antigenotoxicity expressed as capacity of the phytocomplexes to generate, or protect from the generation of gene conversion and point mutation; while SOS-Chromotest evaluate the activation of the bacterial SOS system as measure of genetic damage.
D7 test experimental data showed that neither DEC nor HE exhibit cytotoxic activity at all concentrations tested, and that they did not have genotoxic activity because the values of gene conversion and point mutation remained within the physiological parameters (respectively 0.4-0.8 x 10^{-5} for gene conversion and 0.1-0.5 x 10^{-6} for the point mutation) in contrast to those obtained with positive control (EMS). The evaluation of the antigenotoxic activity aginst a known mutagen (EMS) did not give any appreciable results for both preparations. Both of them generated a sharp increase in the number of survivors in the plates with selective medium (lacking tryptophan for gene conversion and isoleucine for point mutation) treated with EMS, which shows that neither DEC nor HE counteract its mutagenic activity.
The SOS-Chromotest was performed in order to have further evidences of the lack of cytotoxicity and genotoxicity highlighted by the previous test. DEC and HE did not display any citotoxicity or DNA damage with reference to the parameters of the test.

Regarding the antigenotoxic activity, DEC and HE were tested as in a range of concentration following a scheme of progressive dilutions 1:2, up to 1:32. The molecules also identified in the preparations have been tested following the same dilution scheme to include in the concentrations considered to be those present in the preparations. The antigenotoxic potential was tested against a solution 2.5 μg/ml of 4-nitroquinoline N-oxide (4NQO), which resulted in an induction of SOS system equal to the double of the test performed with only 10% DMSO-saline. Preparations of *C. orchioides* showed a dose-response trend but weak antigenotoxic capacity that never reached the 50% inhibition threshold. It should be stressed that the Ayurvedic preparation (DEC) showed higher activity (16.23 ± 0.81%) compared to the hydro-alcoholic extract (11.32 ± 0.47%). Even the pure molecules identified, curculigoside A and orcinol-β-D-glucoside, exhibited a low genorptective potential, showing respectively an inhibition of 24.53 ± 0.86% and 29.81 ± 1.39%.

Antiproliferative activity

C. orchioides preparations were tested for a possible antiproliferative activity using some of the most common cancer cell lines: A549, CaCo2 (Fig. 32), MCF7, HepG2 and LoVo.

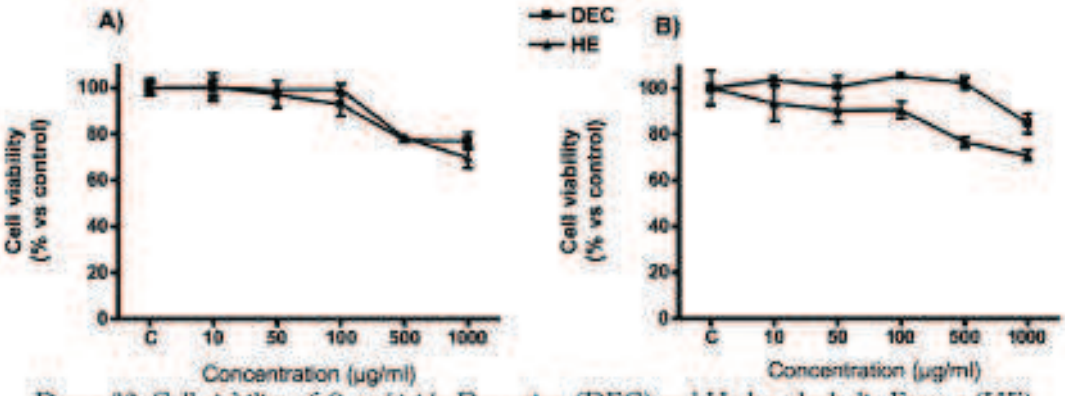


Figure 32. Cell viability of *C. orchioides* Decoction (DEC) and Hydro-alcoholic Extract (HE) against A549 (A) and CaCo-2 (B) cell lines after 24 h. Data were presented as the mean ± SD (n=3).

Among these considered cell lines, tests revealed a weak antiproliferative activity just against HepG2, where both preparations reached IC_{50} value (182.20±0.18 μg/ml for DEC and 200.90±0.23 μg/ml for HE). Considering the other cell lines, HE showed a slightly better potential than DEC without reaching the 50 % inhibition at the maximum concentration tested (1000 μg/ml). Further analyses performed against two leukaemia cell

lines, one drug-sensitive (CCRF-CEM) and one multi-drug-resistant (CEM/ADR5000) confirmed the trend showed by the previous analyses (Tab. 32): both preparations reached IC_{50} inhibition values against the considered cell lines, showing higher activity for HE compared to DEC, but their results were far from the values state by the American National Cancer Institute (Suffness & Pezzuto, 1991).

Plant extracts and pure molecules	CCRF-CEM cell line IC_{50} (µg/ml)			CEM/ADR5000 cell line IC_{50} (µg/ml)		
Curculigo orchioides – DEC	379.17	±	5.85	715.13	±	34.90
Curculigo orchioides – HE	243.57	±	4.21	290.96	±	2.31
curculigoside A	>30			>30		
orcinol-β-O-glucoside	>30			>30		

Table 32. Cytotoxic IC_{50} values of *C. orchioides* preparations, curculigoside A and orcinol-β-d-glucoside against CCRF-CEM and CEM/ADR5000 cell lines. Data were presented as the mean ± SD (n=3).

In particular, *C. orchioides* HE exhibited an IC_{50} of 290.96±2.31 µg/mL against CEM/ADR5000, that its higher than the 113.17±8.29 µg/mL IC_{50} value of doxorubicin (compounds towards which CEM/ADR5000 cell line developed resistance) reported by Kuete et al. (2014).

HEMIDESMUS INDICUS

Chemical characterisation

H. indicus is a cross-functional plant well known and used in the Ayurvedic traditional medicine system. As stated in the general introduction, the aim of the research is contributing to the phytotherapic and normative validation of plant, identifying the most characteristic molecules among the compounds detected in the considered phytocomplexes. An in-depth phytochemical characterisation, including spectrophotometric, GC-MS, RP-HPLC-DAD and NMR analyses, was carried out to reach this target.

As for the other crude drugs, decoction (DEC) and hydro-alcoholic extract (HE) were formulated following the Ayurvedic indication and the those of the European Pharmacopoeia, starting from respectively 10 g and 50 g of dried crude drug. The procedures gave the following yields: 2.56±0.39 g (25.55 %) for DEC and 8.53±0.03 g (17.06 %) for HE. The phytochemical screening started from the evaluation of the total polyphenol content of the considered preparations, performed through spectrophotometric analyses using the Folin-Ciocalteu reagent (Tab. 33).

	DEC	HE
Polyphenols (mg GAE/g)	11.6 ± 0.60	12.34 ± 0.48
Flavonoids (mg HE/g)	2.19 ± 0.20	0.89 ± 0.046
Proanthocyanidins (mg CCIE/g)	0.62 ± 0.04	0.37 ± 0.018

Table 33. Yield of total phenolic quantification of *H. indicus* preparations.

The assay highlighted a slightly higher polyphenols content of HE than DEC, but an opposite trend regarding flavonoids and proanthocyanidins. In particular, HE has a total polyphenol content greater than 6% compared to DEC, but the latter exceeds HE of 59.36% the content of flavonoids, and of 40.38% the content of proanthocyanidins.

The chemical characterisation by RP-HPLC-DAD (Fig. 33) and ^{1}H-NMR (Fig. 34) showed a preponderant sugar composition, in which glucose and fructose were detected.

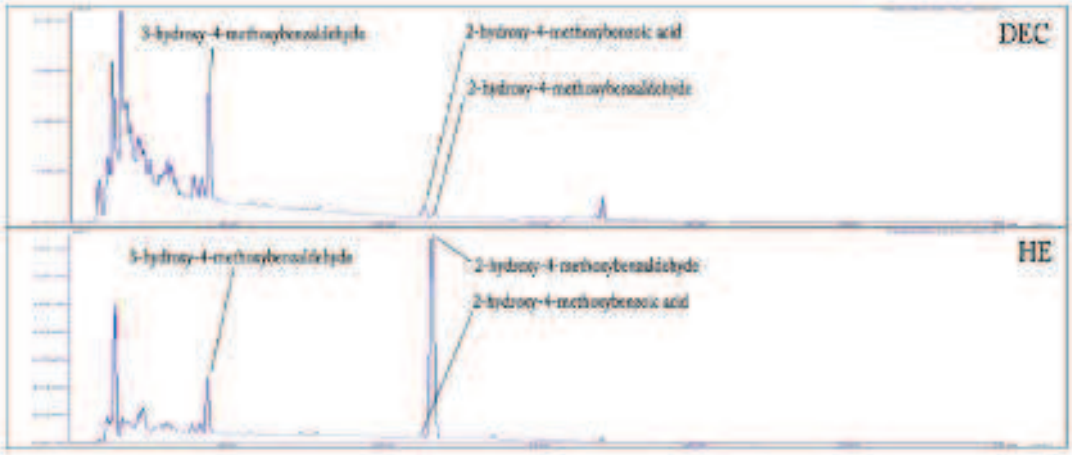


Figure 33. RP-HPLC-DAD chromatograms of *H. indicus* preparations.

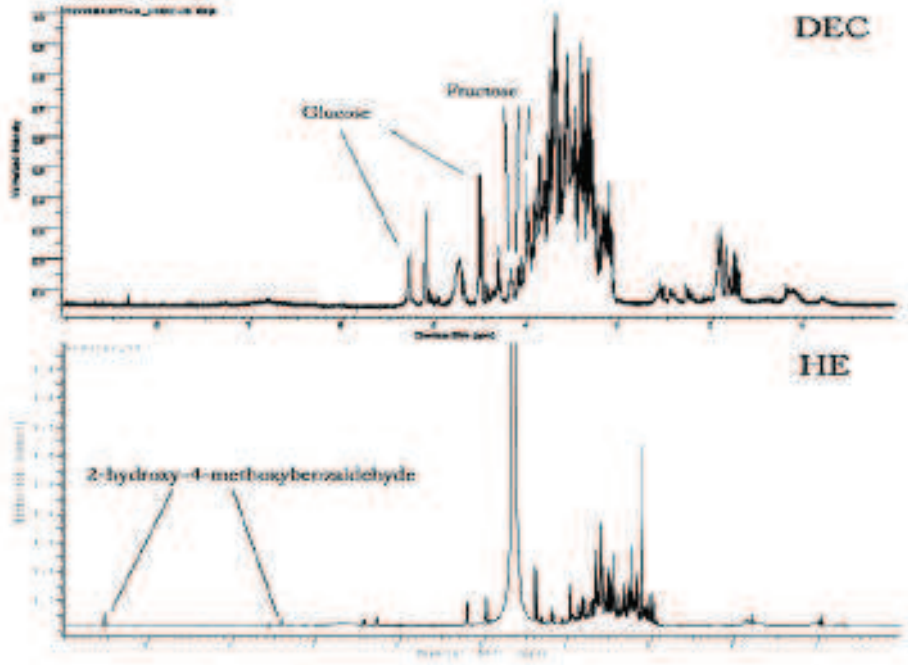


Figure 34. ^{1}H-NMR spectra of *H. indicus* preparations.

The preliminarily RP-HPLC-DAD analyses of both DEC and HE showed a relatively simple chromatograms in which the main secondary metabolites identified and quantified (Tab. 34) were: 2-hydroxy-4-methoxybenzaldehyde (2H4MB), 3-hydroxy-4-methoxybenzaldehyde (3H4MB) and 2-hydroxy-4-methoxybenzoic acid (2H4MBAc).

	DEC (µg/ml)	HE (µg/ml)
2-hydroxy-4-methoxybenzoic acid	23.54±0.23	32.51±1.23
3-hydroxy-4-methoxybenzaldehyde	26.85±0.92	57.10±1.39
2-hydroxy-4-methoxybenzaldehyde	1.72±0.09	214.54±5.17

Table 34. Quantitation of 2-hydroxy-4-methoxybenzoic acid, 3-hydroxy-4-methoxybenzaldehyde and 2-hydroxy-4-methoxybenzaldehyde in *H. indicus* formulations.

If compared with the total polyphenols quantification experimental data (Tab. 33), the amount of this phytomarkers represented about 10 % of the whole polyphenols content, suggesting that other molecules, such as hemidesmins (Das et al., 1992) and derivatives of vanillin isomers (Zhao et al., 2014) were still to identify. In effect, the chromatograms showed the presence of other compounds that posses the typical UV profile of phenolic acids. Moreover, solvents used for the formulations exhibit different specificity towards the two vanillin isomers (2H4MB is more abundant in HE than DEC, as confirmed by ^{1}H-NMR spectra, while 3H4MB showed an opposite trend) giving evidences that the polyphenol composition of the two traditional formulations could differ under the qualitative and quantitative point of view. Literature already reports the presence of vanillin isomer, 2H4MBAc and other molecules in these phytocomplexes, but no information about the quantification are mentioned.

A separation performed using a "gravimetric" column filled with an Amberlite XAD-2 resin (Sigma-Aldrich, Italy) allowed to obtain two complementary phytocomplexes and highlighted the presence of a molecule not identified before: chlorogenic acid (Fig. 35).

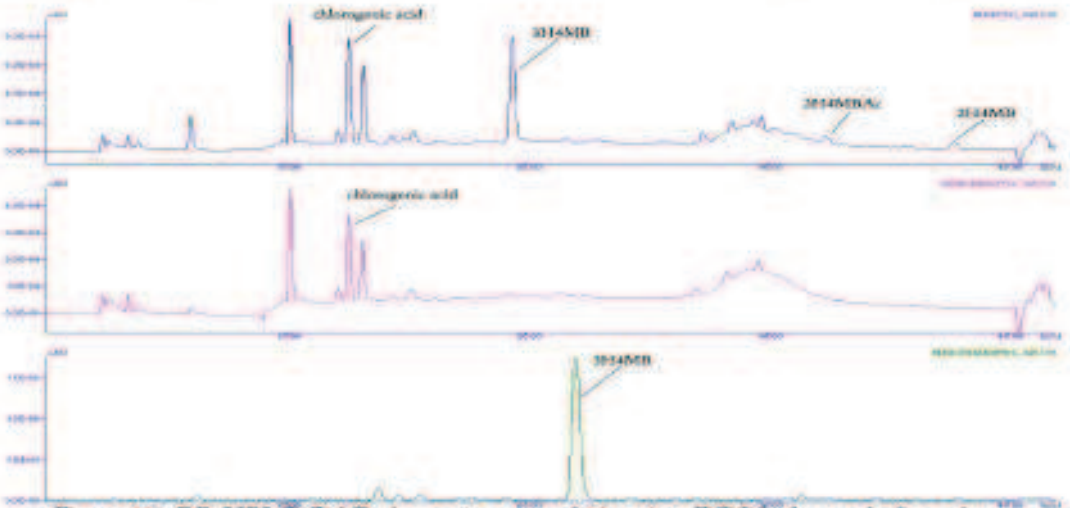


Figure 35. RP-HPLC-DAD chromatograms of *H. indicus* DEC before and after column separation.

The most apolar fraction of DEC after column separation was also investigated by GC-MS (Fig. 36), highlighting a strong sugar component and the presence of lupeol, β-amyrin acetate and lupeol acetate.

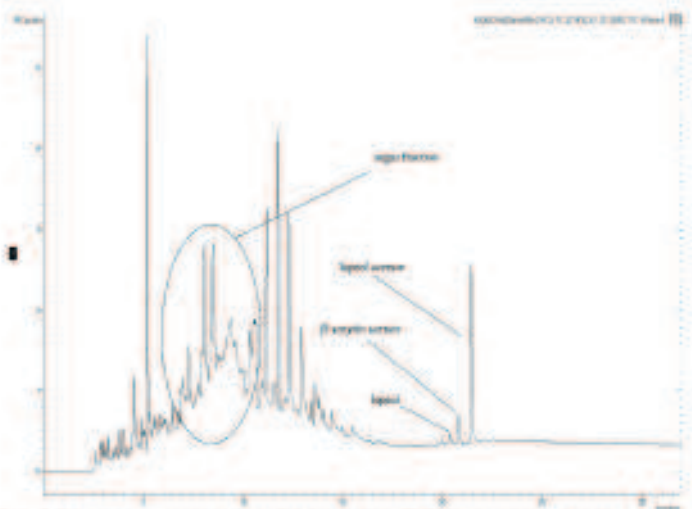


Figure 36. GC-MS chromatograms of *H. indicus* DEC methanolic fraction after column separation.

At this stage, to better analyse the lipophilic fractions of the phytocomplexes, different polarity extractions (soxhlet apparatus, $CHCl_3$ in ultrasounds and SFE) were performed. GC-FID-MS analyses confirmed the presence of already known molecules (Chatterje et al., 2006; Padhy et al., 1973) and highlighted a different extraction specificity of the used techniques. In particular lupeol and β-sitosterol were major constituents of soxhlet and $CHCl_3$ extractions of DEC; while the acetylated compounds (lupeol acetate and β-amyrin acetate) were more abundant in the supercritical CO_2 extraction (Tab. 35).

	DEC $CHCl_3$ extraction (µg/ml)	DEC SFE extraction (µg/ml)	HE $CHCl_3$ extraction (µg/ml)	HE SFE extraction (µg/ml)
lupeol	1.470±0.064	1.071±0.149	n.d.	n.d.
lupeol acetate	24.583±1.320	28.821±0.807	0.125±0.007	0.509±0.086
β-sitosterol	1.660±0.231	0.477±0.077	n.d.	n.d.
β-amyrin acetate	2.947±0.162	3.274±0.085	n.d.	n.d.

Table 35. Quantitation of lupeol, lupeol acetate, β-sitosterol and β-amyrin acetate in *H. indicus* formulations after $CHCl_3$ and supercritical CO_2 extracts (n.d. = not detectable).

Lupeol acetate, in particular, was the only molecule identified in the lipophilic fraction of HE. Soxhlet apparatus extractions of both DEC and HE (characterized by higher polarity solvents than $CHCl_3$ and supercritical CO_2) confirmed the presence of vanillin, its isomers, and allowed the detection of other vanillin derivatives in HE never reported in literature before: vanillic acid, acetovanillone, syringaldehyde and vanillin acetate, in order of retention time.

Biological activity

Antioxidant activity

The antioxidant activity was investigated useing three different assays: ABTS, DPPH and β-carotene bleaching test (BCBT). Results (Tab. 36) showed a different degree of response among the considered assays but they are concordant to highlight the greater antioxidant capacity of HE compared to DEC.

IC_{50} (μg/ml)	ABTS	DPPH	BCBT
DEC	29.41±1.47	82.26±4.11	949.354±0.001
HE	9.44±0.47	69.44±3.47	265.570±0.001
2-hydroxy-4-methoxybenzoic acid	23.03±0.81	n.d.	n.d.
2-hydroxy-4-methoxybenzaldehyde	8.17±0.41	n.d.	n.d.
3-hydroxy-4-methoxybenzaldehyde	1.02±0.05	2356.55±115.83	n.d.
Trolox	2.40±0.12	4.94±0.25	0.035±0.001

Table 36. Antioxidant capacity of *H. indicus* preparation and pure compounds (n.d. = not detectable).

In general ABTS performes better than other test to evaluate the antioxidant activity because of its capacity to be soluble in both aqueous and organic solvents; and because it has less problems related to its structural conformation unlike DPPH. ABTS showed the best IC_{50} value for the vanillin isomer 3-hydroxy-4-methoxybenzaldehyde (1.025±0.055 μg/ml), even lower than Trolox (IC_{50}=2.40±0.12 μg/ml), the vitamin E analogue used as positive control. The activity of 2-hydroxy-4-methoxybenzaldehyde and 2-hydroxy-4-methoxybenzoic were slightly higher than, respectively, HE and DEC activity. These substances may be responsible for, or contribute to, the activity of the two traditional preparations; and the lower activity of DEC compared to HE could be due to their lower concentrations in the Ayurvedic preparation. Considering DPPH test, DEC and HE showed IC_{50} values higher than positive control, confirming the better activity of hydro-alcoholic extract than decoction. Among the pure molecules considered just 3-hydroxy-4-methoxybenzaldehyde and chlorogenic acid showed a radical scavenging activity against DPPH, while for 2-hydroxy-4-methoxybenzaldehyde and 2-hydroxy-4-methoxybenzoic acid was not possible to obtain an IC_{50} value. The β-carotene bleaching test did

not exhibit any interesting results reporting evidences of antioxidant activity just for the two preparations with IC_{50} values bigger than positive control. From the comparison of the three methods is evident the high selectivity of the molecules of DEC and HE for the ABTS assay, in which they obtained IC_{50} values lower than DPPH and BCBT. Literature reports the antioxidant activity (DPPH test) of *H. indicus* root methanolic extract with an EC_{50} value of 18.87 mg/ml (Ravishankara et al., 2002) but, currently, there are no data concerning the preparations DEC and HE.

In order to check and provide evidence of the presence of phytocomplex fractions more active than others, and to verify the contribution of the identified compounds in both preparations, DPPH and ABTS test was performed as HPTLC-bioautographic assay (Fig. 37).

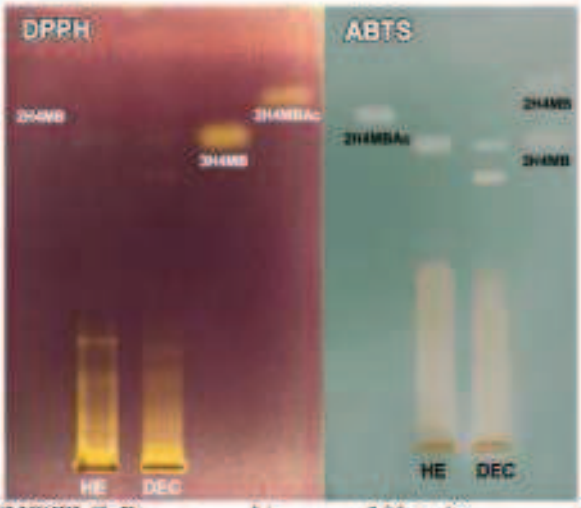


Figure 37. (HP)TLC-Bioautographic assay of *H. indicus* preparations and pure molecules(2H4MB: 2-hydroxy-4-methoxybenzaldehyde; 3H4MB: 3-hydroxy-4-methoxybenzaldehyde; 2H4MBAc: 2-hydroxy-4-methoxybenzoic acid).

The derivatised HPTLC confirmed the higher activity of 3H4MB compared to 2H4MB along with the IC_{50} value obtained by the spectrophotometric analyses, and highlight the presence of other fractions that contribute to the antioxidant activity.

Antimicrobial activity

The antimicrobial activity of DEC and HE was performed on human and phytopathogen bacteria (*E. faecalis, S. aureus, S. epidermidis, E. coli, K. oxytoca, P. aeruginosa*) and yeasts (*C. albicans*). The performed assays did not highlight any sensible activity towards the considered micro-organisms. Literature reports a noteworthy antimicrobial activity

against different microorganisms (Khanna & Kannabiran, 2008), but it consider the saponin fraction obtained with different extractive method.

Genotoxic and antigenotoxic activity

Safety of preparations was investigated through two established tests using *Saccharomyces cerevisiae* D7 strain (D7 test) and *Escherichia coli* PQ37 strain (SOS-Chromotest). Both analyses give indication of the eventual citotoxicity of the preparation/compound (survivor %, expressed as number of grown colonies on complete medium, in D7; % of activation of alkaline phosphatase in SOS-Chromotest), but they differ in the information about their genotoxic potential.

D7 test evaluate genotoxicity and antigenotoxicity expressed as capacity of the phytocomplexes to generate, or protect from the generation, of gene conversion and point mutation. The experimental data (Tab. 37) showed that neither DEC nor HE exhibit cytotoxic activity at all concentrations tested, and that they did not have genotoxic activity because the values of gene conversion and point mutation remained within the physiological parameters (respectively 0.4-0,8 x 10^{-5} for gene conversion and 0.1-0.5 x 10^{-5} for the point mutation) in contrast to those obtained with positive control (EMS). The evaluation of the antigenotoxic activity aginst a known mutagen (EMS) did not give any appreciable results for both preparations. For each of them there was a sharp increase in the number of survivors in the plates with selective medium (lacking tryptophan for gene conversion and isoleucine for point mutation) treated with EMS, which shows that neither DEC nor HE counteract its mutagenic activity.

Genotoxicity DEC

(mg/plate)	Survival%	Conversants x10^5 UFC	Reversants x10^5 UFC
H2O	100.00 ± 1.00	0.39 ± 0.35	0.52 ± 0.17
0.27	102.55 ± 1.55	0.64 ± 0.28	0.15 ± 0.08
0.55	108.92 ± 4.64	0.59 ± 0.39	0.16 ± 0.05
1.06	99.10 ± 2.80	0.66 ± 0.43	0.22 ± 0.06
2.13	104.82 ± 4.30	0.59 ± 0.48	0.22 ± 0.09
4.26	108.24 ± 3.46	0.58 ± 0.26	0.18 ± 0.19
8.52	108.86 ± 5.03	0.68 ± 0.34	0.14 ± 0.05
17.04	105.35 ± 2.82	0.83 ± 0.47	0.15 ± 0.05
34.07	104.30 ± 5.24	0.64 ± 0.26	0.14 ± 0.17
EMS 0.2 %	108.24 ± 6.58	12.79 ± 1.61	17.80 ± 10.10

Antigenotoxicity DEC

(mg/plate)	Survival%	Conversants x10^5 UFC	Reversants x10^5 UFC
H2O	100.00 ± 1.21	0.51 ± 0.04	0.21 ± 0.10
0.27	102.70 ± 2.34	0.54 ± 0.06	0.16 ± 2.60
0.55	99.40 ± 3.19	0.54 ± 0.06	0.14 ± 3.51
1.06	99.10 ± 4.30	0.77 ± 0.20	0.09 ± 0.04
2.13	98.80 ± 1.89	0.60 ± 0.51	0.20 ± 3.55
4.26	104.19 ± 1.04	0.59 ± 0.26	0.25 ± 2.17
8.52	100.02 ± 2.91	0.49 ± 0.50	0.51 ± 3.46
17.04	97.53 ± 2.91	0.48 ± 1.01	0.09 ± 5.40
34.07	99.10 ± 1.04	0.46 ± 0.62	0.09 ± 3.26
EMS 0.2 %	102.40 ± 1.45	0.58 ± 0.46	0.13 ± 2.91

Genotoxicity HE

(mg/plate)	Survival%	Conversants x10^5 UFC	Reversants x10^5 UFC
EtOH	100.00 ± 1.41	0.49 ± 0.08	0.13 ± 0.10
0.10	96.80 ± 5.60	0.69 ± 0.19	0.16 ± 0.13
0.20	100.97 ± 4.17	0.62 ± 0.11	0.14 ± 0.02
0.39	99.10 ± 2.80	0.67 ± 0.08	0.12 ± 0.02
0.78	97.53 ± 5.22	0.59 ± 0.18	0.29 ± 0.15
1.57	106.80 ± 2.89	0.78 ± 0.22	0.18 ± 0.09
3.14	108.40 ± 0.73	0.72 ± 0.19	0.28 ± 0.13
6.27	102.27 ± 2.04	0.76 ± 0.16	0.22 ± 0.12
12.54	97.89 ± 5.38	0.67 ± 0.38	0.23 ± 0.16
EMS 0.2 %	96.79 ± 1.09	13.55 ± 2.09	72.30 ± 3.92

Antigenotoxicity HE

(mg/plate)	Survival%	Conversants x10^5 UFC	Reversants x10^5 UFC
EtOH	100.00 ± 2.37	0.46 ± 0.21	0.21 ± 0.12
0.10	99.38 ± 1.60	13.22 ± 0.94	67.61 ± 3.19
0.20	98.48 ± 2.32	12.46 ± 0.88	67.00 ± 4.13
0.39	98.10 ± 1.15	12.66 ± 1.41	70.00 ± 2.16
0.78	98.08 ± 0.90	13.60 ± 1.03	68.00 ± 2.55
1.57	99.08 ± 1.60	12.80 ± 1.33	68.00 ± 4.60
3.14	100.91 ± 1.62	12.75 ± 1.39	66.10 ± 3.86
6.27	98.78 ± 0.93	13.15 ± 1.16	68.02 ± 2.52
12.54	98.48 ± 0.76	13.10 ± 0.79	67.99 ± 3.44
EMS 0.2 %	100.61 ± 1.09	13.12 ± 0.67	67.06 ± 3.53

Table 37. D7 test: Genotoxic and antigenotoxic activity of *H. indicus* DEC and HE

In order to obtain further evidences of cytotoxicity, genotoxicity and antigenotoxicity of the considered traditional preparations and their most characterising molecules, SOS-chromotest was performed. These assays confirmed the phytocomplexes safety, but it did not show any substantial antigenotoxic capacity of DEC or HE toward the known mutagen 4NQO (Fig. 38); while the data relating tested pure molecules, showed cytotoxicity for the 2-hydroxy-4-methoxybenzaldehyde and the 2-hydroxy-4-methoxybenzoic acid at concentration 4 mg/ml and above.

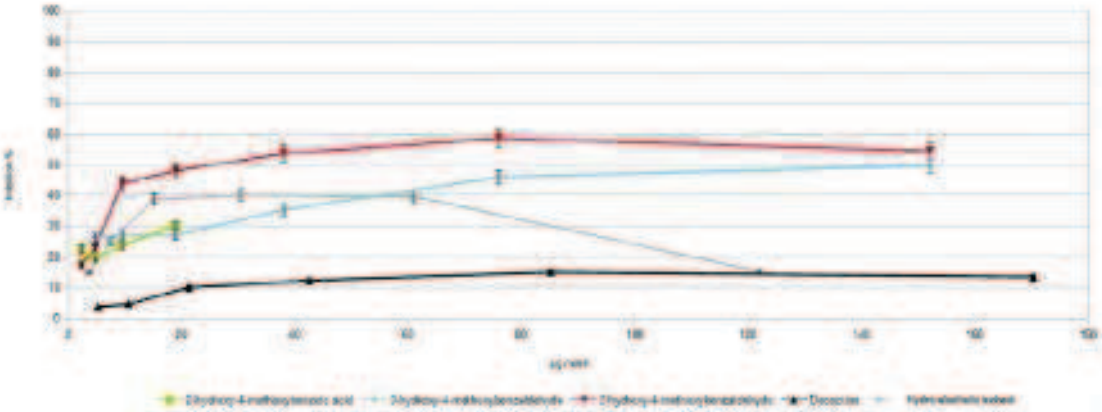


Figure 38. Antigenotoxic activity of H. indicus preparations and major compounds tested with SOS-Chromotest. Data were presented as the mean ± SD (n=3).

The 3-hydroxy-4-methoxybenzaldehyde showed cytotoxicity as well, but lower than previous molecules, because it exhibit cytotoxic at concentration 25 mg/ml and above . Every pure compounds showed a better antigenotoxic activity then DEC and HE, in particular the 2-hydroxy-4-methoxybenzaldehyde exhibit the highest activity decreasing the activation of the bacterial SOS system at the maximum concentration not cytotoxic of the 50.98±2.73 %. After the 2HMB, the rank of antigenotoxic activity continued with the 3-hydroxy-4-methoxybenzaldehyde, with an inhibition of the 37.47±1.61 %, and at the last the 2-hydroxy-4-methoxybenzoic acid showing a genoprotective activity of the 32.60±1.81 %.

Antiproliferative activity

Traditional medical practitioners use *Hemidesmus indicus* to treat several pathologies and, often, poly-herbal preparations containing this plant are used for the treatment of cancer.

In light of this premises, the antiproliferative activity was investigate in the laboratories of the Professor Gianni Sacchetti, Pharmaceutical Biology Department of the University of

Ferrara (Italy), in the facilities of Doctor Professor Thomas Efferth, Institute of Pharmacy and Biochemistry of the University of Mainz (Germany), and of Professor Statti, Pharmacy, Health and Nutrition of the University of Calabria (Italy). The research project considered different cell lines, belonging to the most common type of cancer (CaCo2, A549, MCF7, CCRF-CEM, CEM-ADR5000, HepG2 and LoVo), in order to conduct an in-depth investigation about the antiproliferative capacity of the traditional formulation considered.

Both preparations were tested on every cell lines and their cytotoxicity was not noteworthy, being far from the concentration values, 30 μg/mL for extracts, and 4 μg/mL for pure compounds, stated by the American National Cancer Institute (Suffness & Pezzuto, 1991). DEC, HE and the pure compounds tested resulted inactive against MCF7 and A549 until the concentration of 100 μg/ml included, with the exception of 2-hydroxy-4-methoxybenzaldehyde that, at this concentration, reduced the MCF7 vitality of nearly 20 %.

The results obtained by exposing heterogeneous human epithelial colorectal adenocarcinoma cell (CaCo-2) at growing concentrations of HE showed just a slight reduction in cell viability when compared to the control culture, reduction not significant after 24 hours treatment (Fig. 39).

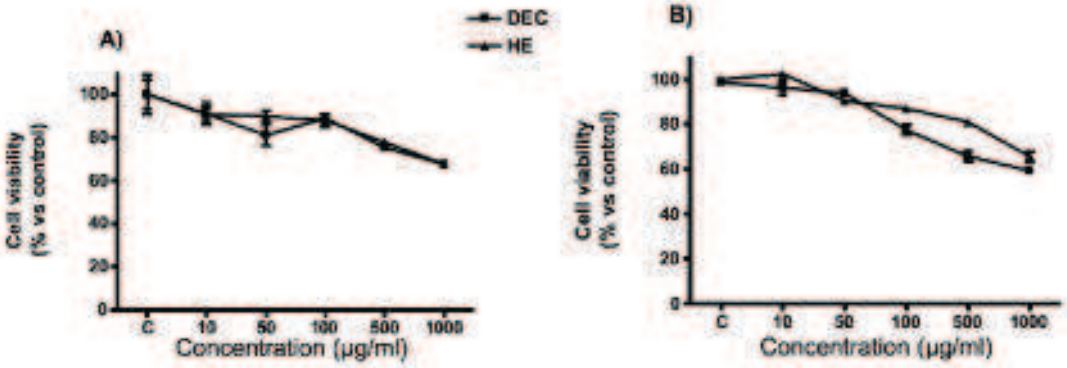


Figure 39. Cell viability of *H. indicus* Decoction (DEC) and Hydro-alcoholic Extract (HE) against A549 (A) and CaCo-2 (B) cell lines after 24 h. Data were presented as the mean ± SD (n=3).

The same cell line treated with growing concentrations of DEC induced a toxic effect with a dose-dependent trend. In particular, cell viability decreased in a statistically significant manner starting from the concentration of 100 μg/ml. As it can be seen from the graphs, the highest HE concentration tested (1000 μg/ml) modify the cell viability index with a different trend than DEC, but they both reach a 40% reduction of viability, demonstrating the comparable activity of the two formulations against this cell line.

Based on the obtained results, the effect of DEC and HE most characterising molecules (2H4MB, 2H4MBAc and 3H4MB for HE and the same molecules plus lupeol acetate for DEC) were tested individually and in combination, using their own concentrations founded in DEC 500 mg/ml (Fig. 40) and HE 100 mg/ml (Fig. 41). After 24 hours treatment, the tested standards did not change CaCo-2 cell viability in a statistical significant way compared to the negative control.

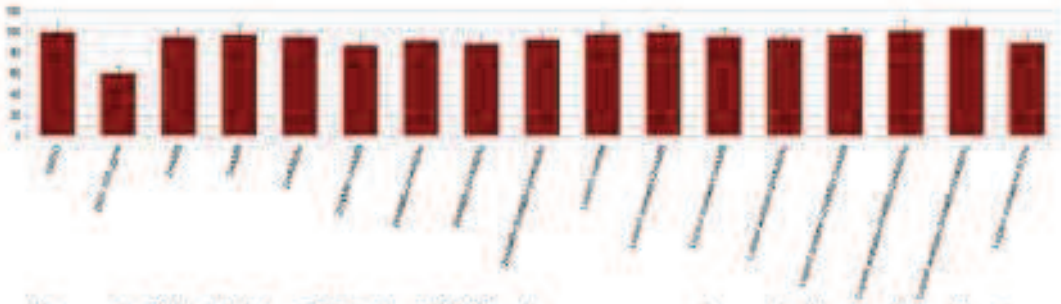


Figure 40. Cell viability of *H. indicus* DEC and pure compounds tested alone and combination against CaCo-2 cell lines after 24 h. Data were presented as the mean ± SD (n=3).

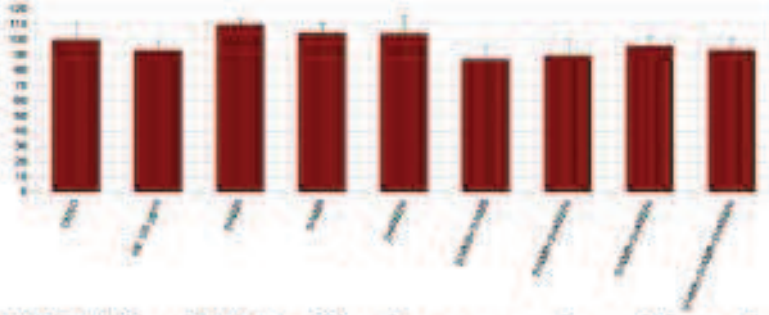


Figure 41. Cell viability of *H. indicus* HE and pure compounds tested alone and combination against CaCo-2 cell lines after 24 h. Data were presented as the mean ± SD (n=3).

In light of this result, it can be assumed that the ayurvedic traditional preparation DEC contains other molecules responsible for its weak antiproliferative effect, or that in the phytocomplex are present synergistic actions not easily ascribable to the considered mixtures.

The analyses of antiproliferative activity performed in the laboratories of Professor G. Statti at the University of Calabria considered HepG2 and LoVo cell lines. The assays showed similar IC_{50} values for both preparations against HepG2 (IC_{50} DEC= 33.52±0.13 µg/ml and IC_{50} HE= 34.50±0.14 µg/ml), but the trend differed agains LoVo were HE exhibited an IC_{50} value close to the one stated by the American National Cancer Institute (Suffness & Pezzuto, 1991) while DEC was inactive.

Preparations and pure compounds were tested on human leukaemia cell lines CCRF-CEM and multi-drug-resistant CEM/ADR5000 in the facilities of Professor T. Efferth at the University of Mainz. The activity of the considered samples against these cell lines were investigated to examine in depth the aspects of cytotoxicity towards leukaemia cell lines already identified in related works (Fimognari et al., 2011; Ferruzzi et al., 2013).

The screening of 13 samples at a single concentration (100 µg/mL for phytocomplexes and 100 µM for molecules) was performed only on the CCRF-CEM cell line. These tests showed interesting values of bioactivity for 6 samples: HE, methanolic fraction after column separation of DEC, methanolic fraction after column separation of HE, apolar fraction after soxhlet extraction of HE, 2H4MB and lupeol. In relation to these results, they were tested on CCRF-CEM and CEM/ADR5000 cell lines in a series of 10 dilutions, showing a dose-dependent trend. The four extracts (HE, methanolic fraction after column separation of DEC, apolar fraction after soxhlet extraction of HE and methanolic fraction after column separation of HE) and the pure compounds (2H4MB and lupeol) showed a reduction of viability on CCRF-CEM cells lower than doxorubicin (0.20 ± 0.06 µM, Kuete et al., 2013), with IC_{50} values reported in Tab. 38.

	IC_{50}	
Sample	CCRF-CEM	CEM/ADR5000
$DEC_{MeOH - XAD2}$	7.36±0.29 µg/mL	22.30±0.98 µg/mL
HE	46.23±1.12 µg/mL	84.85±3.34 µg/mL
$HE_{MeOH - XAD2}$	27.93±0.58 µg/mL	69.70±1.22 µg/mL
$HE_{soxhlet\ apol}$	2.46±0.28 µg/mL	5.76±0.01µg/mL
2H4MB	85.39±1.70 µM	n.d.
lupeol	9.62±0.21 µM	n.d.

Table 38. Cell viability of *H. indicus* HE, extractions and pure compounds against CCRF-CEM and CEM/ADR5000 cell lines after 72 h. Data were presented as the mean ± SD (n=3) (n.d. = not detectable).

The phytocomplexes were also active towards the multi-drug-resistant cell line, exhibiting IC_{50} values higher than respective data obtained against CCRF-CEM cells, but lower than those reported in the literature for doxorubicin (195.12 ± 14.30 μM; Kuete et al., 2013) towards CEM/ADR5000. Considering the data obtained it can be assumed that this cell line is more sensitive to the tested samples compared to doxorubicin to which is resistant, but nonetheless presents phenomena of "cross-resistance" indicated by the IC_{50} experimental values that were about the double compared to those obtained against CEM-CCRF. In general, HE was the only sample that showed antiproliferative activity against every cell line considered and its fractions, obtained first with column separation and after with soxhlet apparatus, exhibited an IC_{50} value in line with the one stated by the American National Cancer Institute (Suffness & Pezzuto, 1991).

CONCLUSIONS and PERSPECTIVES

The Italian and, in general, the EU phytotherapic realities are increasingly adopting herbal traditions belonging to distant and exotic cultures, like Ayurveda, but they implement their use considering cultural parameters unrelated to those traditions, placing them in a typical Hippocratic and allopathic context (Guerrini and Sacchetti, 2012). Following this premise, it is possible to understand that the use of Ayurvedic phytotherapic remedies in Western culture has became estranged from its purpose: entering in the "symptomatology" context it leaves behind the importance of the philosophical concept of "whole man" (combination of body, mind and soul), considering the traditional medications just in terms of their body healing properties and not like a substance able to return the natural human equilibrium. For this reason, one of the goals of scientific research should be, starting from the ethnomedicine knowledge, the investigation of the linear connection between phytocomplex and biological activity in perspective of the "occidental" use (dichotomy symptom/remedy) of the drugs/preparations.

The increased interest of patients (and consumers) in non-conventional medicine, as a way to be more active in their healing process, to decrease the costs and the side effects of the allopathic medications, is driving the regulatory organs to define a better legislation for their use and the alternative remedies trade. The phytochemical standardization of crude drug and botanicals, through the choice of suitable phytomarkers that could define their quality, is one of the crucial points to be able to validate their safety and effectiveness, to move towards a precise legal regulation.

This PhD project was set entirely on chemical and biological characterization of the drugs and preparations covered by the PRIN 2009LR9YLF project (founded by MIUR, Ministry of Instruction, University and Research), with the aim of standardizing the considered phytocomplexes. *Azadirachta indica, Boerhaavia diffusa, Convolvulus pluricaulis, Curculigo orchioides* and *Hemidesmus indicus* and their preparations (decoction, DEC, and hydro-alcoholic extract, HE) were phytochemically investigated with different chromatographic techniques (GC-FID, GC-MS, HPLC and HPTLC), and NMR to identify their main compounds. The study of biological activity was driven by the use of the plants in the millenary ethnomedical Ayurvedic culture, but also from aspects related to modernity as the prevention of the oxidative stress (antioxidant activity), the evaluation of their antigenotoxic potential, and the research for new "lead compounds" for the treatment of neoplastic diseases, keeping in mind the problem of the drug-resistance.

Main results:

- *Azadirachta indica*: identification and quantification of flavonoids, such as rutin, isoquercitrin, quercitrin, kaempferol-3-O-glucoside, kaempferol-3-O-rutinoside in DEC, and same molecules plus quercetin in HE was carried out by HPLC. Rutin quantification was also performed with HPTLC Visualizer and the data comparison with the HPLC results, highlight discrepancies that could be overcome by the method optimisation. Gas chromatographic analyses performed on two different polarity extractions (maceration in chloroform with ultrasound, and SFE) permitted the identification and the quantification of stigmasterol and β-sitosterol. The quantitative evaluation of the identified compounds, by HPLC or GC-FID, is a new feature in the *A. indica* panorama, adding new data to literature. In general, the hydro-alcoholic extract exhibit a higher antioxidant activity than DEC and every flavonoids showed IC_{50} value lower then positive control. D7 and SOS-Chromotest did not highlight any genotoxic activity, confirming the safety of the preparations but they did not showed any noteworthy antigenotoxic capacity neither. Quercetin was the only compound showing antigenotoxic activity in the SOS-Chromotest, but it did not reach the 50% inhibition. The study about the cytotoxicity against all human cancer cell line considered showed data far from those stated by the American National Cancer Institute (Suffness & Pezzuto, 1991), therefore not significant. A paper is in preparations describing the chemical characterisation data and an overview of the biologicals activity tested.
- *Boerhaavia diffusa*: ferulic acid and vanillin were identified and quantified in DEC and in HE, but HE showed the presence of other two characterising molecules: boeravinone B and eupalitin. β-sitosterol was identified and quantified in both preparations, but resulted more abundant in DEC. While literature repost evidences of the boeravinone B, eupalitin and β-sitosterol presence in this crude drug, ferulic acid and vanillin are newly identified, and reported in the publication Tacchini et al. (2015). The evaluation of the antioxidant activity highlight the radical scavenging capacity of ferulic acid against $ABTS^{\bullet+}$ higher than positive control, and the activity of eupalitin towards all the considered tests. Preparations and pure compounds did not show any noteworthy antigenotoxic activity towards the two known mutagen considered. The study of cytotoxicity using DEC and HE

against human cancer cell line considered showed data far from those stated by the American National Cancer Institute (Suffness & Pezzuto, 1991). Eupalitin inhibited the growth of the two leukaemia cell lines, CCRF-CEM (drug-sensitive) and CEM/ADR5000 (multi-drug-resistant), exhibiting not significant IC_{50} values and showing cross-resistance.

- *Convolvulus pluricaulis*: the Ayurvedic preparation DEC was characterised by the presence of phenolic acid: caftaric acid, caffeic acid, p-coumaric acid, iso-ferulic acid and tr-ferulic acid. Stigmasterol, β-sitosterol, lupeol and vanillin isomers and derivatives (2H4MB, 3H4MB and acetovanillone) were also detected in the same preparation. HE showed the presence of p-coumaric acid, vanillin, 2-hydroxy-4-methoxybenzaldehyde, acetovanillone, and vanillic acid but the absence of phytosterols. The antioxidant activity evaluation indicated DEC as the phytocomplex that exhibited activity in every performed test, while HE showed a higher activity than DEC in the ABTS test, no activity against DPPH radical and a lower capacity of blocking the radical propagation than DEC in the β-carotene bleaching test. Regarding the cytotoxic capacity of the considered preparations against A549, CaCo-2, MCF7, LoVo and HepG2 cancer cell lines, the growth inhibition experimental data, compared with the IC_{50} threshold stated by the American National Cancer Institute (<30 μg/ml), are not significant. The tests carried out with DEC and HE extracts against two leukaemia cell lines, drug-sensitive (CCRF-CEM) and multi-drug-resistant (CEM/ADR5000), showed, instead, interesting results. The DEC $CHCl_3$ extract and the HE soxhlet extract exhibit experimental data in line with the one stated by the American National Cancer Institute against the CCRF-CEM cell line, but respectively about 2 and 3 fold bigger in the tests against CEM/ADR5000, showing cross-resistance phenomena. These experimental results are innovative in the bioactivity panorama of *C. pluricaulis* and they could be the starting point to a new research extended to other extraction methods or human cancer cell lines, however, a paper to reporting this data is in preparation.
- *Curculigo orchioides*: Curculigoside A and orcinol-β-D-glucoside were identified and chosen as characterising compounds of the preparations, therefore quantified through HPLC methods. β-sitosterol was also identified and quantified using GC-FID. The hydro-alcoholic extract showed the highest antioxidant activity in every performed test. Regarding the antigenotoxic activity DEC resulted slightly

more active than HE, but the highest activity was showed by orcinol-β-D-glucoside, even if it did not reach the 50 % inhibition at the maximum concentration tested. The evaluation of the cytotoxic activity against human cancer cell line exhibited an opposite trend, seeing DEC more active then HE for A549, CaCo2, and HepG2 . The hydro-alcoholic extract was instead more active against CCRF-CEM and CEM/ADR5000, but the IC_{50} data were far from those stated by the American National Cancer Institute. The pure molecules did not exhibit any activity.

- *Hemidesmus indicus*: the two phytocomplexes were characterised by the main presence of vanillin isomer and derivatives: 2-hydroxy-4-methoxybenzaldehyde (2H4MB), 3-hydroxy-4-methoxybenzaldehyde (3H4MB) and 2-hydroxy-4-methoxybenzoic acid (2H4MBAc). These molecules were quantified and showed their higher concentrations in HE compared to DEC. The GC-MS analyses of different polarity extractions highlighted the presence of lupeol, lupeol acetate, β-sitosterol and β-amyrin acetate. After quantification, they resulted more concentrated in DEC than HE and a different grade of the extraction specificity of the used techniques was observed: lupeol and β-sitosterol were major constituents of soxhlet and $CHCl_3$ extractions; while the acetylated compounds (lupeol acetate and β-amyrin acetate) were more abundant in the supercritical CO_2 extraction. The two preparations showed radical scavenging activity and capacity of blocking the radical propagation. HE showed an higher antioxidant capacity than DEC and 3H4MB was the most active compound. The test of genotoxicity confirmed the safety of the phytocomplexes, but did not highlight any antigenotoxic activity against the considered mutagens. 2H4MB showed to reduce the activation of the SOS system in the SOS-Chromotest indicating an antigenotoxic activity, but without reaching the 50 % inhibition at the maximum concentration tested. Both preparations were tested for their cytotoxic activity against human cancer cell lines (CaCo2, A549, MCF7, CCRF-CEM, CEM-ADR5000, HepG2 and LoVo) but the data were not noteworthy, being far from the concentration values, 30 μg/mL for extracts, and 4 μg/mL for pure compounds, stated by the American National Cancer Institute. The phytocomplex obtained by separation on gravimetric column with of HE and the one obtained by HE soxhlet extraction showed a promising activity with IC_{50} values, respectively, of 27.93±0.58 μg/mL and 2.46±0.28 μg/mL against CCRF-CEM, and 69.70±1.22 μg/mL and

5.76±0.01μg/mL against CEM/ADR5000. In relation to previous published papers regarding the anti leukemic activity of *H. indicus* DEC (Fimognari et al., 2011; Ferruzzi et al., 2013), a paper reporting the experimental results obtained by this PhD project is going to point out the activity of HE towards the drug-sensitive and the drug-resistant leukaemia cell lines here considered.

In conclusion, this research has provided an initial response to the need for standardization of plant compounds derived from the five plants considered, by providing:

- a way to perform the chemical standardization, in terms of the analyses to perform and results to be obtained;
- a link between chemical characterization and biological activity, in some cases showing a non-linear connection, more linked to a phytocomplex rather than to a single molecule or to a mixture of compounds recreated in the laboratory by simulating the contents of the preparations;
- indications of biological activity with particular reference to the antioxidant activity (preventing oxidative stress damage, contributing cause of degenerative diseases and ageing cells), antigenotoxic (protection of the genome by the action of known mutagenic molecules as EMS or 4NQO) and inhibition of the viability of cancer cell lines. The evidence of bioactivity collected, finally, added innovative data to the phytotherapic landscape, confirming the validity of these drugs, and facilitating the process of making a safe trade possible, preventing fraud and misuse.

In the perspective of personal growth this PhD has given me opportunities to come into contact with other Italian (University of Parma, Modena and Cosenza) and foreign (University of Mainz, Germany) research units, with different approaches and research strategies and collaborating in writing scientific papers (Rossi et al., 2013; Prencipe et al., 2014; Tacchini et al., 2015).

Anticancer agents of plants origin have been a mainstay of cancer chemotherapy for about 50 years (Kinghorn et al., 2013). Considering the cytotoxic activity exhibited from *C. pluricaulis* and *H. indicus* extracts against CCRF-CEM cell line, the natural continuation of this research will focus on the investigation of bioactive compound, by bioassay guided fractionation, to use as possible "lead compound" in the formulation of new synthetic molecules to introduce in the cancer prevention and therapy.

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Phytochemical profile and bioactivity of traditional ayurvedic decoctions and hydro-alcoholic macerations of Boerhaavia diffusa L. and Curculigo orchioides Gaertn.

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Phytochemical profile and bioactivity of traditional ayurvedic decoctions and hydro-alcoholic macerations of *Boerhaavia diffusa* L. and *Curculigo orchioides* Gaertn.

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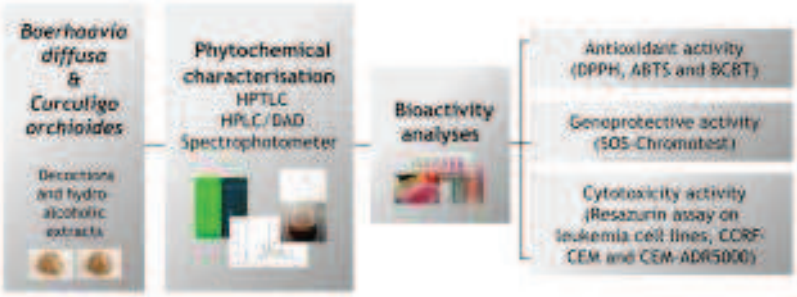

Decoctions (DECs) and hydro-alcoholic extracts (HEs) prepared from roots of *Boerhaavia diffusa* L. (Nyctaginaceae) and *Curculigo orchioides* Gaertn. (Hypoxidaceae) were phytochemically characterised by HPLC-DAD and profiled for their antioxidant, antigenotoxic and cytotoxic activities. *B. diffusa* DEC was rich in ferulic acid and vanillin, while the HE also contained boeravinone B and eupalitin. Both *C. orchioides* HE and DEC displayed the main occurrence of orcinol-β-D-glucoside and curculigoside A. Antioxidant activity was assayed through spectrophotometric DPPH, ABTS and β-carotene bleaching test, and using (HP)TLC bioautographic strategies. For both crude drugs, HE was the best performing preparation. Properly modified SOS-Chromotest evidenced a 10% inhibition by phytocomplexes against 4-nitroquinoline-*N*-oxide, and a higher bioactivity for vanillin (36.60 ± 1.68%) and ferulic acid (35.09 ± 1.53%). *C. orchioides* HE was the preparation which showed higher cytotoxicity against drug-sensitive human T-lymphoblastoid cell line (CCRF-CEM) and multidrug-resistant leukaemia cell line (CEM/ADR5000), and eupalitin was the only pure compound to exhibit an IC_{50} value.

Keywords: *Boerhaavia diffusa*; *Curculigo orchioides*; traditional preparations; chemical fingerprinting; antioxidant activity; genotoxicity; cytotoxicity

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1. Introduction

Ayurveda, the traditional Indian medicinal system, utilises a large number of plants and derived preparations, which are often difficult to characterise through phytochemical, pharmacological and toxicological investigation. This is due to the fact that their efficacy lies more in the synergistic effect of a complex mixture of compounds rather than on a single bioactive molecule. Moreover, these preparations are often formulated by mixing several crude drugs, derived from scarcely known plants, thus adding further complexity to the analysis (Guerrini & Sacchetti 2012). Decoctions (DECs) are frequently employed for various practical reasons, including the availability of water and the concurrent need for sterilisation of both herbal drugs and solvent, a hygienic prophylaxis that must not be overlooked when operating in some geographic and socio-cultural contexts (Kumar et al. 2007). However, most of the current scientific information available about ethnobotanical remedies refers to preparations obtained through approaches more related to Western phytomedicine, e.g. hydro-alcoholic extracts (HEs), whose composition and efficacy may differ from those suggested by traditional healthcare systems (WHO 2010). Moreover, because DECs are more and more frequently suggested in Western countries as a practical approach to Ayurvedic medicine, an in-depth comparison between Ayurvedic orthodox and officially recognised Western phytotherapic preparations is deemed useful for both practitioners and consumers. An accurate chemical characterisation of these preparations represents a fundamental starting point to translate the Ayurvedic concepts in pharmacological and toxicological evidence in order to shed light on Ayurvedic crude drugs and present them in their most effective and safe form to EU markets (Guerrini & Sacchetti 2012). In light of these early assumptions, this paper considers two plants widely used in the Ayurvedic tradition, namely *Boerhaavia diffusa* (Nyctaginaceae) and *Curculigo orchioides* (Hypoxidaceae). Both crude drugs are included in *Rasayana* formulations with the aim to increase life expectancy. In particular, *B. diffusa* and *C. orchioides* are known as traditional remedies for reproductive system deficiencies, liver and renal conditions, and gastrointestinal degenerative disease, and are known for their antioxidant, anti-inflammatory and adaptogenic activities (Rajpoot & Mishra 2011; Nie et al. 2013). These plants are also part of an established Italian project (PRIN 2009LR9YLF) with the aim of contributing to the phytotherapic and normative validation of plants normally used in the Ayurvedic traditional medicinal system, but already employed in EU markets. DEC represents the traditional preparation of these herbal drugs. This paper reports, for the first time, their chemical characterisation combined with biological activity. It reports a preliminary chemical characterisation of *B. diffusa* and *C. orchioides* DECs, together with an evaluation of their antioxidant, antigenotoxic and cytotoxic activities, providing at the same time a direct comparison with their HEs obtained under the Eur. Ph. specifications, in order to highlight which preparation is the most effective.

2. Results and discussion

2.1. *Chemical fingerprinting*

The quantification of the total phenolic content (Table 1) of DECs showed slightly higher values for *B. diffusa* than for *C. orchioides* (22.8%). HEs, instead, evidenced an opposite trend with *C. orchioides* displaying a concentration 71.9% higher than *B. diffusa*. The comparison between

Table 1. Concentrations of total phenols in *B. diffusa* and *C. orchioides*.

	HE (mg of gallic acid/g of crude drug)	DEC (mg of gallic acid/g of crude drug)
B. diffusa	8.66 ± 0.19	8.10 ± 0.44
C. orchioides	30.87 ± 1.69	6.25 ± 0.11

Table 2. Concentrations of the main compounds of *B. diffusa* and *C. orchioides* expressed in μg/mL and in percentage (w/w of dried phytocomplex).

		HE		DEC	
Plants	Compounds	μg/mL	%	μg/mL	%
B. diffusa	Boeravinone B	1.944 ± 0.020	0.014 ± 0.001	/	/
	Eupalitin	2.284 ± 0.055	0.016 ± 0.001	/	/
	Ferulic acid	4.644 ± 0.288	0.032 ± 0.002	6.803 ± 0.022	0.029 ± 0.004
	Vanillin	3.447 ± 0.408	0.024 ± 0.003	1.833 ± 0.087	0.008 ± 0.0004
C. orchioides	Orcinol-β-D-glucoside	232.036 ± 8.489	0.837 ± 0.031	72.932 ± 0.429	0.648 ± 0.004
	Curculigoside A	40.619 ± 6.569	0.146 ± 0.024	7.192 ± 0.033	0.064 ± 0.0003

Note: / indicates compound quantity below the limit of quantification.

B. diffusa DEC and HE showed similar values in total phenolic content while *C. orchioides* presented data fivefold higher for HE than for DEC. Regarding the phenolic content of *C. orchioides* and *B. diffusa* DECs and HEs, very few reports are available and they rely on preparation protocols different from those used in Ayurveda, which makes data hardly comparable (Hua-Bin et al. 2007). The RP-HPLC-DAD characterisation of the phenolic fraction of *B. diffusa* preparations, performed with a fully validated method, showed two different chemical profiles (Figure S1). Vanillin and ferulic acid were the most abundant compounds in DEC, while HE, in addition to these molecules, was characterised by the presence of boeravinone B and eupalitin, which were instead missing in DEC (Table 2). The comparison between formulations highlighted a higher concentration of vanillin in HE than in DEC (46.82%), while ferulic acid had an opposite trend (−31.97%), likely as a consequence of its better solubility in hot water than in alcoholic solution (Merck index 1983). *C. orchioides* preparations highlighted the presence of curculigoside A and orcinol-β-D-glucoside as main compounds in both DEC and HE (Table 2), but both molecules were more abundant in HE than in DEC: 82.29% for curculigoside A and 68.57% for orcinol-β-D-glucoside.

2.2. *Antioxidant activity*

Because of their chemical complexity, the antioxidant evaluation of herbal-derived preparations could lead to scattered results, according to the method adopted. Therefore, the recourse to a pool of methods (ABTS test, DPPH assay and β-carotene bleaching test (BCBT), performed by spectrophotometric and HP-TLC-bioautographic assay for both preparations and pure compounds) is highly advisable (Rossi et al. 2011) (Tables 3 and 4). Only eupalitin, which showed higher antioxidant capacity in the BCBT, highlighted the different outcomes that may

Table 3. Results of antioxidant activity evaluation of *B. diffusa* preparations and pure molecule.

	IC_{50} ABTS (μg/mL)	IC_{50} DPPH (μg/mL)	IC_{50} BCBT (μg/mL)
B. diffusa DEC	150.794 ± 6.241	/	/
B. diffusa HE	33.272 ± 3.406	258.409 ± 11.735	226.179 ± 9.211v
Vanillin	6.058 ± 0.943	/	/
Boeravinone B	4.814 ± 0.011	/	/
Eupalitin	4.402 ± 0.002	15.749 ± 0.120	0.077 ± 0.003
Ferulic acid	1.081 ± 0.043	9.477 ± 0.229	/
Trolox	2.040 ± 0.101	4.090 ± 0.203	0.059 ± 0.002

Note: / indicates that no IC50 value obtained in the concentration range considered.

Table 4. Results of antioxidant activity evaluation of *C. orchioides* preparations and pure molecule.

	IC_{50} ABTS (μg/mL)	IC_{50} DPPH (μg/mL)	IC_{50} BCBT (μg/mL)
C. orchioides DEC	46.895 ± 1.643	217.354 ± 0.310	/
C. orchioides HE	4.435 ± 0.090	35.777 ± 0.624	226.627 ± 10.154
Curculigoside A	4.752 ± 0.197	/	/
Orcinol-β-D-glucoside	4.900 ± 0.011	/	/
Trolox	2.040 ± 0.101	4.090 ± 0.203	0.059 ± 0.002

Note: / indicates that no IC50 value obtained in the concentration range considered.

emerge from methods using different mechanisms. The moderate activity of vanillin, ferulic acid and boeravinone B in the BCBT (respectively, 30.27%, 46.87% and 26.62%) could be explained by the 'polar paradox', since apolar molecules exhibit stronger antioxidant capacity in emulsions because they are concentrated on the lipid–air surface, thus ensuring a higher protection of the emulsion itself (Porter 1993; Frankel et al. 1994). On the other hand, polar antioxidants, remaining in the aqueous phase, are diluted and therefore less effective in protecting the lipid, as already reported by Koleva et al. (2002). For both crude drugs, the higher activity of HE than DEC was evident (about 5-fold for *B. diffusa* and 10-fold *C. orchioides*). ABTS and DPPH (HP)TLC-bioautographic assay (Figure S2) performed with *B. diffusa* preparation displayed the absence of radical scavenging reactions at the Rf corresponding to the most characterising molecules, most probably because of their low concentration in the preparations. ABTS test results for ferulic acid, vanillin and boeravinone B showed IC_{50} values, respectively, 47.01% lower and 66.33% and 57.62% higher than positive control (Trolox). In the DPPH test, data comparison highlighted just a 0.4% difference between experimental ferulic acid IC_{50} value and related data reported in the literature (Terpinc & Abramovic 2010). Concerning DPPH assay and BCBT, these molecules did not show 50% inhibition at the highest concentration tested. Considering *C. orchioides* traditional Ayurvedic preparations, HE showed a wide range of antioxidant capacity, being active as radical scavenger and interfering at the same time with radical propagation (Table 4). In general, both *C. orchioides* preparations exhibited higher antioxidant capacity as radical scavenger than those of *B. diffusa* but showed comparable results in the BCBT, according to the higher phenolic content of *C. orchioides* preparations. (HP)TLC-bioautographic assay highlighted for both *C. orchioides* preparations a higher variety of molecules with antioxidant activity than *B. diffusa* (Figures S2 and S3). IC_{50} values of curculigoside A and orcinol-β-D-glucoside were about twofold greater than positive control in the ABTS test (57.07% for curculigoside A and 58.37% for orcinol-β-D-glucoside), while in the DPPH test and in the BCBT, they did not reach 50% inhibition at the highest concentration tested. We must conclude that, at least for the plants evaluated, the hydro-alcoholic maceration outperforms the Ayurvedic DEC in terms of antioxidant performance and overall polyphenolic content (Li et al. 2007).

2.3. *SOS-Chromotest*

The SOS-Chromotest was performed in order to attest the absence of genotoxicity and to check the possible antigenotoxic activity of preparations and single compounds (Quillardet & Hofnung 1985). DEC and HE did not display any cytotoxicity or DNA damage with reference to the parameters of the SOS-Chromotest. The only exception was boeravinone B, which showed limited cytotoxicity at a concentration about 60-fold higher than the quantity actually present in the HE. *B. diffusa* and *C. orchioides* preparations showed weak antigenotoxic activity with dose-response correlation. The antigenotoxic activity was slightly higher for DEC than HE and for *C. orchioides* compared to *B. diffusa*. The highest antigenotoxic capacity, with dose–response correlation, was shown by ferulic acid (35.09% at the concentration of 10 μg/mL); and vanillin

(36.60% at the concentration of 10 μg/mL), with values in accordance with the literature (Ohta et al. 1986). Even if the antigenotoxic capacity revealed a weak potential, it should be stressed that the Ayurvedic preparation (DEC) displayed a higher capacity than that showed by HE.

2.4. *Resazurin reduction assay*

Every extract reached IC_{50} inhibition values against both considered cell lines (CCRF-CEM and CEM/ADR5000). Curiously, the results of the cytotoxicity evaluation followed the same trend of the results of the antioxidant activity, showing higher activity for the HEs than for DECs. Furthermore, *C. orchioides* preparations exhibited higher activity than *B. diffusa* preparations (Table 5). Considering the HE, *C. orchioides* showed the lowest IC_{50} values: 243.57 ± 4.21 μg/mL against CCRF-CEM and 290.96 ± 2.31 μg/mL against CEM/ADR5000 (been, respectively, 24.12% and 65.74% lower than data obtained for *B. diffusa* HE). Among the molecules considered, the only compound that showed an IC_{50} value of cytotoxic activity was eupalitin. It exhibits IC_{50} values of 13.12 ± 0.76 μg/mL against CCRF-CEM cell line and 23.34 ± 0.33 μg/mL against CEM/ADR5000. Though P-glycoprotein-overexpressing CEM/ADR5000 cells showed a 1.78-fold degree of cross-resistance compared to its drug-sensitive counterpart, they were more sensitive to this compound than doxorubicin (113.17 ± 8.29 μg/mL, Kuete et al. 2014).

3. Experimental

3.1. *Chemicals, bacterial strains and cell lines*

Chromatographic grade solvents used for bioassays were purchased from Sigma-Aldrich Italy (Milano, Italy). Pure compounds used as standard in RP-HPLC-DAD were purchased from Sigma-Aldrich Italy and Extrasynthese, France. *Escherichia coli* PQ37 strain for SOS-Chromotest was purchased from Ecotox (Ontario, Canada), and broth ingredients were from Oxoid and Sigma Aldrich. Drug-sensitive human T-lymphoblastoid cell line (CCRF-CEM) and multidrug-resistant P-glycoprotein over-expressing leukaemia cell line (CEM/ADR5000) were kindly provided by Dr J. Beck (Department of Pediatrics, University of Greifswakl, Greifswald, Germany).

3.2. *Plant material*

Roots of *B. diffusa* (Batch No. 5188, Mfg Date 15 February 2011) and *C. orchioides* (Batch No. 2045, Mgf Date 19 August 2011) were collected from Ram Bagh (Rajasthan, India), authenticated by Dr MR Uniyal, Maharishi Ayurveda Product Ltd., Noida, India; and imported by MAP Italia. After harvesting, the roots were cleaned and cut into small pieces before being

Table 5. Plant extracts and pure molecules IC_{50} values against CCRF-CEM and CEM/ADR5000.

Plant extracts and pure molecules	CCRF-CEM cell line IC_{50} (μg/mL)	CEM/ADR5000 cell line IC_{50} (μg/mL)
B. diffusa – DEC	428.07 ± 4.01	1119.87 ± 45.31
B. diffusa – HE	320.98 ± 17.40	849.33 ± 6.43
C. orchioides – DEC	379.17 ± 5.85	715.13 ± 34.90
C. orchioides – HE	243.57 ± 4.21	290.96 ± 2.31
Eupalitin	13.12 ± 0.76	23.34 ± 0.33
Boeravinone B	>30	>30
Vanillin	>30	>30
Ferulic acid	>30	>30
Curculigoside A	>30	>30
Orcinol-β-D-glucoside	>30	>30

dried. All samples were then ground to a fine powder and kept at −20°C until used for the extractions.

3.3. *Plant DEC and HE preparation*

DEC was prepared in triplicate as previously described by Ferruzzi et al. (2013) according to the procedures reported in the Ayurvedic Pharmacopoeia of India; hydro-alcoholic maceration, instead, followed the procedure reported in the European Pharmacopoeia (2011) as follows: 50 g of ground root was mixed with 450 mL of 30% ethanol–water solution. Mixtures were left for 21 days and were stirred constantly. Extracts were then filtered, lyophilised and stored at −20°C. All the extractions were performed in triplicate.

3.4. *Determination of total phenolics*

Total polyphenolic content was determined using a ThermoSpectronic Helios-γ spectrophotometer, according to previously described methods (Rossi et al. 2012). The content of total polyphenols is expressed as mg of gallic acid equivalent.

3.5. *HPLC analysis of plant preparations*

The analyses were performed using a JASCO modular HPLC system (Tokyo, Japan, model PU 2089) coupled to a diode array apparatus (MD 2010 Plus) linked to an injection valve with a 20 µL sampler loop. The column used was an Eclipse-PLUS-C18 (25 µm × 0.46 cm, i.d., 5 µm) at a flow rate of 1.0 mL/min. The solvents used in the RP-HPLC-DAD analyses were water and 0.5% of formic acid (A), and acetonitrile and methanol, in equal proportion, with 0.5% of formic acid (B). Three different gradients were used to obtain the best resolution for each specific molecule. The detection wavelength for vanillin and ferulic acid was 310 and 270 nm for the other molecules. Samples of DEC and HE were, respectively, dissolved in water and 30% ethanol–water solution. Pure compound samples, instead, were dissolved in methanol, and the calibration curves were made using a range of five concentrations (1.25–50.00 µg/mL for boeravinone B and eupalitin; 1.25–100.00 µg/mL for curculigoside A, ferulic acid and vanillin; 5.00–450.00 µg/mL for orcinol-β-D-glucoside). For each compound, areas were calculated by integration using Borwin ver. 1.22 (JMBS Developments, Grenoble, France).

3.6. *Antioxidant activity*

Radical scavenging and antioxidant properties were performed through different spectrophotometric and (HP)TLC bioautographic assays. DPPH assay, ABTS assay and BCBT were determined spectrophotometrically according to previously described methods (Guerrini et al. 2006, 2009). Trolox (1 mg/mL) was used as positive control. The antioxidant activity of the samples was expressed as IC_{50}, and it was calculated using the inhibition curves obtained by plotting the percentage inhibition against the sample concentration. All the data collected for each assay are the average of three determinations in three independent experiments. (HP)TLC bioautographic assays were performed using ABTS and DPPH radical, to pinpoint the most active compounds from each phytocomplex. Six microlitres of DEC, HE of each herbal drug and methanolic solution of pure compounds were applied in triplicate to a (HP)TLC plate of silica gel (Merck, silica gel 60, F254) as 10 mm wide bands with Linomat V (Camag). The plate was eluted in a chromatographic chamber with a solvent solution composed of ethyl acetate/formic acid/acetic acid/water (100:11:11:20) and another plate in a chromatographic chamber containing a solution of toluene/ethyl acetate/acetic acid (100:90:10). After plate development, the first plate was sprayed with a solution of ABTS (11 mg/10 mL of distilled water, plus 100 µL

of $K_2S_2O_8$ 70 mM solution) and the second with a solution of DPPH (20 mg/100 mL of ethanol). Data reported for each assay are the average of three determinations of three independent experiments.

3.7. *SOS-Chromotest*

Genotoxicity and antigenotoxicity assays were performed as described by Prencipe et al. (2014) using exponential-phase culture of *E. coli* PQ37. All the data collected for each assay are the average of three determinations in three independent experiments.

3.8. *Resazurin reduction assay*

Resazurin reduction assay was performed to assess the cytotoxicity of plant extracts and identified pure molecules towards one sensitive (CCRF-CEM) and one resistant leukaemia (CEM/ADR5000) cell line. The assay was performed in accordance with the method described in Kuete et al. (2014). Fluorescence was measured on an Infinite M2000Pro™ plate reader (Tecan, Germany) using an excitation wavelength of 544 nm and an emission wavelength of 590 nm. Each assay was repeated three times, with six replicates each. The viability was evaluated based on a comparison with untreated cells. IC_{50} values represent sample concentrations required to inhibit 50% of cell proliferation and were calculated from a calibration curve.

3.9. *Statistical analyses*

Results are presented as means ± standard deviations from three different experiments. Collected data were then analysed using one-way ANOVA, where appropriate, to check significant differences.

4. Conclusions

This paper reports the first chemical characterisation and some biological activity of the traditional health preparations of *B. diffusa* and *C. orchioides* DECs and HEs. It highlighted the presence of previously undetected molecules, the antioxidant and antigenotoxic capacity and the possible preventive and therapeutic use of the considered formulations against drug-sensitive and multi-resistant leukaemia cell lines. While the recourse to DEC may represent a more orthodox approach to Ayurvedic herbal drugs, notwithstanding the safer profile involved in the use of pure water as a solvent, the hydro-alcoholic maceration may allow a richer and more effective phytocomplex to be obtained, at least in terms of overall antioxidant protection. The antigenotoxic properties for both preparations from both *C. orchioides* and *B. diffusa* are negligible. The cytotoxicity towards the drug-sensitive human T-lymphoblastoid cell line CCRF-CEM and multidrug-resistant P-glycoprotein over-expressing CEM/ADR5000 leukaemia cell line was not noteworthy, being far from the concentration values (20 μg/mL for extracts, and 4 μg/mL for pure compounds) stated by the American National Cancer Institute (Suffness & Pezzuto 1991). The molecules identified in *C. orchioides* did not show any cytotoxic activity at the tested concentrations, while eupalitin, identified in *B. diffusa*, evidenced an IC_{50} value higher than that of the American National Cancer Institute, but which could be involved in the weak cytotoxicity revealed by *B. diffusa* HE. Even if the cytotoxicity study did not reveal any noteworthy activity of the preparations and pure compounds on the considered cell lines, it could form the basis for further in-depth analyses against other common cancer cell lines.

Supplementary material

Supplementary material relating to this paper is available online, alongside Figures S1–S3.

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Research Paper

In vitro anti-angiogenic effects of *Hemidesmus indicus* in hypoxic and normoxic conditions

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ABSTRACT

Ethnopharmacological relevance: The decoction of the roots of *Hemidesmus indicus* is widely used in the Indian traditional medicine for many purposes and poly-herbal preparations containing *Hemidesmus* are often used by traditional medical practitioners for the treatment of cancer. In the context of anticancer pharmacology, anti-angiogenic therapy has become an effective strategy for inhibiting new vessel formation and contrast tumor growth. These considerations are supported by the evidence that most tumors originate in hypoxic conditions and limitation of oxygen diffusion stimulates the formation of tumor abnormal microvasculature. Aim of this study was to evaluate the *in vitro* anti-angiogenic potential of *Hemidesmus indicus* (0.31–0.93 mg/mL) on human umbilical vein endothelial cells and delineate the main molecular mechanisms involved in its anti-angiogenic activity both in normoxia and hypoxia.

Materials and methods: The decoction of *Hemidesmus indicus* was subjected to an extensive HPLC phytochemical characterization. Its *in vitro* anti-angiogenic potential was investigated in normoxia and hypoxia. Cell proliferation, apoptosis induction, and inhibition of endothelial cell migration and invasion were analyzed by flow cytometry. The endothelial tube formation assay was evaluated in matrix gel. The capillary tube branch points formed were counted using a Motic AE21 microscope and a VisiCam videocamera. The regulation of key factors of the neovascularization process such as VEGF, HIF-1α and VEGFR-2 was explored at mRNA and protein level by real time PCR and flow cytometry, respectively.

Results: Treatment with *Hemidesmus* resulted in a significant inhibition of cell proliferation and tube formation in both normoxia and hypoxia. *Hemidesmus* differently regulated multiple molecular targets related to angiogenesis according to oxygen availability. In normoxia, the inhibition of VEGF was the main responsible for its anti-angiogenic effect; the angiogenesis inhibition induced in hypoxia was regulated by a more complex mechanism involving firstly HIF-1α inhibition, and then VEGF and VEGFR-2 down-regulation. Additionally, the inhibition of endothelial cell migration and invasion by *Hemidesmus* was more pronounced in normoxia than in hypoxia, possibly due to the physiological enhanced induction of invasion characteristic of hypoxia.

Conclusions: Our results indicate that *Hemidesmus* might represent a promising therapeutic strategy for diseases in which the inhibition of angiogenesis could be beneficial, such as cancer. The antiangiogenic activity of *Hemidesmus* is based on multiple interactions with critical steps in the angiogenic cascade. VEGF expression stimulated by HIF-1α as well as endothelial cell migration and differentiation represent important targets of *Hemidesmus* action and might contribute to its cancer therapeutic efficacy that is presently emerging and offer a scientific basis for its use in traditional medicine.

1. Introduction

Angiogenesis covers a key role in the development and spread of tumor. Cancer cells are not able to grow in diameter more than 1–2 mm^3 and metastasize without blood circulation. Tumor cells need blood vessels that bring oxygen and nutrients and remove metabolic wastes to spread. In absence of vascular support, tumors may become necrotic or even apoptotic (Parangi et al., 1996). Most tumors originate in hypoxic conditions and limitation of oxygen diffusion stimulates the formation of tumor abnormal microvasculature (Jain, 2005). The hypoxic condition enhances the transcription of vascular endothelial growth factor (VEGF) by hypoxia

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inducible factor-1α (HIF-1α) (Kuschel et al., 2012). An improved activity of VEGF has been reported in most aggressive cancers and it is related with a poor prognosis (Foekens et al., 2001).

The pivotal role of angiogenesis in tumor spread and metastasis formation provides the rationale for using anti-angiogenic strategies as a form of anticancer treatment. Thus, the inhibition of VEGF-signaling pathway is an interesting therapeutic strategy in the treatment of cancer and the most validated anti-angiogenic strategy targets the VEGF axis. Bevacizumab, the first clinically available angiogenesis inhibitor, directly blocks VEGF, and other drugs such as sunitinib, sorafenib and pazopanib indirectly inhibit VEGF receptor (VEGFR) activity (Cesca et al., 2013). Several angiogenesis inhibitors have been approved by FDA for cancer treatment, but their use is associated with many side effects, among them bleeding is one of the most severe (Elice and Rodeghiero, 2012).

Hemidesmus indicus (L.) R.Br. (HI) belongs to the family of *Asclepiadaceae* and is an Indian weed widely used in the traditional medicine. The plant is a bush, woody, with thick and brown bark, which grows from the upper Gangetic plains East-wards to Assam, throughout Central, Western and Southern India. The decoction of the roots of HI is traditionally used for the treatment of blood diseases, dyspepsia, loss of taste, dyspnea, cough, poison, menorrhagia, fever, and diarrhea (Ayurvedic Pharmacopoeia Committee, 1989; Mary et al., 2003). Moreover, poly-herbal preparations containing HI are used by traditional medical practitioners for the treatment of cancer (Thabrew et al., 2005) and different studies demonstrated the anticancer potential of HI (Costa-Lotufo et al., 2005; Fimognari et al., 2011; Samarakoon et al., 2012; Zarei and Javarappa, 2012; Ferruzzi et al., 2013). Aim of this study was to evaluate the *in vitro* anti-angiogenic potential of HI on human umbilical vein endothelial cells (HUVECs) and delineate the main molecular mechanisms involved in its anti-angiogenic activity both in normoxia and hypoxia.

2. Materials and methods

2.1. Materials and cell cultures

Dimethylsulfoxide (DMSO), fetal bovine serum (FBS), antibiotics (penicillin and streptomycin), trypsin-EDTA, and human recombinant VEGF were obtained from Sigma Aldrich (St. Louis, MO, USA). HUVECs were purchased from Lonza (Basel, Switzerland), cultured in EGM complete medium supplemented with SingleQuots™ (containing hydrocortisone, hEGF, FBS, VEGF, hFGF-B, R3-IGF-1, ascorbic acid, heparin and gentamycin/amphotericin-B, Lonza) and incubated at 37 °C and 5% CO_2 in normoxia (21% O_2) or hypoxia (2.5% O_2). The hypoxia was guarantee by the use of the hypoxic station InVivo$_2$ 200 (Baker Ruskinn, Sanford, MA, USA). To maintain the exponential growth, cells were divided when they reached 80% of confluence in a 25 cm^2 dish. HUVECs at passage between 3 and 8 were used for the experiments.

2.2. Plant materials

HI (voucher #MAPL/20/178) was obtained from Ram Bagh (Rajasthan, India) after its authentication by Dr. MR Uniyal, Maharishi Ayurveda Product Ltd., Noida, India. The ayurvedic crude drug was collected in 2010, in particular, following the indications of Ayurvedic Pharmacopoeia of India (2004), during the balsamic period (January). The decoction was prepared according to the method previously described and agreeing with Ayurvedic Pharmacopoeia (Fimognari et al., 2011). Briefly, 10 g of grinded roots were added to 300 mL of boiling water, and boiled until the suspension reached the volume of 75 mL. The yield of the decoction was 15%. HI decoction was filtered, lyophilized, and stored at room temperature. The experiments were performed by preparing a stock solution of 31 mg/mL. The suspension was centrifuged at 4000 rpm to discard any insoluble material.

2.3. HPLC–MS analysis of plant decoction

The main phytomarkers of HI, namely 2-hydroxy-4-methoxybenzaldehyde, 3-hydroxy-4-methoxybenzaldehyde and 2-hydroxy-4-methoxybenzoic acid (Das and Bisht, 2013), were identified and quantified by HPLC–MS analysis. The reference compounds (all obtained from Sigma) were used as external standards to set up and calculate appropriate calibration curves. The calibration graphs were provided by the regression analysis of peak area of the analytes *vs* the related concentrations.

The analyses of three different batches of HI were performed on a Jasco PU-1585 Liquid Chromatograph (Jasco Corporation, Tokio, Japan) interfaced with a Jasco 1575 UV–vis detector ($\lambda = 254$ nm) and a LCQ-Duo Mass Spectrometer (Thermo Finnigan, San Jose, CA, USA), by a splitting flow *T*-valve. The mass spectrometer is equipped with heated capillary interface and electrospray ionization (ESI) source, operating with an Ion Trap (IT) analyzer. ESI system employed a 4.5 kV (positive polarity) and 5.0 kV (negative polarity) spray voltage and a heated capillary temperature of 200 °C. The sheath gas and the auxiliary gas (nitrogen) flow rates were set to 0.75 and 1.2 L/min, respectively. ESI was optimized using 3-hydroxy-4-methoxybenzaldehyde and 2-hydroxy-4-methoxybenzoic acid for positive and negative polarity, respectively. The mass chromatograms were acquired in total ion current (TIC) modality from 50 to 400 *m/z*, and in MS/MS mode (multiple reaction monitoring) on the ESI generated most abundant ion, corresponding to the pseudomolecular ion; $[M+H]^+$ at 153 *m/z* for 2-hydroxy-4-methoxybenzaldehyde and 3-hydroxy-4-methoxybenzaldehyde, and $[M-H]^-$ at 167 *m/z* for 2-hydroxy-4-methoxybenzoic acid. The relative collision energy varied for the different compounds from 18% to 23%.

Chromatographic analyses were performed on a Phenomenex Gemini C18 column (5 μm, 150 mm × 2.0 mm I.D.) by gradient elution from A (0.1% formic acid in acetonitrile)–B (0.1% formic acid in water) 28:72 (v/v) for 7 min to A-B 55:35 (v/v) in 20 min, at the flow rate of 0.3 mL/min. The re-equilibrium time between runs was 5 min. The injection volume was 50 μL.

2.4. Preparation and GC–FID and GC–MS analyses of lipidic fraction

As previously reported (Das and Bisht, 2013; Fiori et al., 2014) in HI root decoction can be detected not only hydrophilic compounds but also lipophilic ones.

Two g of decoction were exactly weighed into a 25 mL flask and then extracted with 20 mL of chloroform by ultrasound system maceration for 20 min. The residual decoction was centrifuged at 3000 rpm for 20 min. The extraction was performed three times. All supernatants were transferred into a 100 mL round bottom flask, then taken to dryness with a rotary vacuum evaporator. The procedure was repeated on three different batches. The dried extracts have been mixed with 100 μL BSTFA (1% TMCS) (bis (trimethylsilyl)trifluoroacetamide + trimethylchlorosilane) (Sigma-Aldrich) for 45 min at 80 °C. Then 1 μL of solution was directly injected in GC.

Lupeol, lupeol acetate, β-sistosterol, and β-amyrin acetate were identified by GC–MS and then quantified in GC–FID by external standard method. All standards were purchased from Extrasynthese (Genay, France). After derivatization, an appropriate calibration curve was calculated for each reference compound. The calibration graphs were provided by the regression analysis of peak area of the analytes *vs* the related concentrations.

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GC–MS analysis was performed by a gas chromatograph (Model Varian GC-3800, Agilent Technologies Inc., Santa Clara, California, USA) equipped with a VF-5 ms 5% poly- and 95% phenyl-dimethylsiloxane bonded phase column (i.d., 0.25 mm; length, 30 m; film thickness, 0.25 μm, Agilent Technologies Inc.), a mass spectrometer (Model Varian MS-4000, Agilent Technologies Inc.) using electron impact (EI) and hooked to NIST library. Lupeol, lupeol acetate, β-sistosterol, and β-amyrin acetate were identified by comparing their GC retention time and the MS fragmentation pattern. Operating conditions were as follows: injector temperature, 300 °C; carrier (helium) flow rate, 1 mL/min and split ratio, 1:50. Oven temperature was increased from 230 °C to 320 °C at a rate of 5 °C/min, followed by 7 min at 320 °C. The MS conditions were: ionization voltage, 70 eV; emission current, 10 mA; scan rate, 1 scan/s; mass range, 29–600 Da; trap temperature, 150 °C; transfer line temperature, 300 °C.

GC–FID was used for quantitative determination through the normalization method, without using correction factors: the relative peak areas for individual constituents were averaged on three different chromatograms of three independent reactions. The relative percentages were determined using a gas-chromatograph (Model GC-Trace, ThermoQuest Corporation, Atlanta, Georgia, USA) equipped with a FID detector maintained at 350 °C and an autosampler (AS Triplus 3000, Thermo Electron Corporation, Waltham, Massachusetts, USA); all the others GC conditions were the same of GC–MS method.

2.5. Flow cytometry

All flow cytometric analyses were performed using the Guava easyCyte 5HT flow cytometer (Merck Millipore, Hayward, CA, USA).

2.6. Cell viability and proliferation

HUVECs were treated with HI (0.00–0.93 mg/mL) for 6 h and 24 h in normoxia and hypoxia. Viability was determined immediately after the end of treatment. Briefly, HUVECs were mixed with an adequate volume of Guava ViaCount Reagent (containing propidium iodide, Merck Millipore) and allowed to stain 5 min at room temperature before the flow cytometric analysis.

2.7. Analysis of apoptosis

The pro-apoptotic potential of HI (0.00–0.93 mg/mL) was analyzed after treatment of confluent HUVECs for 6 and 24 h in normoxia and hypoxia. Briefly, aliquots of 2×10^4 cells were stained with 100 μL of Guava Nexin Reagent (Merck Millipore), containing Annexin V-phycoerythrin (Annexin V-PE) and 7-amino-actinomycin D (7-AAD). The samples were incubated for 20 min at room temperature in the dark and then analyzed by flow cytometry.

2.8. Endothelial cell tube formation assay

The ability of HI to influence the endothelial cell tube formation was analyzed by using the *In Vitro* Angiogenesis Assay Kit (ECM625, Merck Millipore). The assay is based on culturing cells in an ECMatrix™, a solid gel of basement proteins prepared from the Engelbreth Holm-Swarm (EHS) mouse tumor and consisting of laminin, collagen type IV, heparan sulfate proteoglycans, entactin and nidogen. It also contains growth factors (TGF-β, FGF) and proteolytic enzymes (plasminogen, tPA, MMPs). The gel is optimized for maximal tube-formation. Thus, endothelial cells can rapidly align and form tube-like structures. Briefly, each well, of a 96-well plate, was coated with 50 μL of cold ECMatrix™ solution and the plate was kept at 37 °C for 1 h to allow the solidification of the ECMatrix™. Afterwards, 1×10^4 HUVECs were seeded in each well in a total volume of 150 μL of EGM medium and treated for 6 h in hypoxia or normoxia with various concentrations of HI (0.00–0.93 mg/mL) or VEGF 10 ng/mL as positive control, alone or in association. Pictures from three randomly selected fields were taken using a Motic AE21 microscope (Campbell, CA, USA) and a VisiCam 3.0 videocamera (VWR International PBI, Milan, Italy). The capillary tube branch points formed were counted in four random view-field per well.

2.9. Analysis of angiogenic proteins

After treatment with HI (0.00–0.93 mg/mL) in both oxygen conditions, aliquots of 1×10^6 HUVECs were fixed with a solution of 4% paraformaldehyde and permeabilized with ice cold 90% methanol. Afterward, cells were incubated for 30 min with anti-VEGF (1:500, Abcam, San Francisco, CA, USA) or anti-HIF-1α (1:500, Merck Millipore) antibodies, washed and incubated with the FITC-conjugated secondary antibody (1:500, Sigma). For VEGFR-2, 1×10^6 cells were stained with 2.5 μL of anti-VEGFR-2-PE Cy-5.5 antibody (2.5:10, Biolegend, San Diego, CA, USA). Mean fluorescence intensity was quantified by flow cytometry through the analysis of 10,000 events/sample. In order to exclude non-specific bindings, the fluorescence of the isotype negative control antibody [FITC Mouse IgG1, k (FC), Biolegend] was analyzed.

2.10. RNA extraction and analysis of gene expression

After treatment for 6 h with HI (0.00–0.93 mg/mL) both in normoxia and in hypoxia, total RNA extraction was performed by miRVana™ miRNA Isolation kit (Life Technologies, Carlsbad, CA, USA) according to manufacturer's instructions. Total RNA was reverse transcribed using the High Capacity cDNA reverse transcription kit (Life Technologies). RNA extraction and cDNA transcription were performed as reported above. Relative quantification of VEGF, HIF and VEGFR-2 mRNA as well as 18S and GAPDH, as internal controls, was performed by real time PCR (ABI PRISM 7900HT, Life Technologies) using Universal Master Mix and TaqMan assay (Hs00900054_m1, Hs00153153_m1, Hs00176676_m1, Hs99999901_s1, Hs99999905_m1, respectively) (Life Technologies). Each measure was performed in triplicate. Data were analyzed through $2^{-\Delta\Delta Ct}$ method.

2.11. Migration and invasion assays

Cell migration and invasion assays were performed by QCM™ 24-well Fluorimetric Cell Migration Kit (ECM509; Merck Millipore) and QCM™ 24-well Fluorimetric Cell Invasion Assay Kit (ECM554; Merck Millipore), respectively, according to the manufacturer's instructions. Both assays exploit a polycarbonate membrane with an 8 μm pore size, which in the invasion assay is coated with a thin layer of ECMatrix™ occluding the membrane pores and physically inhibits the passage of non-invasive cells. The two kits possess several common procedure steps. Briefly, HUVECs treated with HI (0.00–0.93 mg/mL) in normoxia and hypoxia were loaded in the upper compartments, while in the lower chambers EGM supplemented with 10% FBS was used as chemoattractant. The plates were incubated for 6 h for the migration and 24 h for the invasion assay. Cells able to migrate through or invade the support were detached from the bottom using a Cell Detachment Buffer, and then fixed and stained with CYQuant GR Dye. The fluorescence of the migrated or invaded cells was evaluated by Infinity F200 Proplate reader (Tecan, Männedorf, Swiss) using 480/520 nm filter set. The migrated and invaded cell number relative variation was obtained comparing the mean fluorescence signal of HI-treated samples with untreated cells.

2.12. Statistical analysis

All experiments were repeated at least three times and results are reported as the mean ± SEM. Differences among treatments were assessed by one-way ANOVA, followed by Dunnett or Bonferroni as

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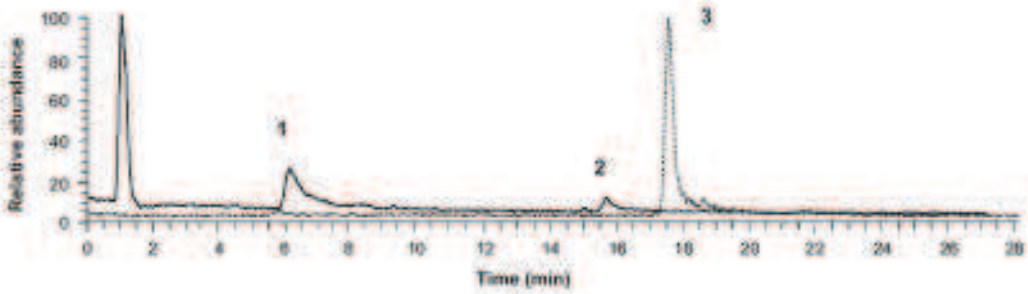

Fig. 1. LC-MS SIM (single ion monitoring) chromatograms of the decoction. Black line: detection in positive polarity of 3-hydroxy-4-methoxybenzaldehyde (1) and 2-hydroxy-4-methoxybenzaldehyde (2). Dashed line: detection in negative polarity of 2-hydroxy-4-methoxybenzoic acid (3).

Table 1
Quantification of lipophilic compounds.

Compound	μg/mL
Lupeol	1.47 ± 0.06
Lupeol acetate	24.58 ± 1.32
β-Sitosterol	1.66 ± 0.23
β-Amyrin acetate	2.95 ± 0.18

post-hoc test. GraphPad InStat version 5.0 (GraphPad Prism, San Diego, CA, USA) was used for all statistical analyses. $P < 0.05$ was considered significant.

3. Results

3.1. HI main phytomarkers

2-Hydroxy-4-methoxybenzaldehyde, 3-hydroxy-4-methoxybenzaldehyde and 2-hydroxy-4-methoxybenzoic acid were identified in HI as reference phytomarkers (Fig. 1). The amount of each phytomarker in the decoction (31 mg/mL) was quantified in: 3.06 ± 0.2 μg/mL for 2-hydroxy-4-methoxybenzaldehyde, 20.40 ± 0.8 μg/mL for 3-hydroxy-4-methoxybenzaldehyde, 20.02 ± 0.7 μg/mL for 2-hydroxy-4-methoxybenzoic acid. Three different batches of HI were analyzed and no significant difference was found in the content of the three phytomarkers (data not shown).

Moreover lupeol, lupeol acetate, β-sistosterol, and β-amyrin acetate were quantified in dried decoction after maceration with chloroform and results are shown in Table 1. All these lipophilic molecules were previously detected in the root (Das and Bisht, 2013), but in our paper were quantified for the first time in the decoction.

3.2. HI affects proliferation of HUVECs

To preliminary exclude any possible effect of HI on angiogenesis connected with the reduction of viability, the cytotoxic potential of HI on HUVECs was screened. In both normoxia and hypoxia, none of the tested concentrations showed a significant reduction in cell viability or apoptosis induction (data not shown). As an example, after 6 h of treatment, the % of viable cells was 100% at the concentration of 0.31 mg/mL, 96% at 0.62 mg/mL, and 89% at 0.93 mg/mL in normoxia; 100% at 0.31 and 0.62 mg/mL, and 98% at 0.93 mg/mL in hypoxia.

In order to determine the effect of HI on HUVEC proliferation, cells were treated with different concentrations (0.00–0.93 mg/mL) for 6 h and 24 h in normoxia (Fig. 2A) and hypoxia (Fig. 2B). Treatment with HI for 6 h induced a reduction of cell proliferation by more than 30% only in normoxia and at the highest tested dose. After 24 h of treatment, a reduction of cell growth by more than 30% was induced by HI 0.62 mg/mL in normoxia, while in hypoxia a similar effect was induced by HI 0.93 mg/mL.

3.3. HI inhibits the formation of new vessels

The ability of HUVECs to migrate, attach each other, and form tube structures on ECMatrix™ is shown in Fig. 3A. The number of branch points counted in an untreated sample in normoxic conditions was lower compared to hypoxia (65 branch points *vs* 77, respectively). HI strongly inhibited tube formation at the highest tested concentrations in both hypoxia and normoxia. For example, in a hypoxic sample we counted 18 and 0 branch points at HI 0.62 mg/mL and 0.93 mg/mL, respectively, and in a normoxic sample 17 and 0 branch points at 0.62 mg/mL and 0.93 mg/mL, respectively. Accordingly, count of the tube branch points formed after HI 0.62 mg/mL treatment showed its ability to significantly reduce the branch point number more in normoxia than in hypoxia (0.17 ± 0.07 *vs* 0.22 ± 0.13) compared to the untreated samples (Fig. 3B). Similar effects were recorded in cells treated with the association HI 0.62 mg/mL-VEGF, with a significant reduction of the branch point number in normoxia (0.21 ± 0.06) and hypoxia (0.24 ± 0.20) compared to the VEGF control (Fig. 3C). Moreover, in samples treated with HI 0.93 mg/mL in both normoxia and hypoxia, HUVECs were not able to form hollow tube-like structures. Thus, branch point could not be determined (Fig. 3B and C). Notably, HI 0.31 mg/mL appeared to stimulate angiogenesis, but the number of tube branches was not significantly increased compared to the untreated and VEGF controls in both O_2 conditions (Fig. 3B and C).

3.4. HI modulates the expression of proteins involved in the angiogenic process

The ability of HI to inhibit angiogenesis was confirmed by testing its ability to induce a post-transcriptional and post-translational modulation of proteins involved in the regulation of vessel formation process. The expression of VEGF, VEGFR-2 and HIF-1α was quantified by measuring the mean fluorescence intensity. In hypoxia and normoxia, HI induced a dose-dependent down-regulation of VEGF, which reached the highest effect at the highest tested concentration (0.81 ± 0.02 in normoxia and 0.77 ± 0.11 in hypoxia) (Fig. 4A). In normoxia, HI did not show any effect on the expression of VEGFR-2 and HIF-1α (Fig. 4B and C). On the other hand, in hypoxia HI down-regulated the expression of all three proteins at all tested doses. In particular, the highest effects on VEGFR-2 and HIF-1α were observed after treatment with HI 0.93 mg/mL, which induced a reduction in their expressions by 40% and 17%, respectively (Fig. 4B and C).

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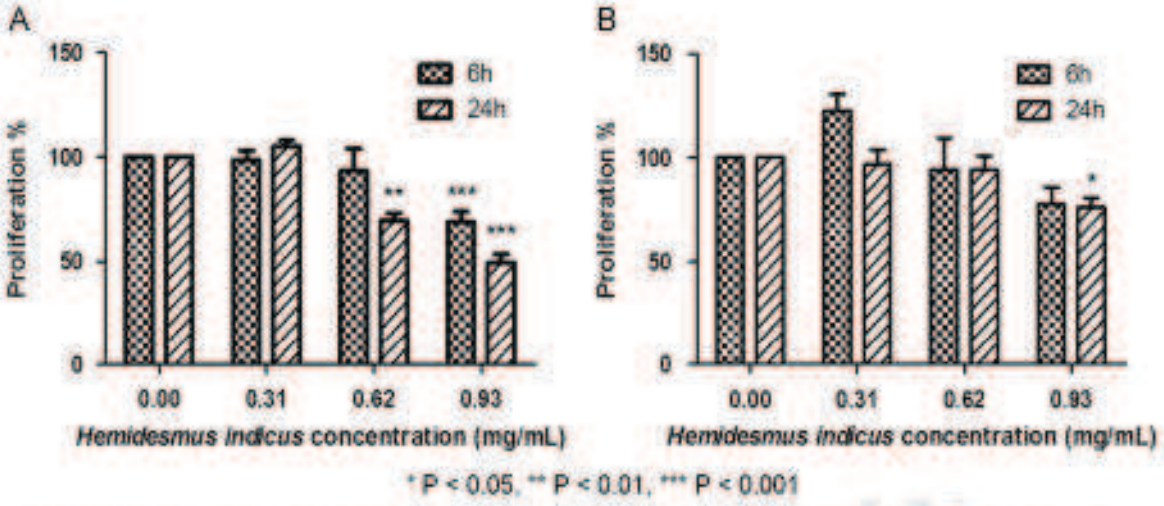

Fig. 2. HUVEC proliferation after treatment with HI (0.00–0.93 mg/mL) for 6 h and 24 h in normoxic (A) and hypoxic (B) conditions.

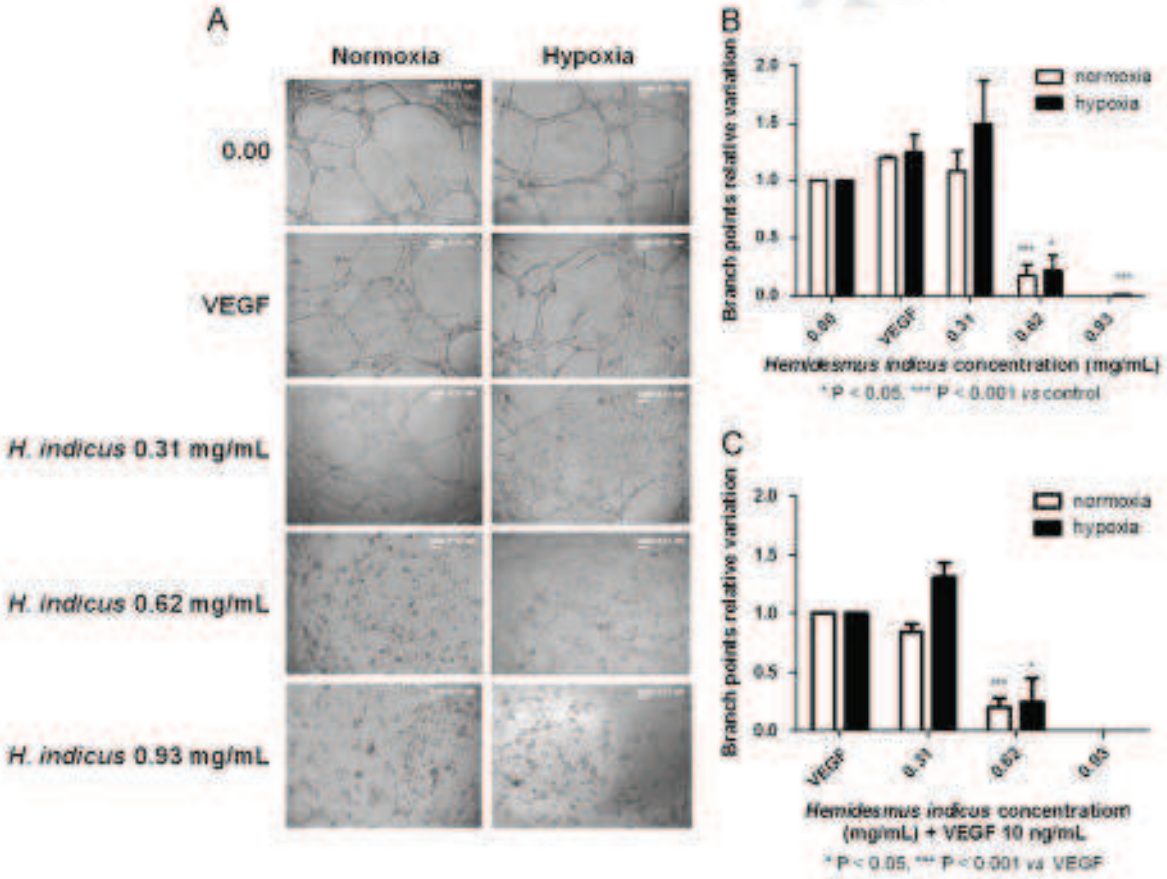

Fig. 3. Microscopic photographs of HUVEC tube formation on ECMatrix™ after HI treatment for 6 h in normoxia and hypoxia (A). (a, a1) Untreated cells; (b, b1) VEGF 10 ng/mL; (c, c1) *Hemidesmus* 0.31 mg/mL; (d, d1) 0.62 mg/mL; (e, e1) 0.93 mg/mL. Branch point relative variation after HUVEC treatment for 6 h in normoxia and hypoxia with HI or VEGF (B) and their association (C).

3.5. HI inhibits VEGF, VEGFR-2 and HIF gene expression

HI modulated VEGF, VEGFR-2 and HIF gene expression. A dose-dependent down-regulation was observed, particularly marked in hypoxia (Fig. 5). The highest inhibitory effect was observed after treatment of HUVECs with HI 0.93 mg/mL, where a stronger down-regulation of HIF-1α and VEGF mRNA was recorded in hypoxia than in normoxia (0.07 ± 0.04 *vs* 0.25 ± 0.06 and 0.50 ± 0.03 *vs* 1.00 ± 0.07, respectively) (Fig. 5C). With regard to VEGFR mRNA, we observed a down-regulation both in normoxia and hypoxia (Fig. 5A).

3.6. HI inhibits cell migration and invasion

Migration plays an important role in angiogenesis and is a prerequisite for tumor-cell invasion and metastasis. We explored whether HI was able to inhibit HUVECs migration and invasion. In normoxia, the inhibition of migration and invasion was more pronounced than in hypoxia (Fig. 6A and B). Actually, HI at the highest tested concentration (0.93 mg/mL) and after 6 h of treatment led to a more pronounced reduction of the migrated cell relative variation in normoxia compared to hypoxia (0.65 ± 0.01 *vs*

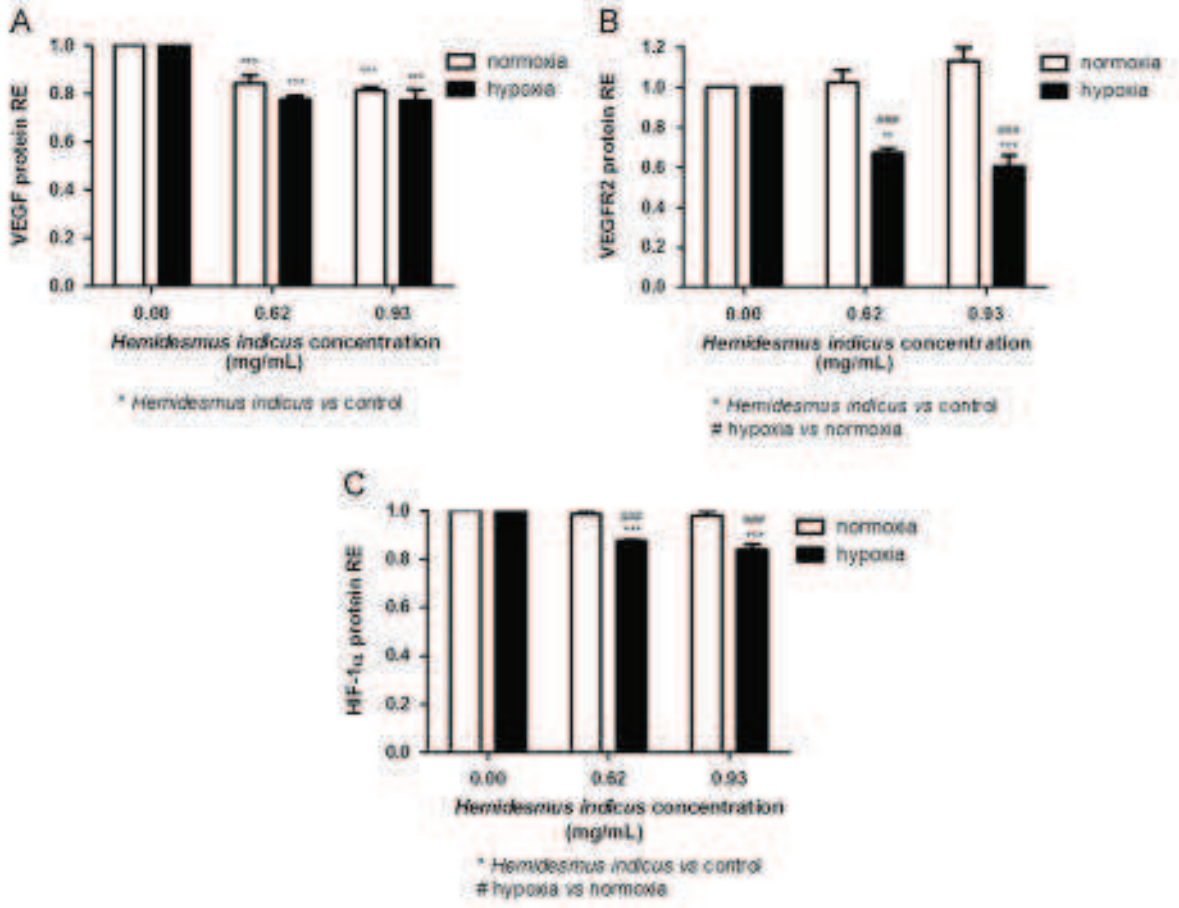

Fig. 4. Relative protein expression (RE) of VEGF (A), VEGFR-2 (B) and HIF-1α (C) in HUVECs after treatment with HI for 6 h.

0.789 ± 0.003, respectively) (Fig. 6A). In the same way, the invaded cell relative variation was 0.50 ± 0.03 in normoxia *vs* 0.63 ± 0.07 in hypoxia (Fig. 6B).

4. Discussion

Here, we provide first evidence that HI exerts antiangiogenic properties that could contribute to its cancer therapeutic potential. The model used covers multiple steps relevant of angiogenesis, such as endothelial cell proliferation, formation of capillary-like structures, quantification of the sprouting, endothelial cell migration and invasion. Of note, those events were analyzed also in hypoxia, which represents a tumor microenvironment strongly stimulating tumor angiogenesis.

Angiogenesis has been identified as a hallmark of tumor progression and anti-angiogenic therapy has become a new anticancer strategy. Nowadays, numerous anti-angiogenic therapeutics are used in association with cytotoxic drugs (Cesca et al., 2013), or in maintenance treatment (Johnsson et al., 2013). Several plant products and extracts, endowed with multiple pharmacological activities, modulate many key factors involved in the complex regulation of the angiogenesis signaling pathway. Among those, fucoidan from *Undaria pinnatifida* (Liu et al., 2012), *Trifolium pretense* L. (Krenn and Paper, 2009), or *Rhizoma rhei* extracts (He et al., 2011) inhibit angiogenesis thorough the modulation of the VEGF pathway.

HI inhibited angiogenesis in normoxia and hypoxia through the regulation of key factors of the neovascularization process. Endothelial cell proliferation is strictly correlated with the angiogenic and metastatic process (Browne et al., 2006). In both O_2 conditions, HI reduced proliferation without showing any cytotoxic effect.

HI inhibited microvessel outgrowth in a dose-dependent manner, also in association with VEGF, a well-know angiogenesis inducer. Of note, however, HI 0.31 mg/mL appeared to stimulate angiogenesis. This evidence is not surprising. Indeed, hormetic dose–responses are often observed with anti-angiogenic agents. In particular, a bell-shaped dose–response can be recorded, where an agent exerts a stimulatory effect at low doses, which is diminished at higher doses. At higher doses, an inhibitory effect may be observed. Agents that exhibit hormetic dose–response include: 5-fluorouracil, ATN-161, bortezomib, cisplatin, endostatin, enterostatin, integrin inhibitors, interferon-α, plasminogen activator-1, rapamycin, rosiglitazone, statins, thrombospondin-1, TGF-α1 and TGF-α3 (Reynolds, 2010).

The expression levels of the principal proteins involved in the angiogenesis regulation (HIF-1α, VEGF and VEGFR-2) and their mRNAs were analyzed after treatment with HI. A down-regulation of HIF-1α protein level was observed in hypoxia, while in normoxia no modulation was reported. At gene level, HI induced a dose-dependent reduction in the expression of HIF-1α, both in normoxia and hypoxia, with the highest down-regulation observed in hypoxia. Because VEGF receptors are mainly expressed on endothelial cells (Terman et al., 1991), we included the high-affinity receptor tyrosine kinase VEGFR-2 to our investigations. Between the two VEGF receptors, VEGFR-2 appears to be the major transducer of VEGF signals in endothelial cells (Jung et al., 2009). In the light of these considerations, inhibition of VEGFR-2 might represent an interesting

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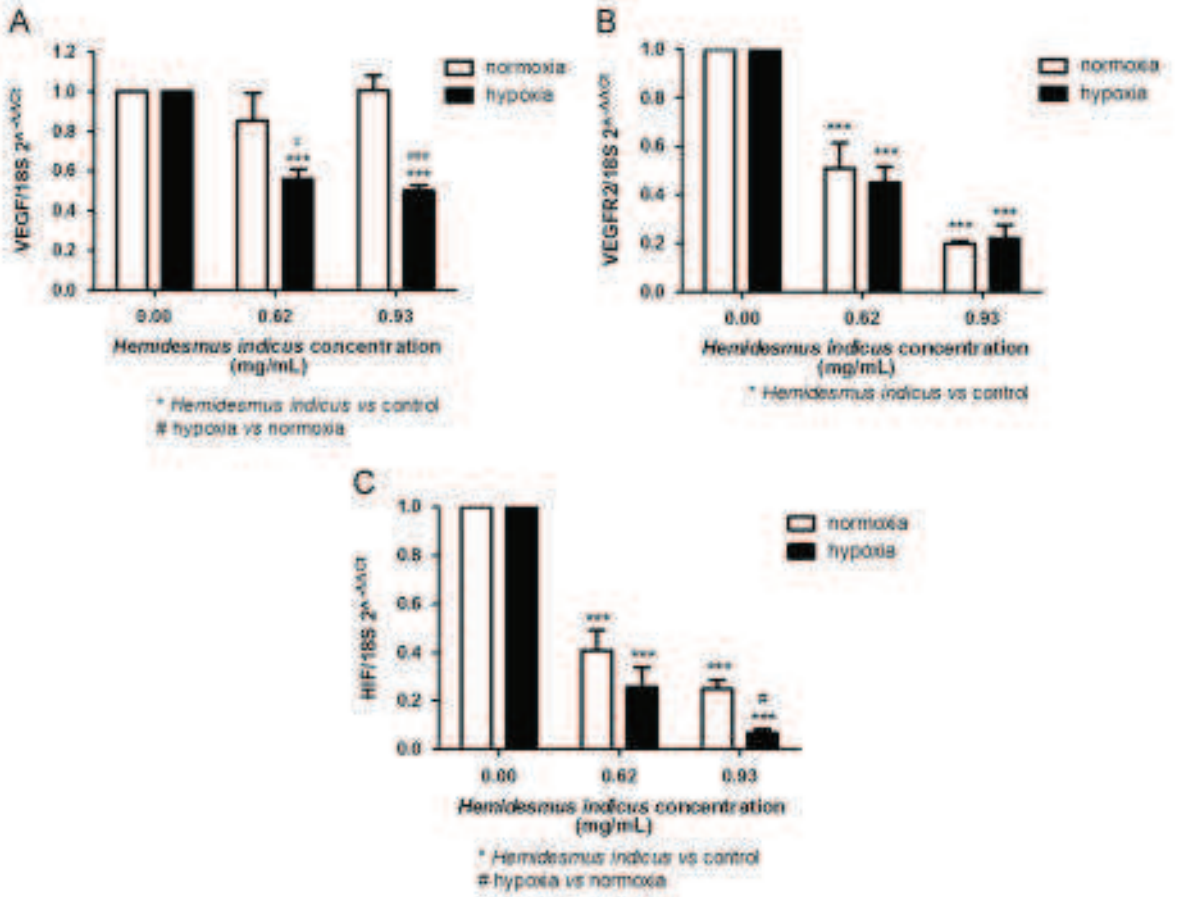

Fig. 5. Relative mRNA expression of VEGF (A), VEGFR-2 (B) and HIF (C) after treatment of HUVECs with HI for 6 h.

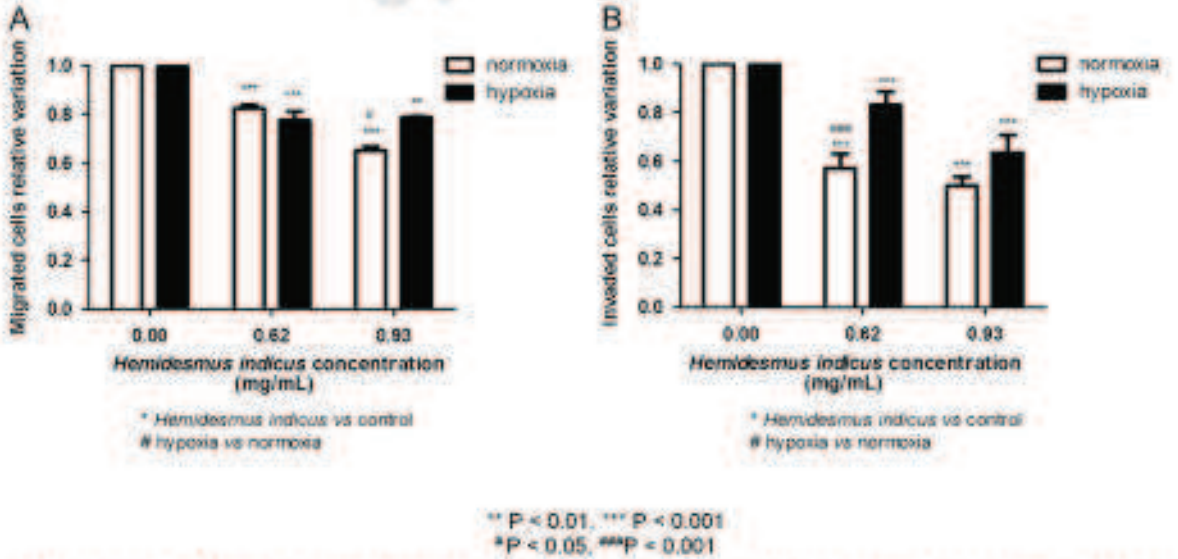

Fig. 6. Relative variation of HUVEC migrated (A) and invaded (B) cell number following HI treatment for 6 or 24 h in normoxic and hypoxic conditions.

approach for anti-angiogenic interventions. HI reduced VEGFR-2 protein expression in hypoxia and down-regulated VEGFR-2 mRNA both in hypoxia and normoxia.

Taken together, our results suggest that the inhibition of angiogenesis by HI is mediated through two distinct mechanisms according to the oxygen availability. In normoxia, the reduced expression of VEGF seems to be the main cause of angiogenesis inhibition. On the other hand, the angiogenesis inhibition induced in hypoxia is regulated by a more complex mechanism involving firstly HIF-1α inhibition, followed by VEGF and VEGFR-2 down-regulation.

Blood vessel formation is the result of multiple molecular events, where VEGF constitutes one of the central factors. It acts as an endothelial mitogen and induces endothelial cell survival, migration, differentiation, and self-assembly (Affara and Robertson, 2004). VEGF

mRNA expression is regulated by HIF-1α and NF-κB (Josko and Mazurek, 2004). HIF-1 is an oxygen-dependent transcriptional activator: in normoxia, HIF-1α is degraded by the proteasome after post-transcriptional modification; under hypoxia, HIF-1α is stable, translocates to the nucleus and favors transcription of >60 target genes, including VEGF (Semenza, 2003). HI strongly reduced the mRNA level of HIF-1 in either normoxia and hypoxia. However, it evokes a differential regulation at protein level: a down-regulation was evident only in hypoxia. A lack of correlation between mRNA and protein abundance is common. Indeed, proteins have very different half-lives due to the result of varied protein synthesis and degradation, and varied post-transcriptional mechanisms involved in turning mRNA into protein. Down-regulation of mRNA concurrent with up-regulation of protein expression may occur when a protein half-life is increased due to stabilization-components involved with the protein's normal turnover or the protein may be stabilized through protein–protein interactions. Further experiments could assess the modulation by HI of microRNAs that moderate the HIF-1α transcriptional program and of the proteasome-mediated degradation of HIF-1 in normoxia.

Of note, previous studies reported that NF-κB is a direct modulator of HIF-1α expression (van Uden et al., 2008) and regulates endothelial cell integrity and vascular homeostasis *in vivo*, also through the modulation of VEGF mRNA expression (Josko and Mazurek, 2004). For example, zerumbone derived from a subtropical ginger, *Zingiber zerumbet* Smith, inhibits tumor angiogenesis via NF-κB in gastric cancer (Tsuboi et al., 2014). Additionally, suppression of NF-κB activity abrogated the NF-κB activation and the pro-angiogenic activities of glioma cells (Jiang et al., 2013). Very recently, it was reported that HI prevented active NF-κB from binding to its nuclear DNA (Guerrini et al., 2014). Consequently, NF-kB-regulated mechanisms, including HIF-1 and VEGF expression, involved in proangiogenic signaling under hypoxia might be sensitive to HI treatment.

Neovascularization influences the dissemination of cancer cells throughout the entire body, eventually leading to metastasis formation. The vascularization level of a solid tumor is an excellent indicator of its metastatic potential (Nishida et al., 2006). In this context, HI significantly suppressed invasion and migration of endothelial cells. These activities were not due to the antiproliferative influence of HI, because they were observed under experimental conditions (0.31 mg/mL in hypoxia and normoxia and 0.62 mg/mL in hypoxia) where cell proliferation was not impeded. Low oxygen conditions accelerate cell invasion (Miyoshi et al., 2006), justifying the less pronounced inhibition of invasion resulted from HI treatments in hypoxia than in normoxia.

HI showed a higher potency in the cellular analysis (*i.e.* proliferation, tube formation, migration and invasion assay) under normoxia condition rather than hypoxia condition, but conversed results in the molecular analysis. This could be due to the typical higher expression of HIF-1α, VEGF and VEGFR2 in hypoxia than in normoxia. Hypoxia treatment significantly enhances VEGF secretion and HIF-1α expression (Kumar et al., 2011) and this can allow an easier identification of subtle molecular effects. Furthermore, the degradation of HIF-1α operated in normoxic conditions by the proteasome may not allow recording the effects of HI on HIF-1α expression.

As to the pharmacological relevance of the concentrations of HI used in our study and taking into account that a decoction is usually more diluted than an extract (Marriott et al., 2006), the active concentrations of HI are well above those indicated by the American National Cancer Institute for a crude extract (Suffness and Pezzuto, 1990).

On the bases of its multiple anticancer mechanisms, HI can represent an interesting botanical drug. The chemical constituents of a botanical drug are not always well defined. In many cases, the active constituent is not identified nor is its biological activity characterized. Accordingly, there is a concern about the therapeutic consistency of marketed batches of a botanical drug (Fimognari et al., 2012). As far as the therapeutic consistency of HI is concerned, our HPLC phytochemical analysis demonstrated the presence of 2-hydroxy-4-methoxybenzaldehyde, 3-hydroxy-4-methoxybenzaldehyde, 2-hydroxy-4-methoxybenzoic acid and for the first time lupeol acetate, together with minor compounds such as lupeol, β-sitosterol and β-amyrin acetate, which can be used as fingerprint. Interestingly, vanillin (4-hydroxy-3-methoxybenzaldehyde) and ethyl vanillin, two structural analogue of 2-hydroxy-4-methoxybenzaldehyde and 3-hydroxy-4-methoxybenzaldehyde, have been shown to possess antiangiogenic activity (Jung et al., 2010; Lirdprapamongkol et al., 2009). The phytochemical analysis performed on three batches of HI demonstrated that the levels of the seven phytomarkers were not statistically different among batches. Of note, the three batches were not statistically different in terms of biological activities.

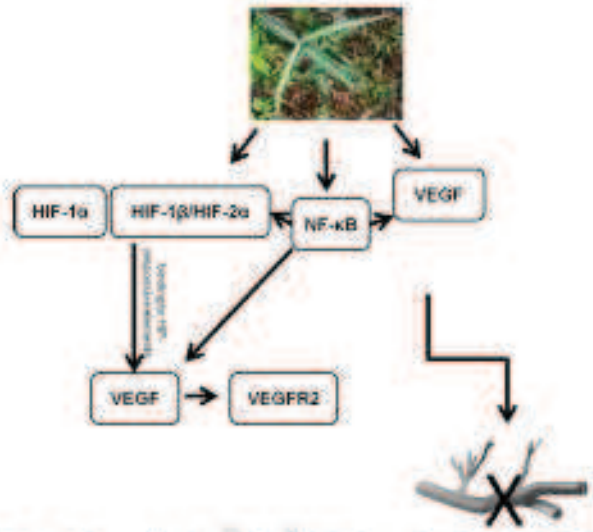

Fig. 7. Possible cascade of events involved in the anti-angiogenic activity of HI.

5. Conclusions

The findings of this study suggest that the antiangiogenic activity of HI is based on multiple interactions with critical steps in the angiogenic cascade (Fig. 7). VEGF expression stimulated by HIF-1α as well as endothelial cell migration and differentiation represent important targets of HI action and might contribute to its cancer therapeutic efficacy that is presently emerging.

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Biological and chemo-diverse characterization of Amazonian (Ecuador) *Citrus* petitgrains

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Summary

Six Amazonian petitgrain samples from *C. nobilis* Lour., *C. aurantium* L., *C. limon* L. and mixture of *Citrus* spp. (Rutaceae), named CN, CA, CL1, CL2, C1 and C2, were chemically characterized by GC-MS and ^{13}C NMR and evaluated for antioxidant acitivity (DPPH and β-carotene bleaching tests), for antimicrobial properties (disk diffusion method) and for antifungal capacity (agar vapour assay). CN, C1, C2 samples evidenced the most interesting results: CN (γ-terpinene/linalool chemotype: 14.3 %/41.6 %, with a considerable amount of thymol: 9.0 %), and C1 (linalool, 18.3 %; sabinene, 11.6 %; thymol, 5.5 %), showed relevant antioxidant activity with both DPPH (IC_{50} = 3.52 and 5.48 mg/ml, respectively) and β-carotene (IC_{50} = 0.387 and 0.491 mg/ml, respectively). Antibacterial properties of CN and C1 against *P. mirabilis* (MIC = 0.61 mg/ml for both) and *B. subtilis* (MIC = 0.61 and 0.44 mg/ml, respectively) were most probably due to thymol. C2 (geranial: 34.7 %, neral: 33.1 %) evidenced a valuable bioactivity against *C. albicans* (MIC = 0.44 mg/ml). The 50 % growth inhibition (IC_{50}) of the dermatophytes *T. mentagrophytes* and *N. cajetani* was reached with amounts of C1, C2 and CN less than 4 µl/plate. Bioactivity of Amazonian *Citrus* spp. CN, C1 and C2 essential oils suggests their potential use as food preservatives or additives in cosmeceuticals as preventive against dermatophytic fungal infections.

Introduction

Herbs and spices have been employed since ancient times as flavouring and preservatives agents for food, but only in the last decade scientific research has focused its interest on essential oils and extracts as natural sources of antimicrobial and antioxidant compounds, as safer alternative additives for food preservations (Burt, 2004; Shaaban et al., 2012).

While *Citrus* essential oils are usually cold-extracted with mechanical systems (as stated in European Pharmacopoeia, VII ed.), the so-called petitgrain oils are obtained by distillation of *Citrus* leaves, buds and small branches from *Citrus* spp. adult plants (Dugo et al., 2010).

The composition of *Citrus* leaves essential oils are not as well defined as the correspondent peel oils (Dugo et al., 2010) and studies concerning these aspects have been often reviewed (Lota et al., 2001a; Lota et al., 2001b; Lota et al., 2002; Dugo et al., 2010). Due to its pleasant and characteristic fragrance, bitter orange (sour orange, bigarade) petitgrain is the most important and appreciated among leaf essential oils: it is widely used in perfumery for preparation of eau de Colognes, lotions and soaps because of its good resistance to alkaline medium. Sour orange plants are cultivated mainly in the Mediterranean countries (France, Italy, Spain) and in Paraguay (Lota et al., 2001a; Dugo et al., 2010). The best known and most employed species is *Citrus aurantium* L. Other *Citrus* spp. petitgrains (lemon, mandarin, etc.) are afforded at small quantities. Lemons (*Citrus limon* in particular) are cultivated in Mediterranean countries, southern California and Argentina while mandarins (other several *Citrus* spp. and hybrids) are cultivated in Mediterranean countries, Japan, Brazil, Argentina, United States (known as tangerines) and Australia (Lota et al., 2000; Dugo et al., 2010).

Although there is extensive literature on *Citrus* spp. petitgrain composition, very few papers concern biological properties or report correlations among phytochemical data, chemodiversity and biological activities (Dugo et al., 2010; Shaaban et al., 2012). In fact it is known that the wide chemodiversity which characterizes this kind of phytocomplexes could be due to several variables, affecting the chemical profile, as geographical origin, time of collection and cultivars (Guerrini et al., 2011). These variables can determine chemical diversities in essential oils obtained from the same species which necessarily could reflect different bioactivities and possible functional uses.

In light of these premises, in Ecuadorian traditional ethnomedicine *Citrus* spp. leaves are used to treat stomachaches (Rios et al., 2007).

The present paper represents the first report about *Citrus* spp. petitgrain essential oils from plants grown at the margin of the Amazonian forest (Ecuador). The aim is to evaluate possible, distinctive bioactivity properties of chemotypes driven due to this peculiar geographical origin. In fact, it is known that the Amazonian biodiverse hot-spot is characterized by an biotic and abiotic set of conditions which can force the secondary plant metabolism to peculiar and unique profiles with corresponding interesting bioactivities (Ryder Wilke et al., 2010; Rossi et al., 2013). Moreover, the ethnobotanical use of *Citrus* spp. leaves is sometimes performed without particular attention to the single species since the shape is similar and they are collected without distinction. This fact contributes to have preparations with mixed-species leaves (mainly as flavouring and anxiolytic agents) (Hanazaki et al., 2000). In the present work, the study of essential oils obtained from mixture of *Citrus* leaves would mime this ethnobotanical evidence. Therefore, different Amazonian *Citrus* spp. petitgrains from single and mixed species leaves were evaluated for their chemical composition through GC, GC-MS and ^{13}C NMR in order to point out the possible chemodiversity aspects related to their geographical origin and to check for functional properties (antimicrobial, antifungal, antioxidant) to valorize their possible applicative uses in food and/or health fields.

Material and methods

Chemicals

All the solvents employed for chemical analyses and bioassays were chromatographic grade. Solvents and pure compounds were all purchased from Sigma-Aldrich Italy (Milano, Italy). *Thymus vulgaris* essential oil, thymol-chemotype employed as reference phytocomplex (Sacchetti et al., 2005), was purchased from Extrasynthese (Genay, France). Microbial culture media were obtained from Oxoid Italia (Garbagnate, Italy).

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Plant material

C. nobilis (named CN), *C. aurantium* (named CA), *C. limon* (named CL1 and CL2) fresh leaves and fresh leaves mixture of genus *Citrus* spp. (named C1 and C2) were purchased by Fundacion Chankuap (Quito, Ecuador), non-governmentive organization which has as main target the valorization of Amazonian sources recovering plant material to directly obtain commercial products from natives, with the cooperation of our research about Ecuadorian Amazonian biodiversity. For what concerns the essential oil mixtures, no information have been given by Fundacion Chankuap regarding the different *Citrus* species employed and their quantitative ratio. Leaves were collected in September 2010 from wild adult plants growing in three different locations on the outskirts of the Wasak'entsa reserve in eastern Ecuador (77°.15" W/2°.35" S) and positively identified by Fundacion Chankuap (Quito, Ecuador). Dried specimens were deposited at the Department of Biology and Evolution, University of Ferrara, Code C1, C2, CA1, CN1, CL1, CL2.

Essential oils isolation

Essential oils were in situ extracted for 8 hours through steam distillation of *C. limon*, *C. aurantium*, *C. nobilis*, mixture of *Citrus* spp. fresh leaves (approximately 10 kg) using a mobile essential oil distiller (Essential Oil Company, Portland, OR, USA) set up following the parameters reported in literature (Horwitz, 2003). Essential oil yields have been achieved through three different distillations of fresh plant material belonging to *Citrus* spp. The petitgrains were dried over anhydrous sodium sulfate and stored in airtight glass vials with Teflon-sealed caps at -18.0±0.5 °C in the absence of light until analysis.

Gas Chromatography

Essential oil samples were analyzed and the relative peak areas for individual constituents averaged. The relative percentages were determined using a ThermoQuest GC-Trace gas-chromatograph equipped with a FID detector and a Varian FactorFour VF-5ms poly-5 % phenyl-95 %-dimethyl-siloxane bonded phase column (i.d., 0.25 mm; length, 30 m; film thickness, 0.15 μm). Operating conditions were as follows: injector temperature 300 °C; FID temperature 300 °C, carrier (Helium) flow rate 1 ml/min and split ratio 1:50. Oven temperature was initially 55 °C and then raised to 100 °C at a rate of 1 °C/min, then raised to 250 °C at a rate of 5 °C/min and finally held at that temperature for 15 min. One μl of each sample dissolved in CH_2Cl_2 was injected. The percentage composition of the oils was computed by the normalization method from the GC peak areas, without using correction factors.

Gas Chromatography-Mass Spectrometry

Essential oil constituents were then analyzed by a Varian GC-3800 gas chromatograph equipped with a Varian MS-4000 mass spectrometer using electron impact and hooked to NIST library.

The conditions were the same reported for GC analysis and the same column was used. The MS conditions were as follows: ionization voltage, 70 eV; emission current, 10 μAmp; scan rate, 1 scan/s; mass range, 29-400 Da; trap temperature, 150 °C, transfer line temperature, 300 °C. The constituents of the volatile oils were identified by comparing their relative retention time, KI and the MS fragmentation pattern with those of other essential oils of known composition, with pure compounds and by matching the MS fragmentation patterns and retention indices with the above mentioned mass spectra libraries and with those in the literature (Adams, 2007). In order to determine the Kovats index of the components, a C_9-C_{22} *n*-alkanes (Sigma-Aldrich) was added to the essential oil before injecting in the GC–MS equipment and analyzed under the same conditions as above.

NMR spectroscopy

^{13}C NMR spectra were recorded at 100.58 MHz and at temperature of 303 K with a Varian Gemini-400 spectrometer. The essential oils were dissolved in $CDCl_3$ (70 mg/0.8 mL) into a 5 mm NMR and solvent signal was used for spectral calibration (central line of triplet at 77.0 ppm). Chemical shifts (ppm) and peak attribution were based on comparisons of the resonances in ^{13}C NMR spectrum of the essential oil with those of pure standards and mixture of these (α-pinene, sabinene, β-pinene, D-limonene, γ-terpinene, linalool, citronellal, 4-terpinenol, citral, thymol) present in our spectral library (Guerrini et al., 2006) or according with those of literature (Kubeczka, 2002); SDBS (Saito et al., 2009).

Biological activities

Antioxidant, antifungal and antimicrobial activities were performed comparing all the data with those obtained with appropriate pure synthetic compounds and/or commercial *Thymus vulgaris* essential oil, in order to have positive control references with single compounds or comparable phytocomplexes reputed for their functional bioactivities. The use of a phytocomplex known for its chemical and biological properties (e.g. thyme essential oil) as a positive reference results particularly indicative of the real functional efficacy of a tested extract (Maietti et al., 2013). Data reported for each assay are the average of three determinations of three independent experiments.

Antifungal and antimicrobial strains

According to previously described methodology (Guerrini et al., 2006; Maietti et al., 2013), *Citrus* petitgrain antifungal and antimicrobial activities were performed with agar vapor method and standard disk diffusion technique respectively.

For antibacterial assays, *Gram*-positive (*Enterococcus faecalis* ATCC 29212, *Staphylococcus aureus* ATCC 29213 and *Bacillus subtilis* ATCC 7003) and *Gram*-negative (*Escherichia coli* ATCC 4350, *Proteus mirabilis* ATCC 29852 and *Klebsiella oxytoca* ATCC 29516) bacterial strains were employed. Antifungal activity was assessed on yeast *Candida albicans* ATCC 48274, on phytopathogen strains (*Botrytis cinerea* Micheli ATCC 48339, *Pythium ultimum* Trow, kindly supplied by Prof. G. D'Ercole (Institute of Vegetal Pathology, University of Bologna, Italy), *Magnaporthe grisea* ATCC 64413) and dermatophyte strains (*Trichophyton mentagrophytes var. mentagrophytes* (Robin) Blanchard CBS (Centraal Bureau Voor Schimmelcultures, Baarn, the Netherlands) 160.66, *Nannizzia cajetani* Ajello IHME (Institute of Hygiene and Epidemiology-Mycology (IHME) Brussels, Belgium) 3441 and *Trichophyton rubrum* (Castellani) Sabouraud IHME 4321).

Antimicrobial activity: disks diffusion method

Mother cultures of each bacteria were set up 24 h before the assays in order to reach the stationary phase of growth. The tests were assessed by inoculating from the mother cultures Petri dishes with proper sterile media with the aim of obtaining the microorganisms concentration 10^6 CFU/ml. For bacteria, aliquots of dimethyl sulfoxide (DMSO) were added to the essential oils in order to obtain a 0.01-50.0 mg/ml concentration range and then deposited on sterile paper disk (6 mm diameter, Difco).

Bioactivity against the yeast *Candida albicans* was also processed. Mother cultures were set up inoculating 100 ml YEPD liquid medium (Yeast Extract and Potato Dextrose) in 250 sterile flasks and

incubated in the dark at 30 °C in order to assess growth curves. From each mother cultures at the stationary phase of growth, broth dilutions were made to obtain the strain concentration of 10^5 CFU/ml to inoculate Petri dishes with agarized YEPD for bioassays. Then, 10 µl of DMSO-essential oil sample solutions were prepared in order to have an assay range 0.01-50.0 mg/ml, and then deposited on sterile paper disk (6 mm diameter, Difco). The Petri dishes were successively incubated at 30 °C in the dark and checked for evaluating the growth inhibition after 48 h. both for bacteria and *Candida* streams, the lowest concentration of each essential oil showing a clear zone of inhibition was taken as the MIC (Minimum Inhibitory Concentration). Negative controls were set up with 10 µl of DMSO in the test solution, while positive ones were assessed with *T. vulgaris* essential oil.

Antifungal activity: agar vapour assay

Biological activity of *Citrus* petitgrains against three phytopathogenic and three dermatophytic fungi was performed by using the agar vapour method (MAIETTI et al., 2013). They were grown in Petri plates (90 mm) supplemented with 15 ml/plate of potato dextrose agar, inoculated with 6 mm plugs from stationary-phase cultures. The plates were then incubated for 24 h at 26 ± 1 °C. Successively, sterilized filter paper discs (diameter 9.0 mm) were absorbed with different volumes of *Citrus* petitgrains samples ranging from 0.20 to 25.00 µl, and placed inside the upper lid of each plate. Plates were kept in an inverted position, tightly sealed with parafilm, and incubated for 7 days at 26 ± 1 °C. Blanks served as a negative control. Commercial *T. vulgaris* essential oil was prepared as described above for petigrain samples, with volumes ranging from 0.20 to 25.00 µl, and considered as a positive control reference phytocomplex. Three replicates were made for each treatment. After 7 days the results were determined as the inhibition of radial growth and expressed as the amount of essential oil that led to 50 % inhibition of growth in each fungal strain (IC_{50}).

Antioxidant activities

Radical scavenging and antioxidant properties of essential oils were performed through different assays, namely the DPPH assay and the β-carotene bleaching test according to previously described methods. This approach permits the antioxidant effectiveness of an essential oil to be more carefully defined, as it is almost impossible to express the antioxidant activity as an absolute value that is universally recognizable, besides being expressed by only one type of assay (MAIETTI et al., 2013). *T. vulgaris* essential oil was used as positive controls. Essential oils antioxidant activity was considered as the IC_{50}, calculated from inhibition curves obtained by plotting the % inhibition against oil concentration. All the data collected for each assay are the average of three determinations for three independent experiments.

Statistical analysis

Relative standard deviations and statistical significance (Student's t test; $P \leq 0.05$), one-way ANOVA and LSD post hoc Fisher's honest significant difference test, were given, where appropriate, for all data collected. All computations were made using the statistical software STATISTICA 6.0 (StatSoft Italia srl).

Results

Chemical fingerprinting

Steam distillation of *Citrus* spp. leaves provided petitgrains with yield from 0.20 ± 0.02 g/100g for CN to 0.29 ± 0.03 g/100g for CL1 and density that covered a range of 0.82-0.92 g/ml (Tab. 1).

The Ecuadorian CA petitgrain exhibited as major components sabinene (38.3 %), *trans*-E-ocimene (6.7 %), linalool (8.8 %); other minor components were 3-carene (8.9 %), D-limonene (7.9 %), β-myrcene (3.4 %), 4-terpinenol (2.5 %), α-pinene (1.9 %), geranal (1.9 %), β-pinene (1.8 %). CL1 essential oil evidenced an high abundance of limonene (52.7 %) and linalool (15.1 %) and as minor compounds citronellal (3.1 %), sabinene (2.7 %) and carvone (2.6 %), instead in CL2 petitgrain predominated sabinene (36.1 %) followed by limonene (24.1 %), linalool (4.7 %), 4-terpinenol (3.9 %), γ-terpinene (3.9 %), citronellal (3.6 %), *trans*-β-ocimene (3.2 %), α-terpinene (2.8 %), β-myrcene (2.6 %). *Citrus nobilis* petitgrain evidenced high abundance of linalool (41.6 %) and appreciable contents of γ-terpinene (14.3 %) and thymol (9.0 %), followed by *trans*-E-ocimene (10.9 %), p-cymene (4.1 %), α-pinene (3.6 %), β-pinene (3.1 %) limonene (2.8 %) as minor compounds. Finally, linalool (18.3 %), sabinene (11.6 %), limonene (11.1 %), γ-terpinene (10.6 %), thymol (5.5 %), β-pinene (4.9 %), *trans*-E-ocimene (4.8 %) and p-cymene (3.4 %) were the most characteristic compounds for C1 petitgrain samples; C2 petitgrain, instead, evidenced geranal (34.7 %) and neral (33.1 %) followed by β-myrcene (5.4 %), linalool (4.7 %), and geraniol (3.1 %) as the most abudant chemicals (Tab. 1).

To contribute to define a metabolomic fingerprinting of *Citrus* spp. essential oils, ^{13}C-Nuclear Magnetic Resonance (NMR) of the most abundant chemical standard compared with whole essential oil spectrum was performed confirming the evidences emerged by GC-MS (supplementary materials, Tab. 5 and Fig. 1). Mono-dimensional ^{13}C spectrum revealed typical and numerous diagnostic signals for characterizing the chemical makeup of carbons and, therefore, of the functional groups typical of the examined molecules.

Antioxidant activities

The essential oils examined evidenced interesting antioxidant properties with sligthly different among the samples (Tab. 2). In particular, CN petitgrain exhibited a good radical antioxidant activity both with DPPH test (IC_{50}=3.52±0.25 mg/ml) and β-carotene bleaching assay (IC_{50}=0.387±0.021 mg/ml). These results are particularly relevant if compared to that obtained with commercial *Thymus vulgaris* essential oil (IC_{50}=1.24±0.10 mg/ml), taken as reference phytocomplex (SACCHETTI et al., 2005). C1 petitgrain, that showed a relative abundance of γ-terpinene (10.6 %) and thymol (5.5 %), probably to relate to the interesting DPPH activity (IC_{50}=5.48±0.45 mg/ml), displayed instead a lower activity in β-carotene bleaching test (IC_{50}=0.491±0.041 mg/ml). Thymol has been tested as pure compound in DPPH and β-carotene bleaching tests suggesting that the antioxidant capacity displayed by essential oils could be mainly due to the presence and the abundance of this substance.

Antimicrobial activity

Evaluation of antibacterial activity (Tab. 3), expressed as MIC (Minimun Inhibitory Concentration), revealed that CN petitgrain was the most active among the *Citrus* spp. phytocomplexes against *Gram*-negative as well as *Gram*-positive bacteria. The most interesting results were against *P. mirabilis* for CN, C1 and CA, against *B. subtilis* for CN and C1 and finally against *E. coli* for CN and CA since MICs were comparable. The antibacterial properties of CN petitgrain were relevant also against *S. aureus* and *E. faecalis* (0.78±0.08 and 0.95±0.09 mg/ml) if compared to the positive control *T. vulgaris*. No remarkable inhibition activity was observed against *K. oxytoca*. C2 petitgrain was instead particularly active against the yeast *C. albicans*, with a MIC of 0.44±0.05 mg/ml.

Tab. 1: Chemical composition of *Citrus* petitgrains

Compound	KI[a]	*RA*[b]					
		CN	CA	CL1	CL2	C1	C2
α-Thujene	930	1.8	0.5	0.2	1.0	1.1	0.1
α-Pinene	939	3.6	1.9	0.7	2.7	2.7	0.2
Sabinene	977	0.4	**38.3**	2.7	**36.1**	**11.6**	0.6
β-Pinene	979	3.1	1.8	0.9	3.4	4.9	0.2
6-Methyl-5-hepten-2-one	986	-	-	0.3	0.1	-	1.9
β-Myrcene	991	0.7	3.4	0.3	2.6	1.1	5.4
α-Phellandrene	1003	0.1	0.6	-	0.2	-	-
p-Mentha-1(7),8-diene	1004	-	-	-	-	0.6	-
3-Carene	1009	-	8.9	-	-	-	-
α -Terpinene	1017	0.4	1.0	0.3	2.8	0.4	-
p-Cymene	1025	4.1	0.5	1.9	0.3	3.4	0.5
D-Limonene	1029	2.8	7.9	52.7	24.1	11.1	0.7
1,8-Cineole	1031	-	-	-	0.2	0.6	-
cis-Z-Ocimene	1031	0.8	0.2	-	0.8	0.4	0.4
trans-E-Ocimene	1037	10.9	6.7	-	3.2	4.8	0.6
γ-Terpinene	1051	14.3	1.6	0.3	3.9	10.6	0.6
cis-Sabinene hydrate	1060	0.1	0.3	0.4	0.8	0.1	-
trans-Linalool oxide	1073	0.1	-	1.5	-	-	-
cis-Linalool oxide	1087	-	-	1.6	-	-	-
Isoterpinolene	1088	-	0.3	-	-	-	-
Terpinolene	1089	1.6	1.7	0.5	0.8	0.9	0.2
p-Cymenene	1091	0.9	-	-	-	0.4	0.2
Linalool	1097	41.6	8.8	15.1	4.7	18.3	4.7
1,3,8-p-Menthatriene	1110	0.3	-	-	-	-	0.3
cis-p-Ment-2-en-1-ol	1122	-	0.1	0.7	-	-	-
cis-Limonene oxide	1138	-	-	0.5	-	-	-
trans-p-Ment-2-en-1-ol	1141	-	-	0.8	-	-	0.3
trans-Limonene oxide	1142	-	-	-	-	0.1	0.5
Isopulegol	1150	-	-	0.2	0.1	0.8	-
Citronellal	1153	-	1.4	3.1	3.6	0.8	0.3
cis-Linalyl oxide	1174	-	-	0.2	-	-	-
trans-Linalyl oxide	1176	-	-	0.1	-	-	-
4-Terpinenol	1177	0.2	2.5	0.7	3.9	0.6	0.2
α-Terpineol	1189	0.2	0.6	0.5	0.3	0.4	0.7
cis-Dihydrocarvone	1193	-	-	0.6	-	-	-
trans- Dihydrocarvone	1201	-	-	0.3	-	-	-
trans-Carveol	1217	-	-	1.4	-	-	-
Citronellol	1226	-	0.3	0.5	0.7	-	0.3
cis-Carveol	1229	-	-	0.6	-	-	-
Nerol	1230	-	0.3	-	0.7	-	-
Neral	1238	-	1.6		0.1	0.2	33.1
Carvone	1243	-	-	2.6	-	-	-
Geraniol	1254	-	0.1	-	-	-	3.1
Geranial	1267	-	1.9	-	0.1	0.3	34.7
Perillaldehyde	1272	-	-	0.4	-	-	1.0

Compound	KI[a]	RA[b]					
		CN	CA	CL1	CL2	C1	C2
Citronellyl formate	1274	-	-	0.9	-	-	-
2-Undecanone	1294	-	-	-	-	-	1.3
Geranyl formate	1298	-	-	0.5	-	-	0.4
Carvacrol	1299	-	-	-	-	-	-
δ-Elemene	1338	-	-	-	0.1	-	-
Citronellyl acetate	1353	-	0.3	0.9	0.2	0.1	-
Neryl acetate	1362	-	0.5	0.8	0.2	0.1	-
Geranyl acetate	1381	-	0.4	-	-	-	0.4
β-Elemene	1391	-	1.4	-	-	0.1	-
Methyl methylantranilate	1406	0.3	0.2	-	-	**13.1**	**3.1**
trans-β-Caryophyllene	1419	0.9	0.8	-	0.6	1.3	0.2
cis-Carvyl propanoate	1422	-	-	0.6	-	-	-
γ-Elemene	1433	-	-	-	-	-	-
trans-α-Bergamotene	1435	-	-	-	-	-	0.2
α-Humulene	1455	0.1	0.4	-	-	0.2	-
β-(E)-Farnesene	1457	-	0.2	-	-	-	-
2-Tridecanone	1470	-	-	-	-	-	0.5
Bicyclogermacrene	1500	0.2	0.2	-	0.3	0.4	-
Germacrene A	1509	-	0.7	-	-	0.2	-
Germacrene B	1561	-	-	-	0.1	-	-
Spathulenol	1578	-	-	-	-	0.1	-
Caryophyllene oxide	1583	-	-	0.5	-	-	-
1-Methoxy-9(E)-octadecen	1651	-	-	-	-	0.7	-
β-Sinensal	1700	-	0.6	-	-	-	-
α-Sinensal	1757	0.3	0.1	-	-	0.2	-
TOTAL IDENTIFIED		99.8	99.0	96.7	98.7	98.8	98.9
Extraction yield (g/100g)		0.20±0.02	0.23±0.01	0.29±0.03	0.29±0.01	0.23±0.01	0.24±0.03

[a] Antilinetic indices calculated on a Varian VF-5ms column
[b] Relative peak area calculated by GC-FID
The major components (bold letters) of samples were identified by ^{13}C NMR

Tab. 2: Antioxidant activity of *Citrus* petitgrains performed by DPPH, β-carotene bleaching assays and compared to commercial *Thymus vulgaris* essential oil and pure compound thymol.

Essential oils	IC_{50} ± SD (mg/ml)	
	DPPH	β-carotene bleaching
CN	3.52 ± 0.25	0.387 ± 0.021
CA	7.12 ± 0.50	0.432 ± 0.037
CL1	9.90 ± 0.71	0.986 ± 0.088
CL2	7.45 ± 0.61	0.521 ± 0.038
C1	5.48 ± 0.45	0.491 ± 0.041
C2	8.41 ± 0.71	0.788 ± 0.066
Thymol	0.60 ± 0.05	0.09 ± 0.011
Thymus vulgaris	1.24 ± 0.10	0.164 ± 0.013

Antifungal activity

The most interesting results concerning antifungal activities (Tab. 4) were exhibited by C2 petitgrain, particularly against dermatophytes species (*T. mentagrophytes*, *N. cajetani*), that showed IC_{50} less than 0.20 μl/plate, comparable to positive control *T. vulgaris* essential oil; however, the concentrations corresponding to the 100 % growth inhibition was better for C2. This essential oil was the most active also against phytopathogens, but less active than the reference standard *T. vulgaris*.

C1 and CN petitgrains also showed good activity against all tested fungi reaching values of 50 % inhibition at concentration comprised from 2 to 8 μl/plate. The most sensitive fungal strain, however, appears to be *T. rubrum*.

The study of activity of citral (mixture of neral/geranial) standard, the most abundant component in C2, against phytopathogens and dermatophytes, confirmed the best activities against *T. mentagrophytes*, *N. cajetani*, *T. rubrum*: in particular, *T. mentagrophytes* was the most sensitive fungus since it evidenced 50 % inhibition at concentration less than 0.20 μl/plate.

Tab. 3: Antimicrobial activity of *Citrus* petitgrains compared to commercial *Thymus vulgaris* essential oil, thymol and citral.

Essential oils	MIC (mg/mL) ± SD						
	Gram-positive bacteria			*Gram*-negative bacteria			Yeast
	S. aureus ATCC29213	*B. subtilis* ATCC7003	*E. faecalis* ATCC29212	*K. oxytoca* ATCC29516	*E. coli* ATCC4350	*P. mirabilis* ATCC29852	*C. albicans* ATCC48274
CN	0.78 ± 0.08	0.61 ± 0.05	0.95 ± 0.09	2.17 ± 0.22	0.61 ± 0.06	0.61 ± 0.05	1.74 ± 0.19
CA	8.46 ± 0.64	1.52 ± 0.16	1.06 ± 0.11	3.38 ± 0.35	0.68 ± 0.07	0.68 ± 0.07	3.38 ± 0.34
CL1	4.06 ± 0.41	3.79 ± 0.37	1.99 ± 0.19	8.12 ± 0.61	6.32 ± 0.62	4.06 ± 0.41	0.88 ± 0.09
CL2	3.50 ± 0.31	1.73 ± 0.17	3.89 ± 0.38	6.05 ± 0.55	3.89 ± 0.38	3.89 ± 0.37	3.46 ± 0.33
C1	1.10 ± 0.12	0.44 ± 0.04	1.93 ± 0.18	4.38 ± 0.42	2.19 ± 0.22	0.61 ± 0.06	1.75 ± 0.18
C2	7.94 ± 0.59	1.94 ± 0.18	3.97 ± 0.37	6.17 ± 0.41	1.76 ± 0.17	3.97 ± 0.39	0.44 ± 0.08
thymol	0.31 ± 0.06	0.28 ± 0.03	0.52 ± 0.05	1.10 ± 0.21	0.29 ± 0.03	0.31 ± 0.02	0.90 ± 0.07
citral	0.50 ± 0.09	0.30 ± 0.07	0.58 ± 0.06	0.20 ± 0.03	0.25 ± 0.05	0.70 ± 0.07	0.42 ± 0.04
T. vulgaris	0.11 ± 0.01	0.11 ± 0.01	0.11 ± 0.01	0.40 ± 0.04	0.06 ± 0.01	0.12 ± 0.01	0.06 ± 0.01

Tab. 4: Antifungal activity of *Citrus* spp. petitgrains compared to commercial *T. vulgaris* essential oil, citral and thymol.

Samples	Phytopathogens			Dermatophytes		
	P. ultimum	*M. grisea*	*B. cinerea*	*N. cajetani*	*T. mentagrophytes var. mentagrophytes*	*T. rubrum*
CN	4.0 ± 0.2	6.3 ± 0.4	3.4 ± 0.3	3.7 ± 0.2	3.6 ± 0.2	2.7 ± 0.2
CA	12.7 ± 0.9	16.1 ± 1.1	15.3 ± 1.2	9.3 ± 0.9	>25	14.2 ± 1.2
CL1	8.3 ± 0.7	>25	20.7 ± 1.5	>25	19.9 ± 1.8	12.0 ± 1.1
CL2	10.0 ± 1.0	>25	18.6 ± 1.5	17.6 ± 1.9	18.3 ± 1.7	16.7 ± 1.4
C1	3.2 ± 0.4	7.9 ± 0.5	6.2 ± 0.4	6.7 ± 0.7	4.8 ± 0.3	2.0 ± 0.2
C2	2.2 ± 0.2	2.4 ± 0.3	1.9 ± 0.2	<0.20[a]	<0.20[b]	1.9 ± 0.1
T. vulgaris	0.40 ± 0.10	0.38 ± 0.08	0.23 ± 0.04	<0.20[c]	<0.20[a]	<0.20[c]
Citral	1.2 ± 0.2	1.3 ± 0.1	1.4 ± 0.1	0.44 ± 0.02	<0.20[b]	0.88 ± 0.05
Thymol	1.1 ± 0.1	2.2 ± 0.3	1.6 ± 0.2	1.1 ± 0.2	1.2 ± 0.4	0.8 ± 0.2

All the values are expressed as IC_{50} (ml/plate) ± standard deviation
[a] 100% growth inhibition at concentration of 2.0 ml/plate
[b] 100% growth inhibition at concentration of 1.0 ml/plate
[c] 100% growth inhibition at concentration of 5.0 ml/plate

Discussion

The purpose of the current study was to compare the chemical composition of Amazonian *Citrus* spp. leaves essential oils with those reported in literature to determine possible different chemotypes and biological activities with the final aim to valorize their commercial use.

The distillation yields were average values among those reported for CA (Blanco Tirado et al., 1995; Lota et al., 2001a) and CN (Lota et al., 2001b); instead for CL petitgrains were lower than those reported for other lemon species (Vékiari et al., 2002).

The Ecuadorian CA petitgrain was comparable to an atypical sabinene/*trans*-E-ocimene chemotype, as previously reported (Lota et al., 2001b). CL1 petitgrain exhibited an atypical composition with high abundance of limonene (52.7 %) and linalool (15.1 %), as reported for the Meyer cultivar (*C. meyeri*) (Lota et al., 2002). CL2 petitgrain could be defined as limonene (24.1 %)/sabinene (36.1 %)/linalool (4.7%) chemotype, standing out the most common lemon chemotype characterized by limonene (17.8-33.5 %), α-pinene (10.5-25.1 %), geranial (8.6-22.6 %), neral (5.9-16.1 %) (Lota et al., 2002). *C. nobilis* petitgrains (CN) evidenced a γ-terpinene/linalool chemotype, because of high abundance of linalool (41.6 %) and appreciable contents of γ-terpinene (14.3 %) and thymol (9.0 %). A similar chemotype was pointed out in a systematic research on petitgrains derived from 58 Corsical mandarin cultivars from different species and 41 cultivars belonging to *C. reticulata* Blanco (Lota et al., 2000; Lota et al., 2001b). Mandarin leaves essential oil composition from plants of different geographical origins, Floridian *C. tangerine* Hort. ex Tan and Israelian *C. reticulata*, evidenced high content of linalool, thymol and γ-terpinene (Attaway et al., 1967; Fleisher and Fleisher, 1990; Fleisher and Fleisher, 1991), while Colombian *C. reticulata* petitgrain was characterized by an high abundance of linalool (52.66 %), but less content of γ-terpinene (1.95 %) (Blanco Tirado et al., 1995).

The chemical composition of *Citrus* spp. leaves essential oils (C1 and C2) does not allow to deduce any consideration about the species employed and their abundance, but it is an important starting point in making suggestions about the comparison between the bio-

Tab. 5: Chemical shifts (^{13}C) of compounds in *Citrus* spp. petitgrains

Compound	Chemical shift ^{13}C
α-Pinene	144.5/116.0/47.0/40.7/38.0/31.4/31.2/26.3/23.0/20.9
Sabinene	154.5/101.5/37.6/32.6/30.1/29.0/27.9/19.8/19.7/16.0
β-Pinene	152.2/105.9/51.6/40.7/40.5/27.0/26.1/23.5/21.8
β-Myrcene	146.2/139.0/131.9/124.3/115.7/113.1/31.6/26.7/25.6/17.6
δ-Carene	131.4/119.4/28.3/23.7/20.8/18.4/16.8/16.6/13.2
p-Cymene	145.9/135.1/129.0/126.2/33.7/24.1/20.9
D-Limonene	150.3/133.8/120.6/108.3/41.0/30.8/30.6/27.9/235/20.8
trans-E-Ocimene	1415/133.7/122.1/110.6/ 27.3/25.7/17.7/11.6
γ-Terpinene	140.6/131.2/118.9/116.0/34.5/31.6/27.5/23.0/21.3
Linalool	145.0/132.0/124.3/111.7/73.5/42.0/27.9/25.7/22.9/17.7
Citronellal	202.7/131.8/124.0/51.0/36.9/27.8/25.7/25.4/19.9
4-Terpinenol	133.8/118.4/71.7/36.8/34.6/30.8/27.0/23.3/16.8
Neral	190.9/163.9/133.7/128.6/122.2/32.6/27.0/25.6/25.1/17.7
Geraniol	139.5/131.4/124.1/123.6/59.4/39.4/26.3/25.7/17.6/16.2
Geranial	191.4/163.9/132.9/127.4/122.5/40.6/25.7/25.6/17.7/17.6
Thymol	152.5/136.6/131.8/126.2/121.5/116.0/26.7/22.7/20.9
Methyl methylantranilate	169.0/151.2/134.7/131.8/115.4/110.6/51.5/29.1

activities of the mixtures and the other essential oils.

However, the studies concerning plants growing in Amazonia are particularly interesting since the Amazonian basin is one of the most important biodiversity hotspots where the ecological conditions and high density and diversity of species per unit area drive the plant secondary metabolism to biosynthetic pathways which are particularly rich in different chemical structures (Ryder Wilkie et al., 2010; Rossi et al., 2013). This aspect could explain the slight differences in chemical composition detected for the essential oils, with particular reference to those belonging to *C. limon* (CL) samples.

The confirmation of the gas chromatographic results by NMR experiments suggests this spectroscopic technique as suitable for the identification, quality control, or fraud detection of essential oils providing their good and fast discrimination. Moreover, these kinds of evidences reinforce the role of non-chromatographic approach as potential tool to discriminate chemotypes, cultivar and hybrids as already suggested elsewhere (Lota et al., 2001b; Guerrini et al., 2006; Guerrini et al., 2011). All these chemical profiles obtained through GC-MS and confirmed by NMR spectroscopy, evidence that Amazonian biodiversity does not induce strong chemodiversity among *Citrus* spp. petitgrains examined, if compared to what related literature reports, even if interesting differences regarding minor compounds were found.

The examined essential oils evidenced that CN petigrain revealed the highest antioxidant activity, if compared to results obtained with commercial *T. vulgaris* essential oil, taken as reference phytocomplex (Sacchetti et al., 2005). C1 petitgrain, with relative abundance of γ-terpinene (10.6 %) and thymol (5.5 %), showed also interesting data. The antioxidant capacity displayed by essential oils could be mainly due to the presence and the abundance of thymol, as experimental results evidenced. However, with particular reference to CN sample, the relevant abundance of γ-terpinene (14.3 %) could be also suggested as responsible of this biological property (Choi et al., 2001), together with the presence of thymol (9.0%) (Ruberto and Baratta, 2000), as well as methyl-N-methylanthranilate (13.1 %) (El-Ghorab et al., 2003).

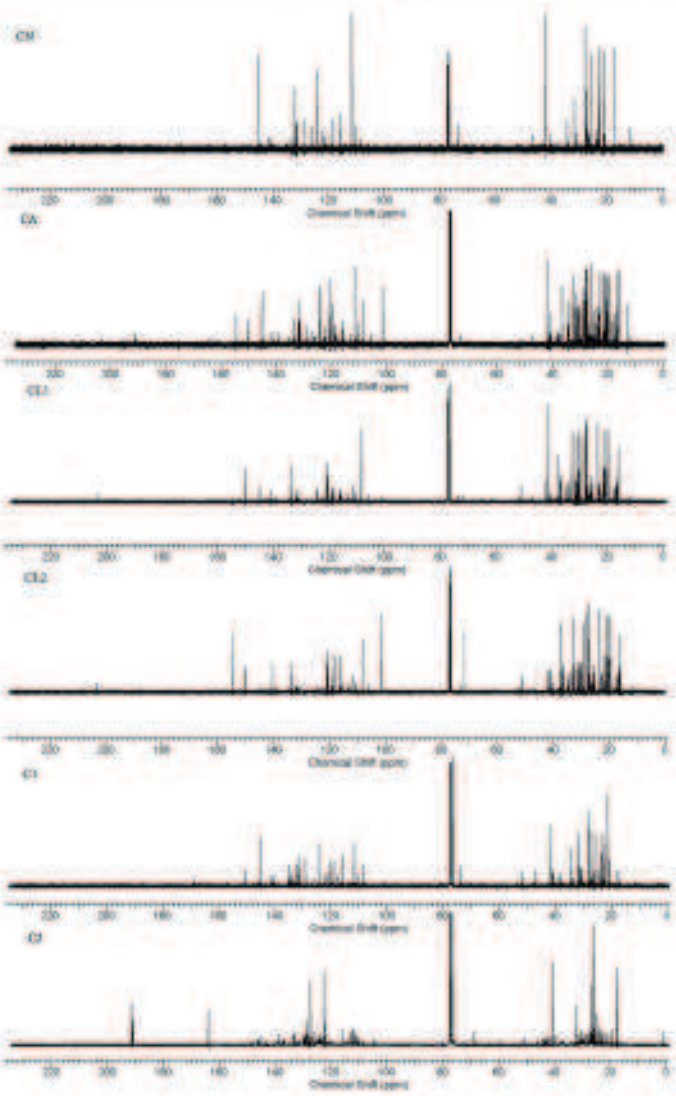

Fig. 1: ^{13}C spectra of *Citrus* spp. petitgrains

CN petitgrain was the most effective against all the bacteria strains: MIC values of CA and C1 samples were instead lower and comparable. The amounts of thymol in CN (9.0 %) and C1 (5.5 %) petitgrains could be one of the possible reasons for the antibacterial activity (Burt, 2004). C2 petitgrain was instead particularly active against the yeast *C. albicans*, with a MIC of 0.44 ± 0.05 mg/ml probably due to the high abundance of neral (33.1 %) and geranial (34.7 %), previously described as anti-*Candida* spp. agents (Silva et al., 2008) and confirmed by our results. Trying to relate antimicrobial activity with chemical data, thymol has been assayed as pure compound, but no remarkable results were obtained. However, it should be stressed that higher antibacterial capacity of thyme essential oil than that of thymol could be due to a sinergic interaction involving more chemicals, thymol included. This suggestion plays certainly a role in the activities displayed by petitgrains.

The most interesting results concerning antifungal activities (Tab. 4) were exhibited by C2 petitgrain due to the high abundance of citral, as confirmed by experimental data. The good activities of C1 and CN petitgrains could be explained with the relative abundance of thymol, tested by us as pure compound and previously described as antifungal agent *in vitro* and *in vivo* against dermatomycoses (Sokovic et al., 2008). Finally, the particular interesting bioactivity of the essential oil mixtures confirmed the amazonian ethnobotany which

often does not discriminate *Citrus* species in using leaves for traditional preparations, emphasizing synergic expression of different extracts/chemical compounds to have better biological performances.

Conclusions

This first report about Amazonian *Citrus* spp. petitgrains evidenced their chemical characterization by GC and GC-MS and remarked the use of NMR as useful tool to characterize and discriminate chemotype for identification, quality control and fraud detection of essential oils (Lota et al., 2001b; Guerrini et al., 2006; Guerrini et al., 2011). However, no remarkable difference emerged with other *Citrus* spp. petitgrains from other geographical regions, even if interesting differences regarding minor compounds were found. In particular Amazonian CN petitgrain, γ-terpinene/linalool chemotype on the basis of chemical composition defined by GC/MS and NMR, and C1 petitgrain revealed both interesting *in vitro* antibacterial and radical scavenging activities. Result highlights that these two essential oils could be potentially employed as food preservatives or functional constituents in food supplements and/or health herbal products.
Moreover the antidermatophytic properties of C1, CN and the most interesting C2 leaf essential oils suggest their possible application in cosmeceuticals as antidermatophytic additives, not only as single essential oil but also as mixtures.

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Hemidesmus indicus induces apoptosis as well as differentiation in a human promyelocytic leukemic cell line

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ABSTRACT

Ethnopharmacological relevance: The decoction of the roots of *Hemidesmus indicus* is widely used in the Indian traditional medicine for the treatment of blood diseases, dyspepsia, loss of taste, dyspnea, cough, poison, menorrhagia, fever, and diarrhea. Poly-herbal preparations containing *Hemidesmus* are often used by traditional medical practitioners for the treatment of cancer. The aim of this study was to investigate the cytodifferentiative, cytostatic and cytotoxic potential of a decoction of *Hemidesmus indicus*'s roots (0.31–3 mg/mL) on a human promyelocytic leukemia cell line (HL-60).

Materials and methods: The decoction of *Hemidesmus indicus* was characterized by HPLC to quantify its main phytomarkers. Induction of apoptosis, cell-cycle analysis, levels of specific membrane differentiation markers were evaluated by flow cytometry. The analysis of cell differentiation by nitroblue tetrazolium (NBT) reducing activity, adherence to the plastic substrate, α-napthyl acetate esterase activity and morphological analysis was performed through light microscopy (LM) and transmission electron microscopy (TEM).

Results: Starting from the concentration of 0.31 mg/ml, *Hemidesmus indicus* induced cytotoxicity and altered cell-cycle progression, through a block in the G0/G1 phase. The decoction caused differentiation of HL-60 cells as shown by NBT reducing activity, adherence to the plastic substrate, α-naphtyl acetate esterase activity, and increasing expression of CD14 and CD15. The morphological analysis by LM and TEM clearly showed the presence of granulocytes and macrophages after *Hemidesmus indicus* treatment.

Conclusions: The cytodifferentiating, cytotoxic and cytostatic activities of *Hemidesmus indicus* offers a scientific basis for its use in traditional medicine. Its potent antileukemic activity provides a pre-clinical evidence for its traditional use in anticancer pharmacology. Further experiments are worthwhile to determine the in vivo anticancer potential of this plant decoction and its components.

1. Introduction

One of the main characteristics of leukemia is the block of cell differentiation at an early stage and the inability of cells to differentiate into functional mature cells (Nowak et al., 2009). The therapy based on the induction of differentiation has favorable outcomes for substances such as all-trans retinoic acid, but the use of a single-target treatment has a limited efficacy and a number of side effects. Given the complexity and heterogeneity of leukemias and the crosstalk among a multitude of signaling pathways, specific combination regimens have been preferentially used in order to enhance the therapeutic efficacy of treatment. An interesting and promising strategy is represented by the multi-target approach, which allows the modulation of several targets simultaneously and results more effective than the inhibition of a single target (Aggarwal et al., 2009). In a curative setting, superior response rates have been achieved from the association of all-trans retinoic acid with arsenic trioxide. Different treatment strategies using these agents, usually in combination with chemotherapy, have provided excellent therapeutic results (Sanz et al., 2009). Moreover, the association with prophylactic steroid or other chemotherapeutic agents has been used to decrease the incidence of the retinoic acid syndrome, a cardio-respiratory distress syndrome that is the most significant toxicity associated with all-trans retinoic acid therapy (Shen et al., 2004; Tallman, 2002).

In the context of multi-target strategy, plant products, based on their intrinsic complexity, could represent an interesting and

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promising approach. They are able to interact with numerous molecular targets thus perturbing biological networks rather than individual targets (Gertsch, 2011).

Hemidesmus indicus (L.) R.Br. (*Asclepiadaceae*) is a traditional Indian medicinal plant. It is a perennial slender lactiferous, prostrate or semierect climber widely distributed throughout India, from upper Gangetic plains east-wards to Assam, throughout Central, Western and Southern India. The traditional name is Sariva, Ananta, Anantamula but it is commonly known as "Indian Sarsaparilla". The drug consists of root with a characteristic pleasant smell of vanilla and acrid taste. The decoction of the roots of *Hemidesmus indicus* is widely used in the traditional medicine for the treatment of blood diseases, dyspepsia, loss of taste, dyspnea, cough, poison, menorrhagia, fever, and diarrhea (Ayurvedic Pharmacopoeia Committee, 1989; Mary et al., 2003). Poly-herbal preparations containing *Hemidesmus* are often used by traditional medical practitioners for the treatment of cancer (Thabrew et al., 2005). Furthermore, different studies demonstrated the anticancer potential of *Hemidesmus indicus*, alone or in combination with other plants (Costa-Lotufo et al., 2005; Samarakoon et al., 2012; Zarei and Javarappa, 2012), but its use in a specific anticancer strategy and its cytodifferentiating effects remain elusive.

The aim of this study was to evaluate some pharmacological activities of *Hemidesmus indicus*, in order to give a scientific basis to its traditional uses. For this purpose, we tested the anticancer activity of the decoction of the plant roots, which represents the most used form in the traditional medicine. In particular, we investigated its cytotoxic, cytostatic and cytodifferentiative potential on HL-60, a human promyelocytic leukemic cell line that can be stimulated to terminally differentiate toward granulocytes and/or macrophages. A phytochemical study was also performed to obtain a chemical characterization of the *Hemidesmus indicus* decoction (HID).

2. Materials and methods

2.1. Plant decoction preparation

Hemidesmus indicus (voucher #MAPL/20/178) was collected from Ram Bagh (Rajasthan, India), and authenticated by Dr. MR Uniyal, Maharishi Ayurveda Product Ltd., Noida, India. The method described in the Ayurvedic Pharmacopoeia of India (Ayurvedic Pharmacopoeia Committee, 1989) was followed for the preparation of HID. In particular, 10 g of grinded roots were mixed with 300 mL of boiling water, allowing the volume of water to reach 75 mL. The yield of the decoction was 15%. HID was then filtered, lyophilized, and stored at room temperature. Immediately before the use, the samples were resuspended in water and centrifuged at 2000 rpm to discard any insoluble material.

2.2. HPLC analysis of plant decoction

HID was subjected to HPLC analysis to quantify its main phytomarkers, namely 2-hydroxy-4-methoxybenzaldehyde, 3-hydroxy-4-methoxybenzaldehyde and 2-hydroxy-4-methoxybenzoic acid (Alam and Gomes, 1998a, 1998b; Das and Singh Bisht, 2012; Sircar et al., 2007). The reference compounds (all obtained from Sigma, St. Louis, MO, USA) were used as external standards to set up and calculate appropriate calibration curves. The analyses were performed using a Jasco modular HPLC (Tokyo, Japan, model PU 2089) coupled to a diode array apparatus (MD 2010 Plus) linked to an injection valve with a 20 mL sampler loop. The column used was a Tracer Extrasil ODS2 (2560.46 cm, *i.d.*, 5 mm) with a flow rate of 1.0 mL/min. The mobile phase consisted of solvent solution B (methanol) and A (water/formic acid = 95:5). The gradient system adopted was characterized by five steps: 1, isocratic, B/30 for 15 min; 2, B raised progressively from 30% to 40% at 20 min; 3, B then raised to 60% at 50 min; 4, B achieved 80% at 55 min and 5, 100% at 60 min. Injection volume was 40.0 μL. Following chromatogram recording, peaks from HID samples were identified by comparing their UV spectra and retention time with those from the pure standards. The identity was also confirmed by 1H NMR on the enriched fraction of the compounds obtained by soxhlet extraction in $CHCl_3$/EtOH 1:1. Dedicated Borwin software (Borwin ver. 1.22, JMBS Developments, Grenoble, France) was used to calculate peak area by integration.

2.3. Standard solution and calibration procedure

Individual stock solutions of 2-hydroxy-4-methoxybenzaldehyde, 3-hydroxy-4-methoxybenzaldehyde and 2-hydroxy-4-methoxybenzoic acid were prepared in water. Six different calibration levels were prepared within the following range: 2–20 mg/mL for 2-hydroxy-4-methoxybenzaldehyde, 1.5–40.0 mg/mL for 3-hydroxy-4-methoxybenzaldehyde, and 1–100 mg/mL for 2-hydroxy-4-methoxybenzoic acid. Each calibration solution was injected into HPLC in triplicate. The calibration graphs were provided by the regression analysis of peak area of the analytes versus the related concentrations. The analysis of HID (31 mg/mL) was performed under the same experimental conditions. The obtained calibration graphs allowed the determination of the concentration of the three components. Three different batches of *Hemidesmus indicus* were tested.

2.4. Cell cultures

Human promyelocytic leukemia cells (HL-60) were obtained from the Istituto Zooprofilattico of Brescia (Italy) and grown in suspension. Cultures were propagated in RPMI 1640 supplemented with 20% heat-inactivated bovine serum, 1% penicillin/streptomycin solution and 1% L-glutamine solution (all obtained from Sigma). To maintain exponential growth, the cultures were divided every third day by dilution to a concentration of 3.5×10^5 cells/mL. To reduce spontaneous differentiation, cells were never allowed to exceed a density of 1.0×10^6 cells/ml. To reduce spontaneous differentiation, cells were never allowed to exceed a density of 1.0×10^6 cells/ml. Cells were treated with different concentrations of HID within the following range: 0–3.1 mg/mL. The stock solution (31 mg/mL) was diluted to 1/10 and the concentration 3.1 mg/mL was obtained. A series of two-fold dilution was used to obtain the lower concentrations.

2.5. Flow cytometry

All flow cytometric analyses were performed by using the easyCyte 5HT flow cytometer (Millipore Guava Technologies, Hayward, CA, USA).

2.6. Cytotoxicity test and analysis of apoptosis

Cytotoxicity test was performed by Guava ViaCount Reagent (Millipore Guava Technologies), containing propidium iodide. The inhibitory concentration causing 50% of cell toxicity following one cell-cycle exposure (30 h) (i.e. IC_{50}) was calculated by interpolation from dose-response curves.

Analysis of apoptosis was performed after 30 and 72 h of treatment with different concentrations of HID. Briefly, aliquots of 2×10^4 cells were stained with 100 μL of Guava Nexin Reagent (Millipore Guava Technologies), a pre-maid cocktail containing

Annexin V-phycoerythrin (Annexin V-PE) and 7-amino-actinomycin D (7-AAD) and, after a 20 min of incubation at room temperature in the dark, the samples were analyzed by flow cytometry. Cytarabine 0.5 μM was used as positive control.

2.7. Cell-cycle analysis

Cell were treated with different concentrations of HID for 24, 30 and 48 h, and then fixed with ice-cold ethanol. Samples were then stained with 200 μL of Guava Cell Cycle Reagent (containing propidium iodide), incubated at room temperature for 30 min in the dark, and analyzed via flow cytometry.

2.8. Analysis of cell differentiation

To evaluate the cytodifferentiative potential of HID, HL-60 were treated with different concentrations of HID for 72 h. After incubation, the cells were screened for the presence of markers or morphologic features of differentiated cells. The following assays were performed to analyze the differentiation forward the granulocyte and/or the macrophage lineage: nitroblue tetrazolium (NBT)-reducing activity, indicative of differentiation along both monocytic and granulocytic lineage (Suh et al., 1995); adherence to the plastic substrate for the macrophagic differentiation (Kim et al., 2001); α-naphthyl acetate esterase activity, a non-specific acid esterase activity that indicates differentiation forward monocytic/macrophage lineage (Suh et al., 1995); and, finally, the detection of CD15 and CD14, two membrane proteins specific for the monocytes/granulocytes or monocytes/macrophages, respectively (Choi et al., 2002; Stocks et al., 1990).

2.8.1. NBT reduction assay

This assay was used to evaluate the ability of HID to produce superoxide when challenged with 12-O,-tetradecanoylphorbol 13-acetate (TPA). The assay was performed according to Catino and Miceli (1988). Approximately 1.0×10^6 cells were treated with *Hemidesmus indicus* and freshly prepared TPA/NBT solution (1 × phosphate buffer containing 0.2% NBT and 200 ng/mL TPA) and incubated for 30 min at 37 °C. The reaction was stopped by placing the samples on ice for 5 min. Cells were then smeared on glass slides. Positive cells reduced NBT yielding intracellular black–blue formazan deposits and this was determined by microscope examination (10× total magnification) of 500 cells. The results were expressed as percentage of positive cells over total cells. DMSO 1% (v/v) was used as positive control.

2.8.2. Adherence to the plastic substrate

Dish-anchored cells were easily distinguished from the undifferentiated suspended cells. Approximately 1.0×10^6 cells were grown in normal cell growth conditions. After 72 h of treatment with different concentrations of HID, the medium was removed and the remaining non-adherent cells were gently washed away with 1× phosphate buffer. The number of adherent cells was counted on a light microscope. Results were reported as number of attached cells (Ahmed et al., 1991). TPA 50 nM was used as positive control.

2.8.3. α-naphthyl acetate esterase activity

Assay for α-naphthyl acetate esterase was performed using the cytochemical kit from Sigma (91-A) (Zhou et al., 1989). Differentiated cells were assessed by microscopic examination of a minimum of 200 cells (in duplicate) for each experiment.

2.8.4. Evaluation of CD15 and CD14

Aliquots of 1.0×10^6 cells were collected to determine CD15 and CD14 expression by flow cytometric analysis. Cells were washed twice in ice-cold 1× phosphate buffer (Sigma), suspended in 20 μL of FITC-conjugated CD15 [3-fucosyl-N-acetyllactosamine (3-FAL)] (Biolegend, San Diego, CA, USA) or in 20 μL of FITC-conjugated CD14 [glycosylphosphatidilinositol (GPT)-linked] (Biolegend), and incubated for 30 min in the dark at 4 °C. After incubation, the cells were washed twice with phosphate buffer, then resuspended in 200 μL of phosphate buffer and analyzed. The mean fluorescence intensity values were calculated. From each sample, 10,000 events were analyzed and non-specific binding was excluded by the isotype negative control antibody [FITC Mouse IgG1, k (FC), (Biolegend)].

2.8.5. TEM analysis

After 72 h of treatment with HID, cells were washed and fixed with 2.5% glutaraldehyde in 0.1 M phosphate buffer (pH 7.3), post-fixed with 1% OsO_4 in the same buffer, alcohol dehydrated and embedded in araldite (Burattini et al., 2010). To obtain better direct ultrastructural observations, semithin sections were stained with 1% toluidine blue at 60 °C and observed by LM. Thin sections were collected on nickel grids, stained with uranyl acetate and lead citrate, and analyzed with a Philips CM 10 electron microscope.

2.9. Statistical analysis

All results are expressed as the mean ± SEM of at least three different experiments. One way ANOVA, followed by Dunnett or Bonferroni as post test was used to evaluate differences between treatments. GraphPad InStat version 5.0 (GraphPad Prism, San Diego, CA, USA) was used for all statistical analyses as well as for IC_{50} value calculation. $P < 0.05$ was considered significant.

3. Results

3.1. HID contains 2-hydroxy-4-methoxybenzaldehyde, 3-hydroxy-4-methoxybenzaldehyde and 2-hydroxy-4-methoxybenzoic acid

HID was found to contain 2-hydroxy-4-methoxybenzaldehyde, 3-hydroxy-4-methoxybenzaldehyde and 2-hydroxy-4-methoxybenzoic acid (Fig. 1). NMR data on the decoction and on the enriched fraction are shown in Supplemental Figs. S1 and S2. The amounts found in the decoction (31 mg/mL) were: 0.0025 ± 0.001 mg/mL for 2-hydroxy-4-methoxybenzaldehyde, 0.0018 ± 0.003 mg/mL for 3-hydroxy-4-methoxybenzaldehyde, 0.0022 ± 0.005 mg/mL for 2-hydroxy-4-methoxybenzoic acid. The analyses were performed on the decoction obtained from three different batches and the difference among the batches in the phytomarker content was not significant (data not shown).

3.2. HID induces cell death

HID decreased HL-60 viability and the IC_{50} value calculated after 30 h of treatment has been 1.52 mg/mL (Fig. 2A). The induction of apoptosis was evaluated for concentrations similar or smaller than the IC_{50} (0–1.55 mg/mL). The incidence of apoptotic cells after 30 h was statistically significant starting from the concentration of 0.62 mg/mL (11.6% vs 4.6% in the control). The highest percentage of apoptotic cells (23.0%) was observed at the highest concentration tested, which resulted higher than that induced by cytarabine. However, a significant fraction of necrotic cells was also recorded starting from the concentration 0.93 mg/mL (Fig. 2B). Flow cytometric histograms are shown in Supplemental Fig. S3.

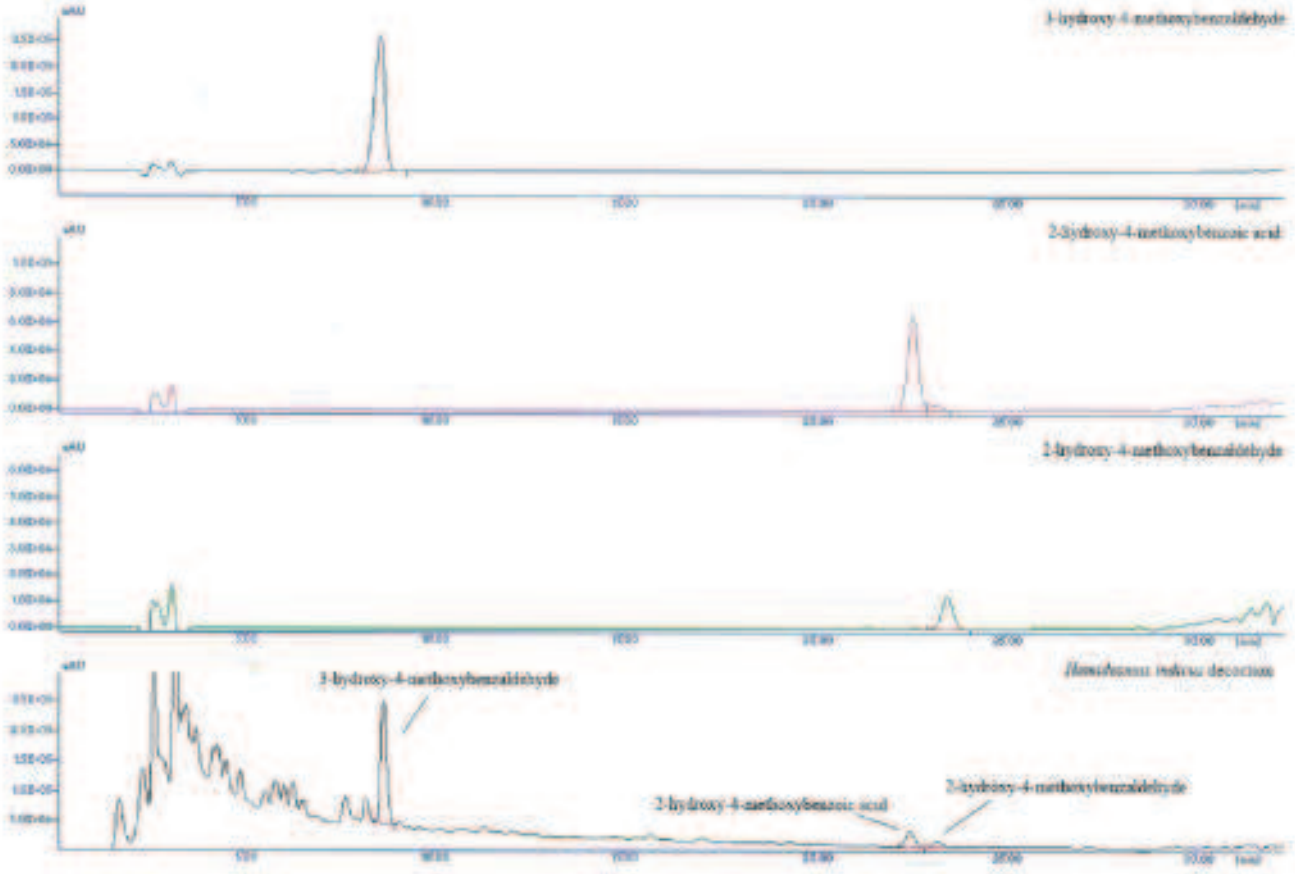

Fig. 1. HPLC chromatogram of HID.

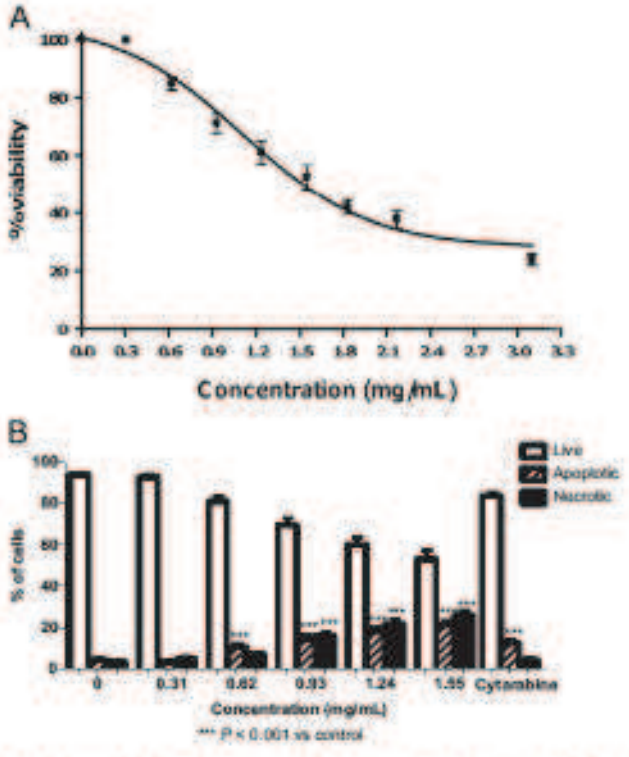

Fig. 2. Cytotoxicity (A) and fraction of viable, apoptotic and necrotic cells (B) after treatment with Hemidesmus indicus at the indicated doses for 30 h.

3.3. HID alters cell-cycle progression

To define the rate at which HID perturbed cell-cycle progression, cell cycle was evaluated at different time points. The effect of HID was time dependent. The early effect, observed at a concentration of 0.47–1.24 mg/mL, appeared as an increase in the percentage of cells in G0/G1 and S phases starting from the concentration 0.62 mg/mL, accompanied by a compensatory decrease in the cells in G2/M phase. The highest effect was observed at the highest dose tested, where the percentage of cells in G0/G1 phase reached 23%, in S phase 31% and in G2/M phase about -53% (Fig. 3). Longer exposure (30 h) led to an increase of cells in the G0/G1 and S phases. The effect was significant starting from the concentration of 0.93 mg/mL *Hemidesmus* (G0/G1: 28%; S: 14%). A decrease of cells in G2/M phase starting from 0.62 mg/mL was also observed (from −15% to −59%) (Fig. 3). Prolonged exposure (48 h) appeared as a marked increase in the proportion of cells in G0/G1 phase starting from 0.47 mg/mL (about 6%), that reached the highest level at 1.24 mg/mL (about 50%). A decrease in the percentage of cells in S and G2/M phases was also recorded (Fig. 3).

3.4. HID induces HL-60 differentiation

The analysis of cytodifferentiation has to be performed at concentrations where cell viability is higher than 80% (Kong et al., 1999). For the analysis of cytodifferentiation induced by HID after 72 h of treatment, HL-60 cells were treated with up to 0.62 mg/mL

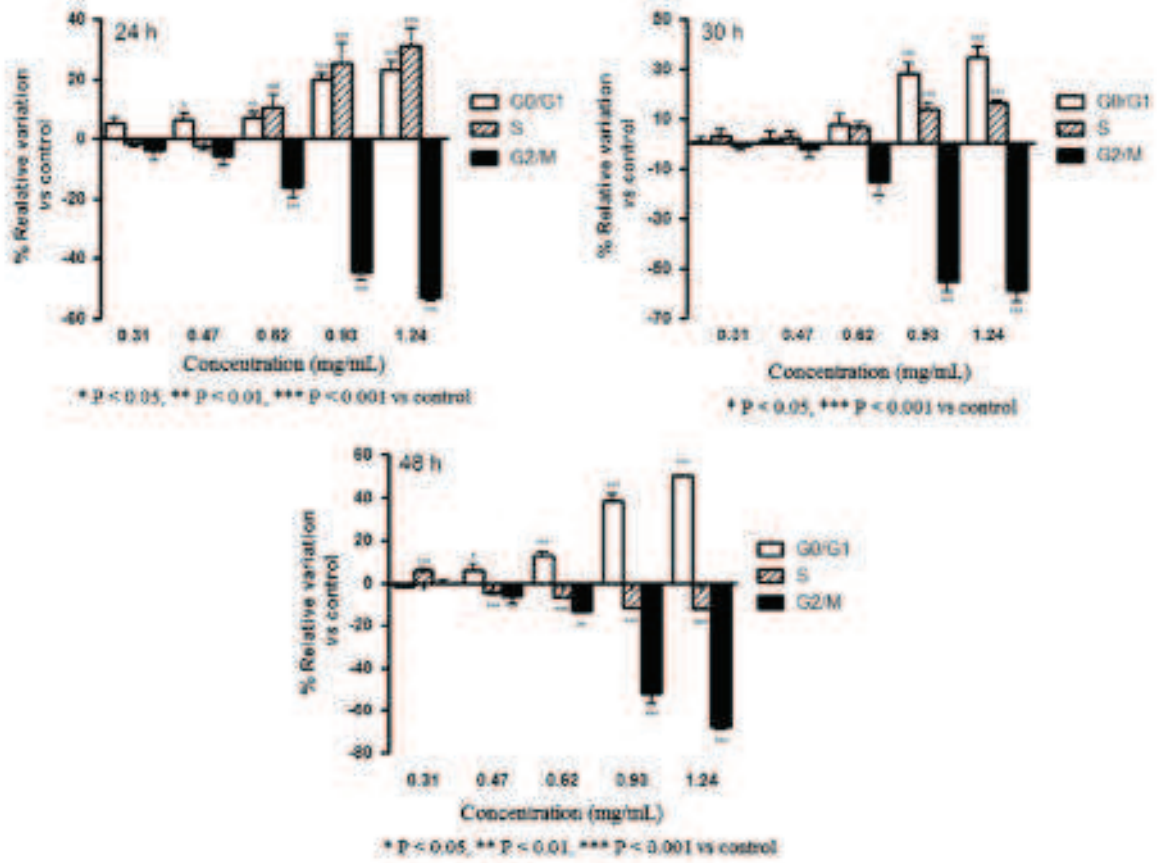

Fig. 3. Effects of *Hemidesmus indicus* on cell-cycle distribution following 24 h (A), 30 h (B) and 48 h (C) of treatment with *Hemidesmus* expressed as percentage of relative variation compared to untreated cells (control).

of HID, where we recorded a cell viability of 82%. At 0.93 mg/mL cell viability was 48%.

NBT reduction, a marker for granulocyte/monocyte differentiation, revealed that HID induced a dose-dependent differentiation of HL-60 cells (Fig. 4A). After treatment with HID 0.62 mg/mL, the fraction of NBT-positive cells was 36.56% ± 0.78 compared to 5.62% ± 0.47 in the control, thus comparable to DMSO (43.88% ± 0.80), used as positive control (Fig. 4A). HID 0.62 mg/mL also induced an increase in the number of adherent cells by over 8-fold (Fig. 4B). This suggests that HID is able to stimulate differentiation into monocyte/macrophage. The increased number of α-naphthyl-acetate-esterase-positive cells confirmed the differentiation toward the monocyte/macrophage. The highest effect was observed at the highest concentration of HID (23.50% ± 0.84 vs 3.00% ± 0.52 in the control) (Fig. 4C).

To confirm the ability of HID to induce differentiation, the expression of two specific monocyte/granulocyte and monocyte/macrophage markers, CD15 and CD14 respectively, was analyzed. Cells treated with 0.62 mg/mL of HID showed a CD15 mean fluorescence of 2.32-fold higher than untreated cells (Fig. 4D). The mean fluorescence of CD14 was 2.45-fold higher compared to the control at 0.62 mg/mL and 1.50-fold higher at 0.31 mg/mL (Fig. 4E).

The cytodifferentiative potential of HID was further detailed by TEM analysis. Fig. 5 shows cell differentiation towards granulocyte and/or macrophage lineages. Myeloid cell granule maturation, at ultrastructural level, occurs through a progressive condensation of granule content from a loose, scarce electron-dense substance, to a dense compact material. In HID-treated HL60 cells, 0.2–0.5 μm immature granules, scattered throughout the cytoplasm and consistently absent in control cells, are clearly recognizable in A. Occasional 0.1–0.3 μm granules, containing a uniform, electron-dense material, can be also revealed and correspond to a further differentiated granule form (A, B). The presence of larger empty vacuoles (C), typical a phagocytic process, suggests a progressive macrophage differentiation, as also confirmed by the observation of large cells, with flattened polymorph nucleus, phagocyting necrotic ones (D).

4. Discussion

The stimulation of differentiation is a recognized alternative in the treatment of cancer, by generating cells with limited or no replicative capacity able to enter in the apoptotic pathways (Nowak et al., 2009). It is well known that the only successful differentiation therapy in the clinic still remains treatment with all-trans retinoic acid and arsenic trioxide (Mi et al., 2012). At molecular level, all-trans retinoic acid and arsenic trioxide are able to synergistically modulate multiple downstream pathways/cascades. This combination therapy, compared to the traditional anthracycline-based regimen, results in lower toxicity, improvement of long-term outcome, higher survival rates, with >90% patients disease-free after 5 years by the end of treatment (Ablain and De The, 2011; Mi et al., 2012). A multi-target approach thus represents an attractive therapeutic strategy in the oncological field.

Botanical drugs are multi-component systems of known and possibly active compounds able to interact with different pharmacologically-relevant targets. In this context, the in vitro anti-leukemic effect observed in our study for HID deserves attention. HID is able to induce cytodifferentiating, cytostatic and cytotoxic effects. Accordingly to a recent study performed on a different leukemic cell line (Fimognari et al., 2011), treatment of HL-60 cultures with HID caused apoptosis and cell-cycle inhibition, which may represent an

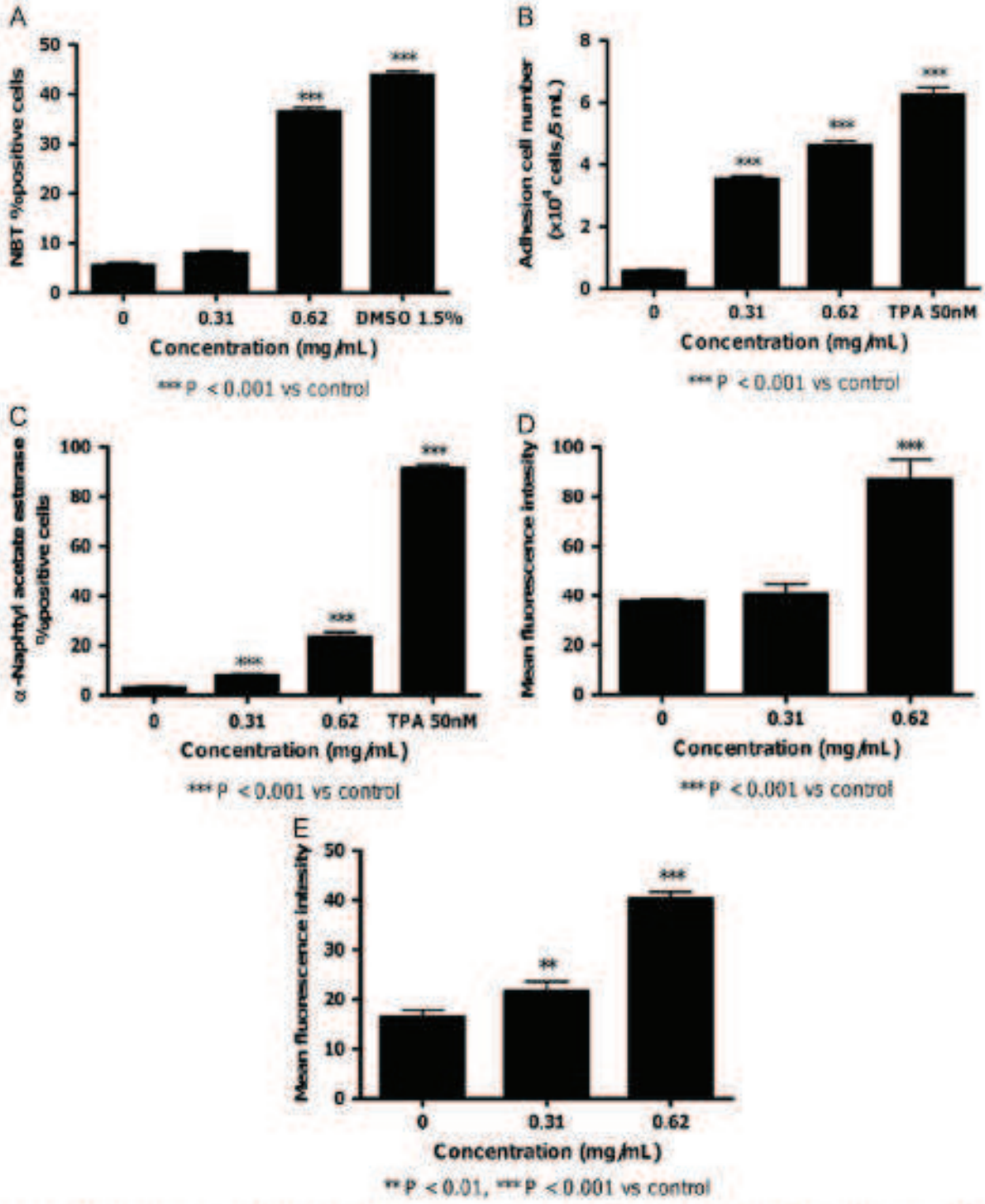

Fig. 4. Dose-dependent effects of *Hemidesmus indicus* on HL-60 cell differentiation as evaluated by NBT-reducing ability (A), cell adhesion (B), α-naphthyl acetate esterase activity (C) and expression of CD15 (D) and CD14 (E) markers.

event only partly dependent on cytodifferentiation in our experimental conditions. In fact, after 30 h of treatment with HID, we had already recorded an increase in the apoptotic cell fraction and after 24 h of treatment we had already observed a block in cell-cycle progression. The early appearance of cell-cycle-inhibition and apoptosis compared with cytodifferentiation clearly indicates that cytotoxicity and cytostasis events are primary direct effects due to HID and are not due to activation of apoptosis and/or inhibition of cell proliferation as a consequence of cell's differentiation. Although reported in vitro, those effects are worthy of comparing with the effects recorded for all-trans retinoic acid on the same cell line. No induction of apoptosis was observed on HL-60 cells after 48 h of treatment with all-trans retinoic acid. By 72 h, a modest fraction of cells incubated with all-trans retinoic acid became apoptotic (Otake et al., 2005). These results are consistent with the evidence that apoptosis induced by all-trans retinoic acid is a consequence of its cytodifferentiative potential (Lawson and Berliner, 1999) and does not represent an independent event.

The treatment of HL-60 with HID altered cell-cycle progression though the induction of a dose- and time-dependent accumulation

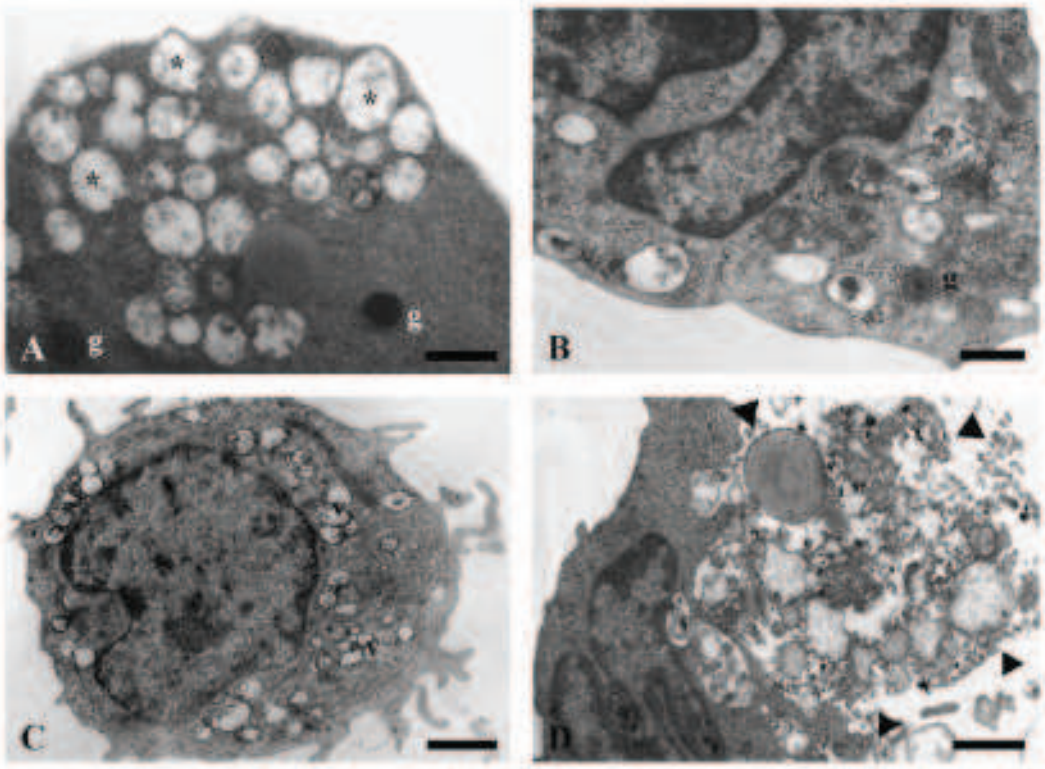

Fig. 5. TEM of HL-60 cells after *Hemidesmus indicus* treatment. Forming (*) and mature (g) granules indicate the granulocytic differentiation (A,B). Cytoplasmic vacuoles (v) and phagocytosis patterns (►) evidentiate the macrophagic properties (C,D). A,B, bar = 5 μm; C,D, bar = 1 μm.

of cells in the G0/G1 phase evident after 24 h of treatment. This is a critical point because cells need to exit from cell cycle before differentiating and this decision is commonly made in G1 phase (Wang et al., 2006). By comparing our results with those obtained with all-trans retinoic acid and arsenic trioxide on the same cell line, we observe that the antiproliferative effects of *Hemidesmus* are much earlier than those of all-trans retinoic acid and arsenic trioxide, evident after 4 and days of incubation, respectively (Jiang et al., 2008).

HL-60 cells have the potential to differentiate into macrophages or in granulocytes (Birnie, 1988). Our biological data support the conclusion that HID significantly induced HL-60 to differentiate along the macrophage and granulocyte lineage. The subsequent morphological analysis by TEM confirmed the cytodifferentiation ability of this traditional plant. The ability of HID to induce differentiation into granulocytes resulted in a less extent compared to the data concerning the differentiation inducted by all-trans retinoic acid, but in a greater extent compared to arsenic acid (Jiang et al., 2008).

Taken together, our results showed the capability of HID to induce a plethora of effects, all contributing to its antileukemic effect. This aspect appears important because the therapeutic success of drugs used in the acute promyelocytic leukemia results from the balance of self renewal, apoptosis and differentiation. Indeed, in clinical practice, retinoic acid by itself only rarely yields prolonged remission, even thought it induce massive differentiation. Otherwise, in vivo studies on arsenic trioxide reported an initial induction of apoptosis followed by slow blast differentiation (Ablain and De The, 2011).

As to the pharmacological relevance of the concentrations of HID used in the present study, the active concentrations of HID are well above 30 μg/mL. Suffness and Pezzuto (1990) indicated a crude extract promising for further purification based on IC_{50} values lower than 30 μg/mL after an exposure time of 72 h. However, we tested the decoction of *Hemidesmus indicus* because it represents the traditional preparation. The decoction is usually more diluted than the extract (Marriott et al., 2006). For example, the content of ethanol and water soluble principles and the content of total phenolic compound in different preparations of *Acacia aroma* leaf are as follows: 29.95 mg/ml in tincture, 178 mg/ml in fluid extract, 48.45 mg/ml in alcoholature, 8.5 mg/ml in decoction (Arias et al., 2004). The concentrations of soluble principles in the fluid extract of *Acacia aroma* is about 21-fold higher than those in the decoction. Moreover, it is worth to note that the IC_{50} value for HID after 72 h of treatment is about 0.93 mg/mL and that we observed the most interesting activities of HID in the concentration range 0.31–0.62 mg/mL, where we recorded a significant induction of apoptosis, cytostatic and cytodifferentiating effects with a moderate and no statistically significant induction of necrotic events. By using that range of concentrations of HID and the ratio between the content of soluble principles in the extract and that of soluble principles in the decoction reported for A. aroma, the concentrations of HID able to evoke a biological effect in our experimental system are similar or even lower than those indicated by Suffness and Pezzuto (1990).

The increasing interest on botanical drugs is supported by the FDA publication of industry guidelines for botanical drug products (2004) and by the approval of the first botanical prescription drug, an extract of green tea leaves (Veregen®), for the perianal and genital condyloma (2006). Botanical drugs are multi-component systems where all chemical constituents are not well defined. For this reason, they can pose a number of problems, among which therapeutic consistency is the most important. This aspect makes it necessary to minimize the differences between the batches by restricting and controlling the cultivars and determining a reliable fingerprint for the product. In our study, HID was subjected to an HPLC analysis to quantify its main phytomarkers, namely

2-hydroxy-4-methoxybenzaldehyde, 3-hydroxy-4-methoxybenzaldehyde and 2-hydroxy-4-methoxybenzoic acid, which can be used as fingerprint. We did not observe any significant difference between the different batches used for the study.

5. Conclusions

Due to its multiple effects, *Hemidesmus indicus* represents a promising candidate in the therapy against promyelocytic leukemia. Based on its ability to target simultaneously multiple molecular mechanisms, it can represent a strategy not only for undifferentiated tumor cells. Even though additional studies are required to pinpoint the signaling pathways involved in its anticancer activity, our results might substantiate the use of this plant in the traditional medicine to treat cancer.

Acknowledgment

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at http://dx.doi.org/10.1016/j.jep.2013.02.009.

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Croton lechleri Müll. Arg. (Euphorbiaceae) stem bark essential oil as possible mutagen-protective food ingredient against heterocyclic amines from cooked food

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ABSTRACT

The Amazonian *Croton lechleri* stem bark essential oil was tested for its anti-mutagenic potential by performing the Ames test against heterocyclic amines (HCAs), in continuing research on applicative functional profile of this phytocomplex as food ingredient (Rossi et al., 2011). *Salmonella typhimurium* strain TA98 was used with and without metabolic activation (S9 mix). The anti-mutagenic properties was assayed with the following HCAs: 2-amino-3-methylimidazo-[4,5-*f*]quinoline (IQ), 2-amino-3,4-dimethylimidazo-[4,5-*f*]quinoline (MeIQ), 2-amino-3,8-dimethylimidazo-[4,5-*f*]quinoxaline (MeIQx), the imidazoles 2-amino-6-methyldipyrido-[1,2-*a*:3′,2′-*d*]imidazole (Glu-P-1) and 2-aminodipirydo-[1,2-*a*:3′,2′-*d*]imidazole (Glu-P-2). All HCAs with S9 induced mutagenicity at 10^{-10} mol/plate. Without S9, IQ and MeIQ showed mutagenicity at 10^{-8} mol/plate, MeIQx and Glu-P-1 at 10^{-5} mol/plate, while Glu-P-2 was inactive. In presence of HACs (10^{-9} mol/plate), *C. lechleri* essential oil was tested for mutagen-protective properties (concentration range: 0.01–0.10 mg/plate) taking the Highest Uneffective Dose (HUD) as threshold reference. With S9 mix, *C. lechleri* essential oil displayed a significant reduction of revertants at 0.05 mg/plate, from 21% to 34%. The essential oil showed mutagen-protective efficacy against IQ and MeIQ tested as direct mutagens (10^{-7} mol/plate), with a revertants percentage reduction of 39% and 40%, respectively. No anti-mutagen capacity was noted for MeIQx and Glu-P-1 (10^{-5} mol/plate). Since HACs are known as possible colon and liver cancer inducers, *C. lechleri* essential oil was tested for its cytotoxicity and anti-proliferative capacity against LoVo and HepG2 cancer cell lines showing IC_{50} of 74.95 ± 0.05 μg/ml (LoVo) and 82.28 ± 0.03 μg/ml (HepG2), displaying a promising role of this essential oil as a functional food ingredient with interesting mutagen preventing properties.

1. Introduction

Plants are constantly monitored in the search for new therapeutic agents to treat disorders and diseases based on ethnobotanical, ethnopharmacological, chemosystematic and ecological information. In spite of the literature which emphasises biological applications of phytocomplexes and/or bioactive pure molecules, many natural derivatives remain largely untapped (Bezerra et al., 2009). However, this research contributes to the chemical and biological knowledge of those plants which are rarely or never studied but which may potentially contribute to improving the efficacy and safety of pharmaceuticals and health products. Among these studies, those concerning Amazonian plants are particularly interesting since the Amazonian basin is one of the most important biodiversity hotspots, where the ecological conditions and high density and diversity of species per unit area drive the plant secondary metabolism to biosynthetic pathways which are particularly rich in different chemical structures (Hopkins, 2007).

In recent years the research on plant extracts has been performed through two main binaries, i.e., chemical characterization and biological activity, the latter diversified in a plethora of biocapacities from those which are antibiotic to those which are anti-cancerogenic, taking into account efficacy and safety aspects. Particularly interesting as plant derivatives are the essential oils, as traditional herbal products are employed in many different applications (cosmetics, foods, pharmaceuticals, agriculture, etc.). As with many other plant derived products, they are investigated also for their capacity to prevent cancer onset as anti-mutagenic tools. One of the most important way through which humans come in contact with carcinogens is diet. In fact, the human diet contains a large number and variety of mutagens and carcinogens, and many of them have an action mechanism involving the generation of mostly oxygen radicals. Heterocyclic amines (HACs), produced mainly from household cooking of food rich in proteins, are known as possible human carcinogens as asserted by epidemiological and

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risk assessment studies (Saito, Sakai, & Nagase, 2006). The carcinogenic risk induced by these compounds is due not only to the level of exposure depending on the diet, but also to the fact that they interfere with uptake and biotransformations of dietary factors. In fact, the research into the aetiology of neoplasia has focused its attention on the complex mixture of chemical entities characterising the diet which can inhibit or modulate the onset and development of cancer. Moreover, somatic mutations are recognised not only as an initiating event in the development of sporadic cancers, but also as key steps in the development of atherosclerosis and a large number of chronic diseases in humans such as diabetes and neurodegenerative diseases (Berić, Nikoliv, Stanojević, Vuković-Gačić, & Knežević-Vukčević, 2008; Ferguson, 2010). Even if the best approach to decrease the incidence of cancer and related diseases is to avoid contact with carcinogens and mutagens, exposure to such compounds is however unavoidable in a large number of cases (Saito et al., 2006). There is abundant evidence that chemical mutagens and carcinogens can be inhibited by a plethora of naturally occurring minor constituents of plant origin, including spices, fruits and beverages, vegetables, etc. Therefore, chemical characterization of phytocomplexes, fractions and pure compounds and investigation into their bioactivities as tools to prevent mutagenesis and/or carcinogenesis are of great and increasing interest (Shishu, Singla, & Kaur, 2003). For example, there are many papers which report the mutagenic and antimutagenic potential of essential oils as phytocomplex and of terpenes (mono-, sesqui- and diterpenes), either isolated pure compounds against common environmental mutagens, or processed foods containing them, by mainly performing the Ames test employing *Salmonella typhimurium* strains (Aydin, Başaran, & Başaran, 2005; Bakkali, Averbeck, Averbeck, & Idaomar, 2008; Berić et al., 2008; Ipek et al., 2005; Saito et al., 2006; Sghaier et al., 2010; Vuković-Gačić, Nikčević, Berić-Bjedov, Knežević-Vukčević, & Simić, 2006).

The anti-mutagenic properties of the essential oils may be due to different factors, e.g., (i) the inhibition of mutagen penetration into the cells, (ii) activation of cell antioxidant enzymes, (iii) mutagen neutralisation by direct scavenging activity or inactivation of radicals produced by mutagens, (iv) inhibition of metabolic conversion of pro-mutagens into mutagens by microsomal enzyme pools, (v) activation of enzymatic detoxification of mutagens, (vi) involvement interference with DNA repair systems, (vii) general and unspecified hepatoprotective activity (Bakkali et al., 2008; Edris, 2007). Moreover, this kind of growing interest in the use of essential oils needs however to assess their cytotoxic and genotoxic potential, identify possible toxic/mutagenic components, and try to display an almost complete profile of risks and benefits of employing these herbal derivatives as health promoters (Bakkali et al., 2008, and references therein).

Among the genus *Croton*, *C. cajucara* was investigated for its mutagenicity and antimutagenicity, in particular methanolic bark extracts of it, giving important evidence, using the micronucleus test, even if no specific evidence was identified about the putative chemicals responsible (Dos Santos et al., 2008). *C. regelianus* was instead studied for the possible antitumor role of its essential oil from leaves using different tumor cell lines, identifying the role of ascaridole and edoperoxides in exerting antiproliferative capacity against *in vitro* cancer cells and suggesting an action mechanism similar to that of artemisinin related compounds (Bezerra et al., 2009). Finally, *C. flavens* essential oil from leaves was tested for anticancer (i.e., antiproliferative) activity against human lung carcinoma and human colon adenocarcinoma cell lines, suggesting the sesquiterpenes α-cadinol, β-elemene and α-humulene as the compounds most responsible for the cytotoxic properties of the phytocomplex (Sylvestre, Pichette, Longtin, Nagau, & Legault, 2006). As a preliminary step of a research pathway that laid the foundation of the in-depth investigation reported here, we recently demonstrated the non-mutagenic activity of *C. lechleri* bark essential oil (Ames test and *S. cerevisiae* D7 assay). The anti-mutagenic properties of the terpene phytocomplex were also tested against ethyl methane sulphonate (EMS) employing *S. cerevisiae* D7 strain, and against 2-nitrofluorene, sodium azide and 2-aminoanthracene for TA98 and TA100 Ames strains (Rossi et al., 2011).

Croton lechleri Müll. Arg. (sin. *Croton draconoides* Müll. Arg.), a small-sized Amazonian tree belonging to the *Euphorbiaceae* family, is mainly known for traditional uses of its sap which was investigated in depth from a chemical and bioactivity perspective. In our previous related research, *C. lechleri* stem bark essential oil was studied for its chemical composition and bioactivities, suggesting its employment as a functional food constituent (Rossi et al., 2011, and references therein). In continuing research on *C. lechleri* essential oil's applicative functional profile, the anti-mutagenic potential was here reported by performing the Ames test against heterocyclic amines, known to be indirect mutation inducers produced during cooking of protein-rich foods (Robbana-Barnat, Rabache, Rialland, & Fradin, 1996). In fact, the Ames assay is well established and it is an effective strategy to check phytocomplexes or pure compounds for their potential chemopreventive role. It is also an ubiquitously accepted mutagenicity and antimutagenicity test and a good predictive tool for carcinogens (83% of mutagens found in the Ames test are also carcinogens) (Ames, Durston, Yamasaki, & Lee, 1973; Edenharder, Worf-Wandelburg, Decker, & Platt, 1999; Rossi et al., 2011). Cytotoxic and antiproliferative activity was then assayed against human colon carcinoma (LoVo) and human hepatocellular carcinoma (HepG2) cells, to evaluate possible further anticancer evidence of this Amazonian plant derivative.

2. Materials and methods

2.1. Plant material and isolation of essential oil

Three different stocks of *C. lechleri* Müll. Arg. stem barks collected in September 2006 from wild adult trees growing in the outskirts of the Juyukamentsa village (Morona-Santiago province, Ecuador) were subjected to steam distillation and the essential oil obtained was then treated to prevent degradations, as previously reported (Rossi et al., 2011).

2.2. Chemical characterization of the essential oil

The essential oil was chemically characterised through gas chromatography (GC), gas chromatography–mass spectrometry (GC–MS) and the chemical structure of the main compounds was confirmed by GC–MS and proton Nuclear Magnetic Resonance (^{1}H NMR) (Rossi et al., 2011).

2.3. Chemicals

Chromatographic grade solvents and pure compounds used for bioassays were purchased from Sigma–Aldrich Italy (Milano, Italy) except for the heterocyclic amines (HCAs), namely the quinolines 2-amino-3-methylimidazo-[4,5-*f*]quinoline (IQ), 2-amino-3,4-dimethylimidazo-[4,5-*f*]quinoline (MeIQ), 2-amino-3,8-dimethylimidazo-[4,5-*f*]quinoxaline (MeIQx), the imidazoles 2-amino-6-methyldipyrido-[1,2-*a*:3′,2′-*d*]imidazole (Glu-P-1) and 2-aminodipirydo-[1,2-*a*:3′,2′-*d*]imidazole (Glu-P-2) supplied by Toronto Research Chemicals Inc. (Toronto, Canada). Dimethyl sulfoxide (DMSO, Sigma-Aldrich Italy) was used as solvent for HCAs and *C. lechleri* essential oil dilutions. All the microbial culture media were from Oxoid Italia (Garbagnate, Italy). Lyophilized post-mitochondrial supernatant S9 fraction (Aroclor 1254-induced,

Sprague-Dawley male rat liver in 0.154 M KCl solution), commonly used for the activation of promutagens to mutagenic metabolites, was purchased from Molecular Toxicology, Inc. (Boone, NC, USA) and stored at −80 °C. The components of S9 mix were: 8 mM $MgCl_2$, 32.5 mM KCl, 5 mM G6P, 4 mM NADP, 0.1 m sodium phosphate buffer pH 7.4 and S9 at the concentration of 0.68 mg/mL of mix.

2.4. Ames test: Mutagen-protective activity of C. lechleri essential oil against HACs

2.4.1. Tester bacterial strain

To evaluate the mutagen-protection capacity of *C. lechleri* stem barks essential oil in presence of the HACs previously cited, the tester strains *S. typhimurium* TA98 and TA100 kindly supplied by Prof. R. Barale (Pisa University, Italy) were used. For all assays, an inoculum of thawed permanent culture was added to 20 ml of Nutrient Broth and incubated a 37 °C in an orbital shaker (120 rpm) until reaching a microbial concentration of approximately 2×10^8 bacteria/ml.

2.4.2. C. lechleri essential oil cytotoxicity: Highest Uneffective Dose (HUD)

To correctly set up the assay to test the possible mutagen-protection capacity of the essential oil, the Highest Uneffective Dose (HUD) of the *C. lechleri* essential oil was determined in order to define the range of essential oil concentration avoiding cytotoxic interferences. Following the indications previously reported (Rossi et al., 2011), the *C. lechleri* HUD was determined for the wide concentration range from 0.01 to 10.00 mg/plate, with and without metabolic activation (S9 mix) with the object to avoid overlapping of the cytotoxic and antimutagenic results which would be therefore indistinguishable. In other words, HUD is of crucial importance to set up mutagen-protection experiments to confirm that the dose-dependent disappearance of the mutant colonies is not a result of cell-killing (Maron & Ames, 1983; Rossi et al., 2011). The essential oils were diluted in DMSO, mixed with 2 ml of molten top agar and plated with 0.1 ml of the diluted culture. Histidine/biotin agar plates were enriched with 10 μmoles of L-histidine and 0.05 μmol of biotin by incorporating these nutrients into the soft agar overlay. Triplicate plates were poured for each dose of solution. Negative controls were set up with 100 μl/plate of DMSO with or without S9 mix. The colony-forming units (CFU) were assessed after the plates were incubated at 37 °C for 48 h and compared with that of control where no test samples were added. HUD for *C. lechleri* essential oil was evaluated by visual estimation (colonies counting) of three independent experiments and integrated by statistical analyses.

2.4.3. Mutagenic activity of heterocyclic amines (HCAs)

The mutagenic activity of the HCAs was determined on the basis of the counted CFU in plates treated with IQ, MeIQ, MeIQx, Glu-P-1 and Glu-P-2 at concentrations comprising between 10^{-10} and 10^{-5} mol/plate (Edenharder et al., 1999). The results were then compared to those recorded in negative control plates to check significant evidence of direct and indirect induced mutagenicity by HCAs. Therefore, to reach the target, mutagenic assays were performed in triplicate with and without metabolic activation (S9 mix).

2.4.4. Mutagen protection of C. lechleri essential in presence of HCAs

The potential mutagen protection of *C. lechleri* essential oil with TA98 tester strain system was determined for a concentration range from 0.01 to 0.10 mg/plate in presence of HCAs. Negative controls were set up with 100 μl/plate of DMSO with or without S9 mix. HCAs were all used at 10^{-9} mol/plate to check the protective capacity of the essential oil in presence of a clear indirect mutagenicity mediated by the presence of S9 mix. Given the different concentration at which the different HCAs induced direct mutagenicity (without S9 mix), the protective activity of the essential oil was checked in presence of IQ and MeIQ at 10^{-7} mol/plate, while for MeIQx, Glu-P-1 and Glu-P-2 the concentration tested was 10^{-5} mol/plate. The inhibition rate for mutagenic induction was computed considering the data obtained from three independent experiments, with and without metabolic activation, according to the formula: inhibition rate (%) = $(A - B) \times 100/A \pm$ standard deviation, where A are revertants in positive control, and B are revertants in the essential oil samples, having subtracted the spontaneous revertants. Data were expressed also as CFU/plate ± standard deviation.

2.5. DNA methyltransferase inhibition

C. lechleri essential oil (10^{-4} to 10^{-1} mg/ml concentration range) was analysed for the DNA methyltransferase 1 activity by the EpiQuik™ DNA methyltransferase assay kit and instructions provided by the manufacturer (EpiQuik DNA Methyltransferase 1 Activity/Inhibitor Screening Assay Kit, Epigentek Group Inc., New York, NY, USA). This kit yields accurate measurements of methylcytosine content as a percentage of total cytosine content. Briefly, in an assay with this kit, the unique cytosine-rich DNA substrate is stably coated on the strip wells. These wells are specifically treated to have a high DNA absorption ability. The Dnmt1 enzyme transfers a methyl group to cytosine from S-adenosyl-L-methionine to methylate DNA substrate. The methylated DNA can be recognised with anti-5-methylcytosine antibody. The ratio or amount of methylated DNA, which is proportional to enzyme activity, can then be colorimetrically quantified through an enzyme-linked immunosorbent assay-like reaction using 5-methylcytosine antibody. The amount of methylated DNA is proportional to the OD intensity and the degree of DNA methylation can be calculated using the following formula:

$$\%\text{Methylation} = [\text{OD(sample} - \text{blank})/2\text{OD(positive control} - \text{blank})] \times 100$$

where OD represents the optical density, blank is buffer without DNA, positive control is methylated control DNA.

2.6. MTT [3-(4,5-dimethylthiazol-2-yl)-2,5 diphenyltetrazolium bromide] assay

Two cancer cell lines, human colon carcinoma LoVo (ECACC No.: 87060101) and human hepatocellular carcinoma HepG2 (ATCC No.: CRL-11997) were used. The LoVo and HepG2 cells were cultured in RPMI 1640 and D-MEM medium, respectively, both supplemented with 10% foetal bovine serum, 1% L-glutamine and 1% penicillin/streptomycin.

The 3-(4,5-dimethylthiazol-2-yl)-2,5 diphenyltetrazolium bromide assay (MTT, Sigma, Italy) was used to determine the ability of metabolically active cells to reduce the yellow tetrazolium salt (MTT) forming insoluble purple formazan crystals. The assay for each sample concentration was performed in triplicates and the culture plates were kept at 37 °C with 5% (v/v) CO_2 for one day. After incubation, 100 μl of medium was removed from each well. Subsequently, 100 μl of 0.5% w/v MTT, dissolved in phosphate buffered saline, was added to each well and allowed to incubate for a further 4 h. After 4 h of incubation, 100 μl of DMSO was added to each well to dissolve the formazan crystals. Absorbance values at 570 nm were measured with a microplate reader (GDV DV 990 B/V, Roma, Italy) and expressed as IC_{50}, which is the concentration to reduce the absorbance of treated cells by 50% considered as cytotoxicity with reference to the control (untreated cells).

Cells counts and viability were performed using a standard trypan blue cell counting technique. The cell concentration was adjusted to 2×10^4 cells/ml. 100 µl of the above concentration were cultured in 96-well plates for one day to become nearly confluent. Concentrations ranging from 0.05 to 1 mg/ml of the samples were prepared from the stock solutions by serial dilution in cellular specific medium to give a volume of 100 µl in each well of a microliter plate (96-well). Then cells were cultured with vehicle, essential oil for 24 h.

2.7. Statistical analysis

Relative standard deviations and statistical significance (Student's *t* test; $P \leq 0.05$) were given where appropriate for all data collected. One-way ANOVA and LSD post hoc Fisher's honest significant difference test were used for comparing the bioactive effects of different *C. lechleri* essential oil samples. Student's *t* test ($P \leq 0.05$) combined with the Highest Uneffective Dose (HUD) comparison was used to interpret the results of a significant decrease in the number of *Salmonella* revertants. When the modulator dose concentration is statistically effective and it ranges below or coincides with the HUD, the samples were considered to present sign of the effect (antimutagenicity). All computations were made using the statistical software STATISTICA 6.0 (StatSoft Italia srl).

3. Results and discussion

C. lechleri essential oil was preliminarily investigated for its chemical composition and bioactivities, for its possible employ as functional food ingredient (Rossi et al., 2011). In particular, the essential oil was characterised as sesquiterpene chemotype, sesquicineole (17.18%), α-calacorene (11.22%), 1,10-di-epi-cubenol (4.72%), β-calacorene (4.31%) and epicedrol (4.07%) being the most abundant compounds accounting for 76.93% of the total (95.84%). The 18.89% of the remaining characterised part is composed of monoterpenes, in particular limonene (4.17%), borneol (2.66%), p-cymene (2.59%) and α-pinene (2.00%) (Fig. 1). Starting from the bioactivity evidences reported for the non-mutagenicity and for the mutagen-protective capacity (Rossi et al., 2011), *C. lechleri* essential oil was subjected to the Highest Uneffective Dose (HUD) evaluation in order to determine the maximum concentration of the terpenic phytocomplex which does not induce cytotoxicity (Table 1).

Therefore, the HUD for toxic effect, with and without metabolic activation through S9 mix, for TA98 and TA100 strains was settled at 0.1 and 0.01 mg/plate respectively (significance $P < 0.05$ according to *t* test; Fig. 1). This kind of result points out the higher sensitivity of TA100 than TA98 in respect to the toxicity of essential oils. This evidence could be due to the higher permeability of TA100 bacterial cell wall than TA98 towards mono- and sesquiterpenes (Ipek et al., 2005; Vuković-Gačić et al., 2006).

Given the fact that *C. lechleri* essential oil did not exhibit mutagen protective activity for TA100 against the direct mutagen sodium azide and indirect mutagen 2-aminoanthracene at lower concentration than HUD (0.01 mg/plate) (Rossi et al., 2011), and in light of a similar approach reported by related papers (Arimoto-Kobayashi & Hayatsu, 2003), the *S. typhimurium* TA98 strain with and without metabolic activation (S9 mix) was adopted for the following assays with heterocyclic amines (HACs) (Tables 2 and 3). Moreover, the specific mutagenic activity of HACs is much higher in TA98 strain than in TA100, indicating that heterocyclic amines induce frame shift mutations (Frederiksen, 2005).

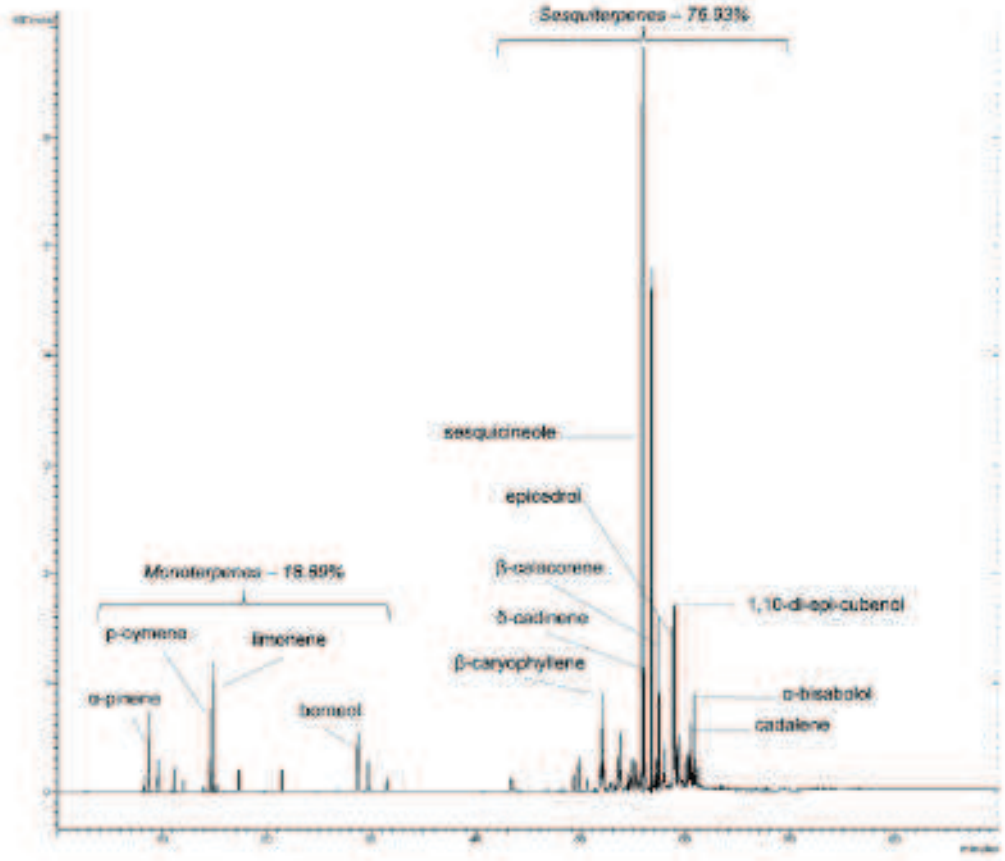

Fig. 1. Gas chromatographic spectrum of Croton lechleri stem bark essential oil. Sesquiterpenes were the most abundant compounds being the 76.93% of the total, while monoterpenes accounted for 18.89%.

Table 1
Highest Uneffective Dose (HUD) tested with and without metabolic activation (S9 mix). HUD represents the maximum concentration of *Croton lechleri* essential oil (*C. l.* e.o.) DMSO diluted which does not induce cytotoxic evidences in *S. typhimurium* TA98 and TA100 strains cultures. The HUD data are essential to interpret the results of significant decrease in the number of *Salmonella* revertants. Negative controls (*C. l.* e.o. = 0.000 mg/plate) have been set up with 100 μl/plate of DMSO. The results are expressed both as survival percentage ± standard deviation (s.d.) and Colony Forming Units (CFU)/plate ± standard deviation.

C. l. e.o. (mg/plate)	CFU/plate	Survival%	CFU/plate	Survival%	CFU/plate	Survival%	CFU/plate	Survival%
	TA98 without S9 mix		TA98 with S9 mix		TA100 without S9 mix		TA100 with S9 mix	
0.000	1682 ± 67	100.00 ± 3.98	3303 ± 61	100.00 ± 1.85	3433 ± 44	100.00 ± 1.28	3587 ± 46	100.00 ± 1.85
0.010	1708 ± 51	101.54 ± 3.03	3337 ± 39	101.03 ± 1.18	3421 ± 52	99.65 ± 1.52	3617 ± 42	101.03 ± 1.18
0.025	1695 ± 47	100.77 ± 2.77	3361 ± 52	101.76 ± 1.55	2452 ± 46*	71.42 ± 1.87*	1814 ± 33*	50.57 ± 1.82*
0.050	1676 ± 41	99.64 ± 2.44	3367 ± 57	101.94 ± 1.73	1516 ± 22*	44.16 ± 1.45*	902 ± 26*	25.15 ± 1.88*
0.075	1634 ± 38	97.15 ± 2.33	3315 ± 48	100.36 ± 1.45	1034 ± 23*	30.12 ± 2.12*	698 ± 23*	19.46 ± 1.29*
0.100	1568 ± 33	93.22 ± 1.96	3289 ± 54	99.57 ± 1.96	128 ± 19*	3.73 ± 1.48*	457 ± 18*	12.74 ± 0.93*
0.250	834 ± 15*	49.58 ± 1.79*	1256 ± 23*	38.03 ± 1.83*	94 ± 12*	2.74 ± 1.27*	127 ± 12*	3.54 ± 0.33*
0.500	31 ± 12*	1.84 ± 0.71*	634 ± 24*	19.19 ± 0.73*	7 ± 2*	0.21 ± 0.05*	16 ± 3*	0.45 ± 0.08*
1.000	–*	0.00 ± 0.00*	94 ± 7*	4.12 ± 0.23*	–*	0.00 ± 0.00*	–*	0.00 ± 0.00*
5.000–10.000[a]	–*	0.00 ± 0.00*	–*	0.00 ± 0.00*	–*	0.00 ± 0.00*	–*	0.00 ± 0.00*

(–) no Colony Forming Units (CFU) has been detected because of the cytotoxicity expressed by the *C. lechleri* essential oil.
[a] *C. lechleri* essential oil has been tested until the concentration of 10.00 mg/plate giving always cytotoxicity evidences.
* Significant evidences (cytotoxicity) in light of Student's *t*-test results.

Table 2
Ames test (*Salmonella typhimurium*, strain TA98) to assay mutagen induction (revertants his+/plate) with metabolic activation (S9 mix) of the Heterocyclic amines (HCAs) 2-amino-3-methylimidazo-[4,5-*f*]quinoline (IQ), 2-amino-3,4-dimethylimidazo-[4,5-*f*]quinoline (MeIQ), 2-amino-3,8-dimethylimidazo-[4,5-*f*]quinoxaline (MeIQx), the imidazoles 2-amino-6-methyldipyrido-[1,2-*a*:3′,2′-*d*]imidazole (Glu-P-1) and 2-aminodipirydo-[1,2-*a*:3′,2′-*d*]imidazole (Glu-P-2) tested at 10^{-10} to 10^{-5} mol/plate concentration range. HCAs have been diluted in DMSO; negative controls (HCAs = 0 mol/plate) have been set up with 100 μl/plate of DMSO. The results are expressed as Colony Forming Units (CFU)/plate ± standard deviation (s.d.). All the values resulted significant according to Ames computation (Maron & Ames, 1983), except for those achieved for negative controls.

HCAs (mol/plate)	TA98 with S9 mix				
	IQ CFU/plate ± s.d.	MeIQ CFU/plate ± s.d.	MeIQx CFU/plate ± s.d.	Glu-P-1 CFU/plate ± s.d.	Glu-P-2 CFU/plate ± s.d.
0	59 ± 4	59 ± 4	59 ± 4	59 ± 4	59 ± 4
10^{-10}	1060 ± 28	2162 ± 39	561 ± 21	490 ± 35	268 ± 18
10^{-09}	2526 ± 153	2812 ± 64	1434 ± 77	1254 ± 34	1322 ± 139
10^{-08}	4155 ± 169	2864 ± 37	2479 ± 24	4029 ± 154	3320 ± 193
10^{-07}	3824 ± 117	3166 ± 67	3450 ± 31	3463 ± 151	3026 ± 161
10^{-06}	2990 ± 105	2762 ± 73	3069 ± 53	2956 ± 158	2395 ± 116
10^{-05}	2687 ± 107	2228 ± 84	2562 ± 47	2587 ± 108	1838 ± 104

Table 3
Ames test (*Salmonella typhimurium*, strain TA98) to assay mutagen induction (revertants his+/plate) without metabolic activation (S9 mix) of the Heterocyclic amines (HCAs) 2-amino-3-methylimidazo-[4,5-*f*]quinoline (IQ), 2-amino-3,4-dimethylimidazo-[4,5-*f*]quinoline (MeIQ), 2-amino-3,8-dimethylimidazo-[4,5-*f*]quinoxaline (MeIQx), the imidazoles 2-amino-6-methyldipyrido-[1,2-*a*:3′,2′-*d*]imidazole (Glu-P-1) and 2-aminodipirydo-[1,2-*a*:3′,2′-*d*]imidazole (Glu-P-2) tested at 10^{-10} to 10^{-5} mol/plate concentration range. HCAs have been diluted in DMSO; negative controls (HCAs = 0 mol/plate) have been set up with 100 μl/plate of DMSO. The results are expressed as Colony Forming Units (CFU)/plate ± standard deviation.

HCAs (mol/plate)	TA98 without S9 mix				
	IQ CFU/plate ± s.d.	MeIQ CFU/plate ± s.d.	MeIQx CFU/plate ± s.d.	Glu-P-1 CFU/plate ± s.d.	Glu-P-2 CFU/plate ± s.d.
0	42 ± 4	42 ± 4	25 ± 2	25 ± 2	25 ± 2
10^{-10}	48 ± 8	45 ± 6	26 ± 2	31 ± 3	26 ± 3
10^{-09}	56 ± 6	51 ± 8	31 ± 3	29 ± 6	27 ± 5
10^{-08}	230 ± 14*	413 ± 39*	29 ± 5	28 ± 3	29 ± 7
10^{-07}	1907 ± 45*	1257 ± 38*	26 ± 6	24 ± 4	23 ± 4
10^{-06}	1404 ± 52*	341 ± 27*	35 ± 5	40 ± 2	38 ± 6
10^{-05}	1334 ± 25*	278 ± 26*	115 ± 9*	94 ± 9*	42 ± 5

* Significant values according to Ames computation (Maron & Ames, 1983).

The mutagen protective capacity of *C. lechleri* essential oil against direct and indirect mutagens (nitrofluorene and 2-aminoanthracene) on TA98 (Rossi et al., 2011) led us to investigate the same capacity employing heterocyclic amines (HCAs). Heterocyclic amines, produced during the cooking process of proteins rich foods, i.e., meat (beef, pork, lamb, and chicken), residues after cooking meat, beef flavors, fish, are potent carcinogenic mutagens when metabolically activated (Arimoto-Kobayashi & Hayatsu, 2003; Aydin et al., 2005; Robbana-Barnat et al., 1996). Although the quantification of HACs in a typical Western diet proves to be difficult, the exposure level to these chemical compounds can be considered similar, to a good approximation, to that of nitrosamines and benzo[a]pyrene (Robbana-Barnat et al., 1996). However, since the real risk to humans is still a matter of debate, WHO and National Health Organizations suggest minimizing the exposure. The HACs formation during cooking at temperatures above 200 °C is mainly due both to pyrolysis of amino acids and to different reaction sequences having amino acids, carbohydrates and creatinine as substrates. The multi-target carcinogen activity of HACs was demonstrated in different laboratory animal models, and the

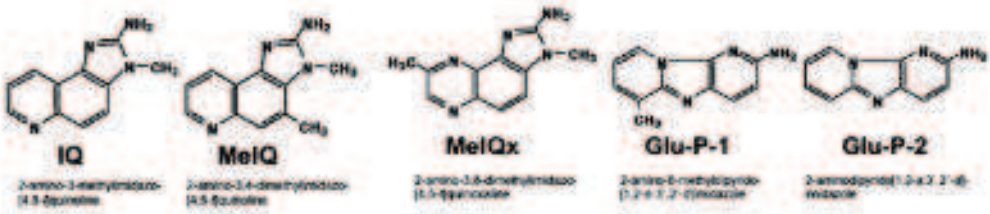

Fig. 2. Chemical structure of the tested Heterocyclic amines (HCAs) 2-amino-3-methylimidazo-[4,5-*f*]quinoline (IQ), 2-amino-3,4-dimethylimidazo-[4,5-*f*]quinoline (MeIQ), 2-amino-3,8-dimethylimidazo-[4,5-*f*]quinoxaline (MeIQx), the imidazoles 2-amino-6-methyldipyrido-[1,2-*a*:3′,2′-*d*]imidazole (Glu-P-1) and 2-aminodipirydo-[1,2-*a*:3′,2′-*d*]imidazole (Glu-P-2).

HACs synergic capacity was related to inducing amino-α-carboniles adducts formation in DNA of cells belonging to stomach, liver, colon, kidneys, prostate and skin tissues (Aydin et al., 2006, and references therein). The HACs considered were the quinolines 2-amino-3-methylimidazo-[4,5-*f*]quinoline (IQ), 2-amino-3,4-dimethylimidazo-[4,5-*f*]quinoline (MeIQ), 2-amino-3,8-dimethylimidazo-[4,5-*f*]quinoxaline (MeIQx), the imidazoles 2-amino-6-methyldipyrido-[1,2-*a*:3′,2′-*d*]imidazole (Glu-P-1) and 2-aminodipirydo-[1,2-*a*:3′,2′-*d*]imidazole (Glu-P-2) (Fig. 2). Even if heterocyclic amines are generally reputed to be indirect mutagens, they were used to induce mutagenicity on TA98 strain with and without S9 mix (Tables 2 and 3). All HCAs in presence of S9 mix induced mutagenicity already at 10^{-10} mol/plate, with significant revertants values according to Ames computation (Maron & Ames, 1983), confirming related literature data (Table 2; Edenharder et al., 1999). Interesting evidence emerged by the same assay without metabolic activation, with a direct significant mutagenicity of IQ and MeIQ at 10^{-8} mol/plate (Table 3). In the same conditions, MeIQx and Glu-P-1 gave revertants at 10^{-5} mol/plate while Glu-P-2 resulted inactive as direct mutagen (Table 3). Given the fact that the concentration of 10^{-8} mol/plate is consistent with the amount of HACs in cooked food (Frederiksen, 2006), the results have important implications on the debate about the relationship between heterocyclic amines mutagenesis/cancerogenesis risk and the diet. Results on HUD and to HACs mutagenicity, with and without metabolic activation, drove the mutagen-protective activity of *C. lechleri* essential oil (0.01–0.1 mg/plate concentration range) against all the HACs metabolically activated with S9 mix tested at 10^{-9} mol/plate (Table 4). *C. lechleri* essential oil induced a significant decrease in revertants colonies at 0.05 mg/plate with all HACs. In fact, the reduction was almost of 22% for IQ, 29% for MeIQ, 31% for MeIQx, 20% for Glu-P-1 and of 23% for Glu-P-2. At the highest concentration tested of essential oil (0.1 mg/plate), the highest mutagenic protection was expressed with a revertants reduction of about 69% for Glu-P-2, followed by MeIQx (59%), IQ (42.19%), Glu-P-1 (33%) and MeIQ (30%).

In light of their direct mutagenic activity (Table 3), IQ and MeIQ was tested at 10^{-7} mol/plate to check the possible genotoxic protective activity of *C. lechleri* essential oil (Table 5). As for the metabolic activated conditions, a significant protective activity of essential oil was expressed at 0.05 mg/plate without S9, with a reduction of revertants colonies percentage of almost 39% and 40% for IQ and MeIQ respectively. At 0.1 mg/plate, *C. lecheri* essential oil induced a revertants reduction of 46% for IQ and of 41% for MeIQ in treated *S. typhimurium* TA98 strains. On the contrary, TA98 strains treated with MeIQx and Glu-P-1 at 10^{-5} mol/plate did not exhibit a reduction of revertants colonies in the presence of *C. lechleri* essential oil. Glu-P-2 was not assayed because it did not induce mutagenicity without metabolic activation (Table 6).

The fact that IQ and MeIQ displayed a moderate direct-acting mutagenic activity still inhibited by *C. lecheri* essential oil, leads us to suggest that the essential oil may be involved both early and late in mutagenesis, i.e., by inhibiting metabolic activation via P450, and by either reacting with an ultimate mutagen or blocking its access to DNA. As a matter of fact, the antimutagenic action without S9 mix cannot be regarded as an effective proof to discharge the hypothesis of a promutagen metabolic activation inhibition. Indeed, the antimutagenic effects of flavonoids, chalcones and structurally related compounds on the activity of IQ were proved only when cytosol activation is involved without hexogen S9 fraction addition (Hatch, Lightstone, & Colvin, 2000). This could be the result of inhibition of either N-OH-IQ formation by a non-450 route or a different pathway of IQ activation. MeIQx, as a case in point, is activated through metabolic pathway under the action of *N*-acetyltransferase forming N2-(guanine-8-yl-MeIQx) adduct with C8 position of guanine (Ochiai et al., 1993). To confirm the hypothesis of a HCAs endogen activation by *S. typhimurium* TA98, an acetyltransferase enzyme was partially purified from this strain and characterised as *O*-acetyltransferase (Saito, Shinohara, Kamataki, & Kato, 1985).

The chemical composition of *C. lechleri* stem bark essential oil (Fig. 1; Rossi et al., 2011) can help to suggest which compounds are responsible for the mutagenic protection. However, the bioactivity of an essential oil, as well as of other kind of phytocomplexes, is hardly due to a single active compound, but it is rather ascribed to a synergic activity of different kinds of chemicals that may not be necessarily the most abundant (Bakkali et al., 2008; Edris, 2007; Voon, Bhat, & Rusul, 2012). Terpenes and their derivatives were found to be potentially useful in the prevention and therapy of several diseases, including cancer. For example, d-limonene (4.20% in *C. lechleri* essential oil) and linalool (0.82% in *C. lechleri* essential oil) are claimed to inhibit, in a dose-dependent manner, the development of mammary, liver, skin, lung, colon, fore-stomach, prostate, cervical and pancreatic carcinomas (Cheng, Shieh, Chiang, Chang, & Chiang, 2007). β-Caryophyllene, a sesquiterpene with interesting protective capacity against oxidative stress (Rossi et al., 2011; Zheng, Kenney, & Lam, 1992) was suggested as a promising potential anticarcinogenic agent. The antimutagenic effects of linalool and β-caryophyllene were also evaluated by the bacterial reverse mutation assay on *S. typhimurium* TA98 and TA100 (Di Sotto, Evandri, & Mazzanti, 2008). Linalool is devoid of antimutagenic activity against 2-nitrofluorene, sodium azide, methyl methane sulfonate and 2-aminoanthracene. In contrast, β-caryophyllene showed a strong antimutagenic activity against 2-nitrofluorene. To our knowledge, related literature does not report any data about inhibition of IQ, MeIQ, MeIQx, Glu-P-1 and Glu-P-2 mutagenicity in the bacterial reverse mutation assay on *S. typhimurium* by terpenes. Even so, it is possible to make suggestions about the correlations between the chemical composition of *C. lechleri* bark essential oil and other structurally related compounds already studied for antimutagenic effect against HCAs. Interesting suggestions came from a quantitative structure–activity relationship (QSAR) database, developed by the combination of inhibitory process results of flavonoids against HCAs mutagenesis with structural evidences, *ab initio* quantum chemical, hydropathic, and antioxidant factors (Hatch et al., 2000). Limited quantitative outcomes emerged from this research strategy supporting the evidence that the inhibition

Table 4
Ames test (Salmonella typhimurium, strain TA98) with metabolic activation (S9 mix) to assay mutagen protective activity of *Croton lechleri* essential oil (*C. l.* e.o.; 0.01–0.10 mg/plate concentration range) in presence of 1×10^{-9} mol/plate of the heterocyclic amines (HCAs): 2-amino-3-methylimidazo-[4,5-*f*]quinoline (IQ), 2-amino-3,8-dimethylimidazo-[4,5-*f*]quinoxaline (MeIQx), the imidazoles 2-amino-6-methyldipyrido-[1,2-*a*:3′,2′-*d*]imidazole (Glu-P-1) and 2-aminodipyrido-[1,2-*a*:3′,2′-*d*]imidazole (Glu-P-2). Negative controls (*C. l.* e.o. = 0.000 mg/plate) have been set up with 100 μl/plate of DMSO. The results are expressed both as revertants percentage (Rev.%) ± standard deviation (s.d.) and Colony Forming Units (CFU)/plate ± standard deviation.

C. l. e.o. (mg/plate)	TA98 with S9 mix									
	HCAs 10^{-9} mol/plate									
	IQ*		MeIQ		MeIQx		Glu-P-1		Glu-P-2	
	Rev.% ± s.d.	CFU/plate ± s.d.	Rev.% ± s.d.	CFU/plate ± s.d.	Rev.% ± s.d.	CFU/plate ± s.d.	Rev.% ± s.d.	CFU/plate ± s.d.	Rev.% ± s.d.	CFU/plate ± s.d.
0.000	100.00 ± 5.62	2307 ± 70	100.00 ± 5.58	3672 ± 97	100.00 ± 5.42	1106 ± 51	100.00 ± 5.56	1170 ± 52	100.00 ± 5.63	1267 ± 41
0.010	102.56 ± 5.11	2366 ± 51	94.21 ± 5.52	3459 ± 95	95.42 ± 6.51	1056 ± 51	92.99 ± 5.43	1083 ± 34	96.03 ± 5.30	1217 ± 30
0.025	98.09 ± 4.89	2263 ± 32	90.32 ± 5.07	3427 ± 95	92.94 ± 5.03	1028 ± 51	92.13 ± 4.98	1078 ± 44	94.32 ± 5.15	1195 ± 24
0.050	78.41 ± 2.72*	1809 ± 27*	72.13 ± 4.13*	2649 ± 93*	68.64 ± 4.18*	759 ± 51*	79.28 ± 4.58*	926 ± 38*	77.36 ± 4.22*	981 ± 20*
0.075	68.66 ± 2.04*	1584 ± 23*	70.86 ± 3.98*	2602 ± 93*	54.70 ± 3.80*	605 ± 35*	72.95 ± 4.07*	853 ± 51*	54.77 ± 3.82*	694 ± 18*
0.100	57.81 ± 1.42*	1333 ± 32*	69.85 ± 3.72*	2565 ± 87*	41.25 ± 2.11*	456 ± 11*	66.77 ± 3.81*	781 ± 41*	31.46 ± 2.59*	399 ± 29*

* Significant values (Student *t* test).

Table 5
Ames test (*Salmonella typhimurium*, strain TA98) without metabolic activation (S9 mix) to assay mutagen protective activity of *Croton lechleri* essential oil (*C. l.* e.o.; 0.01–1.00 mg/plate concentration range) in presence of 1×10^{-7} mol/plate of the heterocyclic amines (HCAs) 2-amino-3-methylimidazo-[4,5-*f*]quinoline (IQ) and 2-amino-3,4-dimethylimidazo-[4,5-*f*]quinoline (MeIQ). Negative controls (*C. l.* e.o. = 0.000 mg/plate) have been set up with 100 μl/plate of DMSO. The results are expressed both as revertants percentage (Rev.%) ± standard deviation (s.d.) and Colony Forming Units (CFU)/plate ± standard deviation.

C. l. e.o. (mg/plate)	TA98 without S9 mix			
	HCAs 10^{-7} mol/plate			
	IQ		MeIQ	
	Rev.% ± s.d.	CFU/plate ± s.d.	Rev.% ± s.d.	CFU/plate ± s.d.
0.000	100.00 ± 5.80	1772 ± 46	100.00 ± 5.48	1566 ± 55
0.010	99.29 ± 5.11	1759 ± 40	99.04 ± 5.02	1551 ± 40
0.025	95.48 ± 4.84	1692 ± 38	95.92 ± 4.88	1502 ± 39
0.050	61.16 ± 2.92*	1084 ± 24*	59.64 ± 3.53*	934 ± 44*
0.075	58.19 ± 2.78*	1002 ± 22*	59.06 ± 2.82*•	925 ± 32*
0.100	54.25 ± 2.42*	961 ± 31*	58.80 ± 3.78*	921 ± 27*

* Significant values (Student *t* test).

Table 6
Ames test (*Salmonella typhimurium*, strain TA98) without metabolic activation (S9 mix) to assay mutagen protective activity of *Croton lechleri* essential oil (*C. l.* e.o.; 0.01–1.00 mg/plate concentration range) in presence of 1×10^{-5} mol/plate of the heterocyclic amines (HCAs) 2-amino-3,8-dimethylimidazo-[4,5-*f*]quinoxaline (MeIQx), the imidazoles 2-amino-6-methyldipyrido-[1,2-*a*:3′,2′-*d*]imidazole (Glu-P-1) and 2-aminodipirydo-[1,2-*a*:3′,2′-*d*]imidazole (Glu-P-2). Negative controls (*C. l.* e.o. = 0.000 mg/plate) have been set up with 100 μl/plate of DMSO. The results are expressed both as revertants percentage (Rev.%) ± standard deviation (s.d.) and Colony Forming Units (CFU)/plate ± standard deviation.

C. l. e.o. (mg/plate)	TA98 without S9 mix			
	HCAs 10^{-5} mol/plate			
	MeIQx		Glu-P-1	
	Rev.% ± s.d.	CFU/plate ± s.d.	Rev.% ± s.d.	CFU/plate ± s.d.
0.000	100.00 ± 3.90	90 ± 3	100.00 ± 4.25	93 ± 4
0.010	100.74 ± 5.21	91 ± 8	96.76 ± 5.03	90 ± 8
0.025	103.33 ± 5.48	93 ± 8	98.92 ± 5.19	92 ± 5
0.050	105.19 ± 5.58	95 ± 8	101.08 ± 5.58	94 ± 5
0.075	98.89 ± 5.18	89 ± 7	102.15 ± 5.64	95 ± 5
0.100	97.42 ± 5.12	88 ± 7	106.83 ± 5.81	99 ± 15

of heterocyclic amine mutagenesis by flavonoids involves the interference with the cytochrome P450 pathway, caused by non-covalent, or eventually covalent, bindings. The widely discussed antioxidant and radical scavenging properties of flavonoids appear to be unrelated to the inhibition of mutagenesis. In fact, the major variables controlling the inhibitory efficacy of the flavonoids in the *S. typhimurium* assay are near-planarity, hydrophobicity and dipole moment.

Relevance of planarity was confirmed in chalcones and structurally related compounds acting as heterocyclic amine mutagenesis inhibitors (Edenharder et al., 1999). If planarity is considered as a structural feature useful as anti mutagenic activity identifier, sesquicineole, the most abundant chemical in *C. lechleri* bark essential oil (17.29%), would be characterised as an effective HCAs mutagenicity inhibitor. On the contrary, α-calacorene (11.29% in *C. lechleri* essential oil) could be structurally related with the inhibitory potency of the flavonoids and chalcones.

Hypothesising that *C. lechleri* essential oil protective activity would be involved in DNA methylation processes, methyltransferase 1 inhibition of essential oil (10^{-4} to 10^{-1} mg/ml concentration range) was tested three times by the EpiQuik™ DNA methyltransferase assay kit. No significant differences between control and treated samples

Table 7
Cytotoxicity expressed as growth inhibition percentage (Inh.%) and IC_{50} (μg/ml) of *Croton lechleri* essential oil (*C. l.* e.o.) against human colon carcinoma LoVo (ECACC No.: 87060101) and human hepatocellular carcinoma HepG2 (ATCC No.: CRL-11997) cell lines. Exposure time 24 h ± s.d. (n = 3). Vinblastine (2 g/ml) has been used as positive control.

C. l. e.o. (μg/ml)	LoVo (Inh.%)	HepG2 (Inh.%)
1000	99.12 ± 0.06	88.46 ± 0.04
500	98.97 ± 0.05	87.56 ± 0.04
250	98.92 ± 0.05	85.86 ± 0.03
100	72.66 ± 0.04	63.39 ± 0.02
50	23.53 ± 0.02	20.76 ± 0.01
IC_{50} (μg/ml)	74.95 ± 0.05	82.28 ± 0.03

were found (data not shown), pointing out that the essential oil is not involved in any significant inhibitory effect of DNA methylation.

Given the fact that the Ames assay is an ubiquitously accepted mutagenicity and anti-mutagenicity test and a good predictive tool for carcinogens (83% of mutagens found in the Ames test are also carcinogens) (Ames et al., 1973; Edenharder et al., 1999; Rossi et al., 2011), the stem bark *C. lechleri* essential oil was evaluated for its *in vitro* cytotoxic properties on two human cancer cell lines: colon carcinoma LoVo and hepatocellular carcinoma HepG2. Both human cell lines were capable of forming a homogeneous monolayer on plastic substratum of the culture wells, set for the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. The cytotoxic effect of essential oil on the growth of human tumor cell lines, expressed as IC_{50} (Table 7), point out the human colon carcinoma LoVo as the most responsive cell line (IC_{50} = 74.95 μg/ml) with interesting values consistent with those highlighted in other related studies about the antitumor activity of *Croton* spp. essential oils (Bezerra et al., 2009; Sylvestre et al., 2006). This essential oils cytotoxicity can be related to the bioactivity of different terpene-rich extracts from other *Croton* species (Dao et al., 2010; Phan, Phan, Hamada, & Otsuka, 2005). In light of this evidence it could be speculated that the cytotoxic activity of the *C. lechleri* stem bark essential oil would be mainly determined by the most abundant sesquiterpene fractions. In particular, considering the antioxidant capacity reported in a previous related paper (Rossi et al., 2011) as discriminant tool in suggesting the most responsible compounds, the sesquiterpene α- and β-calacorene, δ-cadinene, cadalene, caryophyllene oxide, 1,10-di-epi-cubenol and epicedrol would probably be the most involved compounds in exerting cytotoxicity of the *C. lechleri* stem bark essential oil.

4. Conclusion

C. lechleri stem bark essential showed interesting anti-mutagenic properties at concentrations consistent with its safe use as additive functional phytocomplex in foods (for e.g., as relish for high protein cooked foods). Based on this evidence, a new threshold of HCAs as indirect mutagens was evaluated, together with the determination of direct mutagen capacity of some HCAs at concentrations consistent with their presence in cooked foods. Data reported in this paper also demonstrate that *C. lechleri* stem bark essential oil offers an effective protection against the mutagenic potential of HCAs with and without metabolic activation. In light of the results achieved with the Ames test, and the cytotoxic and antiproliferative assays against colon and hepatocellular carcinomas, it is hard to suggest the potential risk caused by this essential oil in humans, since complex metabolic activation reactions are not adequately represented in *in vitro* assays with exogenous homogenate enzymes. Nevertheless, the data reported in this study provide the framework for the development of new anticancer drugs.

5. Conflict of Interest

The authors declare that there are no conflicts of interest.

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Inhibition of Cancer Cell Proliferation and Antiradical Effects of Decoction, Hydroalcoholic Extract, and Principal Constituents of *Hemidesmus indicus* R. Br.

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Indian Sarsaparilla (*Hemidesmus indicus* R. Br.) is widely used in Indian traditional medicine. In the present work, we explored the effects of decoction, traditional Ayurvedic preparation, and hydroalcoholic extract, a phytocomplex more traditionally studied and commercialized as food supplement in western medicine, from the roots as possible source of chemicals with new functional potential linked to their nutritional uses. The antiproliferative and antioxidant properties were assayed. To test antiproliferative affects, different cancer cell lines, growing both as monolayers (CaCo2, MCF-7, A549, K562, MDA-MB-231, Jurkat, HepG2, and LoVo) and in suspension (K562 and Jurkat) were used. The decoction showed strong activity on HepG2 cells, while the hydroalcoholic extracts were active on HepG2, LoVo, MCF-7, K562, and Jurkat cell lines. Weak inhibition of cancer cell proliferation was observed for the principal constituents of the preparations: 2-hydroxy-4-methoxybenzaldehyde, 2-hydroxy-4-methoxybenzoic acid, and 3-hydroxy-4-methoxybenzaldehyde that were tested alone. The antiradical activity was tested with 2,2-diphenyl-1-picrylhydrazyl and 2,2′-azinobis(3-ethylbenzothiazoline-6-sulfonic acid)diammonium salt tests and inhibition of nitric oxide production in lipopolysaccharide-stimulated RAW 264.7 macrophages. Interesting result has also been obtained for hydroalcoholic extract regarding genoprotective potential (58.79% of inhibition at 37.5 μg/mL). Copyright © 2015 John Wiley & Sons, Ltd.

Keywords: Ayurveda; antitumor activity; genoprotective potential; antioxidants; nitric oxide inhibitors.

Abbreviations: DPPH, 2,2-Diphenyl-1-picrylhydrazyl; ABTS, 2,2′-Azinobis(3-ethylbenzothiazoline-6-sulfonic acid)diammonium salt; CaCo2, Human colorectal adenocarcinoma cells; MCF-7, Human estrogen receptor (ER)-positive breast adenocarcinoma cells; A549, Human lung carcinoma cells; HepG2, Human hepatocellular carcinoma cells; LoVo, Human colon cardinoma cells; MDA-MB-231, Human estrogen receptor (ER)-negative breast adenocarcinoma cells; Jurkat, Human T-lymphoid leukemia cells; K562, Human chronic myeloid leukemia cells

INTRODUCTION

Hemidesmus indicus R. Br. (Asclepiadaceae), also known as Indian sarsaparilla, is a common weed found all over India. Its root is widely used in ayurvedic traditional medicine, and it is an ingredient in its typical preparations alone or in combination with other plants (Ayurvedic Pharmacopoeia Committee, 1989).

Hemidesmus indicus roots have a wide variety of ethnomedicinal uses, the most important of which is probably the treatment of dysentery and diarrhea, but it is also used for other infections, skin disease, menorrhagia, postpartum recovery, stomach ulcer and gastric ailments, fever, headache, pain and inflammation, sore mouth, venereal disease including gonorrhea and syphilis, impotence, and as a blood purifier, cooling tonic and appetite stimulant, and to promote health and vitality and to neutralize snake bite and scorpion sting (Das *et al.*, 2003; Austin, 2008). Under a pharmacological point of view, *H. indicus* has been studied for the first time in 1962, when the diuretic potential of its roots has been explored (Satoskar *et al.*, 1962). Since then, four reviews and numerous other specific articles on the pharmacology of *H. indicus* have been published (Austin, 2008; Aneja *et al.*, 2008; George *et al.*, 2008; Das and Bisht, 2013), suggesting a wide range of beneficial effects, including chemopreventive and antitumour activity, hepatoprotection, free radical scavenging and antioxidant activity, cardioprotection, neuroprotection, antithrombotic and hypolipidemic effects, renal protection, antiulcer activity, and anti-infective and antiinflammatory activities through *in vitro* and *in vivo* research strategies (Das and Bisht, 2013). The pharmacological studies referred particularly to the decoction of the *H. indicus* roots, which is the preparation traditionally indicated in Pharmacopeia and Ayurvedic medicine. In this paper, we have compared the decoction with a hydroalcoholic extract, a traditional product used in western medicine.

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The major shortcoming in a large number of experimental and clinical studies is the absence of phytochemical standardization of the administrated preparations. Therefore, the aim of this research was to standardize the extracts, to define and quantify some specific markers and then, as required by International Agencies for the use approval of a drug as medicinal plant or food supplement, to highlight the correlation between chemical information and biological–therapeutic activities. For this reason, we have firstly performed the standardization of *H. indicus* root decoction and hydroalcoholic extract, and then, we have determined the *in vitro* antitumor activity against a panel of cancer cell lines in order to highlight their possible selective cytotoxic effects on cancer cells, comparing the obtained results with related literature data for decoction against HepG2 (Thabrew *et al.*, 2005; Samarakoon *et al.*, 2012) and contributing to extend the researches toward other cell lines not previously studied. Finally, in light of acquired evidences, regarding the chemical and functional characterization of each single preparation, suggestion about new therapeutic potential of *H. indicus* extracts was pointed out.

MATERIALS AND METHODS

Plant materials. The ayurvedic crude drug (roots) was collected in 2010 from Ram Bagh (Rajasthan, India), in particular, following the indications of Ayurvedic Pharmacopoeia of India (2004) during the balsamic period that is for *H. indicus* R. Br. roots in January (winter). This crude drug was authenticated by Dr. M. R Uniyal, Maharishi Ayurveda Product Ltd., Noida, India.

Chemicals. Gallic acid, hyperoside, cyanidin chloride, 2,2-diphenyl-1-picrylhydrazyl (DPPH), 2,2′-azinobis(3-ethylbenzothiazoline-6-sulfonic acid)diammonium salt (ABTS), Folin–Ciocalteau reagent, RPMI (acronym of Roswell Park Memorial Institute) 1640 medium, fetal bovine serum (FBS), L-glutamine, penicillin/streptomycin, trypan blue, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), lipase from porcine pancreas Type II, 4-nitrophenyl octanoate, orlistat, Griess reagent (1% sulphonamide and 0.1% *N*-(1-naphtyl) ethylenediaminedihydrochloride in 2.5% H_3PO_4), and (L-NG-Nitroarginine Methyl Ester) L-NAME were obtained from Sigma-Aldrich S.p.a. (Milano, Italy). MCF-7 and A549 were purchased by Istituto Zooprofilattico Sperimentale della Lombardia e dell'Emilia-Romagna, Brescia, Italy; K562, CaCo2, MDA-MB-231, Jurkat, HepG2, LoVo, and RAW 264.7 were from American Type Culture Collection (University Boulevard, Manassas, VA 20110 USA). References substances (2-hydroxy-4-methoxybenzaldehyde, 3-hydroxy-4-methoxybenzaldehyde, and 2-hydroxy-4-methoxybenzoic acid) in HPLC analysis are from Sigma-Aldrich S.p.a. All other reagents, of analytical grade, were supplied by VWR International s.r.l. (Milan, Italy).

Extraction and preparation of formulations. The decoction was obtained by mixing 10 g of grinded roots with 300 mL of boiling water, allowing the volume of water to reach 75 mL, according to the method previously described and agreed with Ayurvedic Pharmacopoeia (Ferruzzi *et al.*, 2013). The hydroalcoholic extract was prepared suspending 50 g of dried grinded roots in 450 mL of ethanolic solution 30% (v/v ethanol/water) and stirred for 21 days at 25 °C (Préparations homéopathiques (1038), Pharmacopeé française, 11e edition). The two extracts were then filtered, lyophilized in an Edwards E–C Modulyo lyophilizer, and stored in the dark at −20 °C. Both formulations were prepared 10 times to ensure the best statistical standardization. Resulting powders were then redissolved according to the corresponding assay conditions and checked for the amount of phytomarkers by HPLC before starting experiments. Vouchers of the lyophilized extracts were deposited in Department of Life Sciences and Biotechnology (SVeB) of the University of Ferrara and, respectively, coded as HEI01D and HEI01E.

Determination of total phenolics, anthocyanins, and flavonoids content and free radical scavenging activity. The determination of the total polyphenolic, flavonoidic, and procyanidin content in decoction and hydroalcoholic extract was performed using a Helios-γ spectrophotometer (Thermo Spectronic, Cambridge, UK), according to previously described methods (Rossi *et al.*, 2012). Total polyphenols were expressed as gallic acid, flavonoids as hyperoside, and procyanidins as cyanidin chloride.

Radical scavenging properties were performed in different assays, DPPH and ABTS tests, according to previously described methods (Rossi *et al.*, 2012) to determine the IC_{50} value using ThermoSpectronic Helios-γ spectrophotometer.

HPLC and nuclear magnetic resonance analyses. The decoction and the hydroalcoholic extract were subjected to HPLC analysis to quantify its main phytomarkers: 2-hydroxy-4-methoxybenzaldehyde, 3-hydroxy-4-methoxybenzaldehyde, and 2-hydroxy-4-methoxybenzoic acid. The reference compounds were used as external standards to set up and calculate appropriate calibration curves. The analyses were performed using a Jasco modular HPLC (model PU 2089, Tokyo, Japan) coupled to a diode array apparatus (MD 2010 Plus) according to the method described by Ferruzzi *et al.* (2013). To ensure the best standardization process of the two extracts, nuclear magnetic resonance fingerprinting has been acquired as described in our previous paper (Ferruzzi *et al.* 2013).

Cell line and cell culture. The human cancer cell lines MDA-MB-231, MCF-7, HepG2, CaCo2, and A549 were grown in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% FBS and 2 mM L-glutamine; LoVo in RPMI-1640 medium supplemented with 10% of FBS, 1% L-glutamine, and 1% antibiotic solution (penicillin/streptomycin); and K562 and Jurkat in RPMI-1640 medium supplemented with 10% FBS, 50 U/mL penicillin, and 50 ug/mL streptomycin. Cells were maintained in a humidified atmosphere of 5% CO_2 at 37 °C. After 4–5 days, cells were removed from culture flask and centrifuged at 1500 rpm for 10 min. The medium was then removed and cells resuspended with fresh medium. Two types of tests were performed:

Viability assay, for A549, CaCo2, HepG2, and LoVo cells, using a standard trypan blue cell counting technique and determination of cell proliferation using a ZF Coulter Counter (Coulter Electronics, Hialeah, FL, USA) for all the other cell lines. The murine monocytic macrophage cell line RAW 264.7 was used to determine the inhibition of nitric oxide (NO) production. The cells were grown in Dulbecco's Modified Eagle's Medium in the same conditions as described earlier. Cell monolayers were subcultured onto 96 well culture plates (1×10^5 cells/well) used for experiments 24 h later.

Evaluation of antiproliferative and cytotoxic effects. Cells growing in suspension (K562 and Jurkat) were seeded at an initial concentration of 3×10^{-4} cells/mL and cultured in the presence of increasing concentrations of compounds. Non-treated cells were considered as control. Cell growth was usually determined after 3, 4, and 5 days of culture as cell number per mL, using a ZF Coulter Counter (Coulter Electronics, Hialeah, FL, USA). These time points were selected because between days 3 and 5, untreated controls K562 and Jurkat cells are on the log phase of cell growth (Bianchi *et al.*, 2000). Adherent cells (MDA-231 and MCF-7) were cultured at an initial concentration of 1.5×10^{-4} cells/mL, treated with increasing concentrations of compounds and after 72 h washed with sterile phosphate-buffered saline 1X and trypsinized. Cell growth was monitored as described for K562 and Jurkat cell lines.

For the others cell lines (HepG2, LoVo, CaCo2, and A549) the MTT assay, reported previously (Marrelli *et al.*, 2013), was used to estimate cell number indirectly. Cell monolayers were subcultured onto 96 well culture plates (2×10^4 cells/well) and treated with serial concentrations of the samples. After 24 h of incubation, 100 µL of medium were removed from each well. Subsequently, 100 µL of 0.5% w/v MTT, dissolved in phosphate-buffered saline, was added to each well and allowed to incubate further for 4 h. After 4 h of incubation, 100 µL of dimethyl sulfoxide was added to each well to dissolve the formazan crystals. Absorbance values at 550 nm were measured with a microplate reader (GDV DV 990 B/V, Roma, Italy). Cytotoxicity was expressed as LD50, which is the concentration needed to reduce the absorbance of treated cells by 50% with reference to the control (untreated cells). Doxorubicin was taken as positive control.

Inhibition of nitric oxide production in lipopolysaccharide-stimulated RAW 264.7 cells. The murine monocytic macrophage cells RAW 264.7 were cultured with different concentrations of extracts for 24 h, after addition of LPSs (final concentration of 1 µg/mL) for antiinflammatory tests. The presence of nitrite, a stable oxidized product of NO, was determined in cell culture media 24 h later by the Griess reagent (1% sulfanamide and 0.1% *N*-(1-naphtyl)ethylenediaminedihydrochloride in 2.5% H_3PO_4) as previously described (Conforti *et al.*, 2012). About 100 µL of cell culture supernatant was combined with 100 µL of Griess reagent in a 96 well plate followed by spectrophotometric measurement at 550 nm using a microplate reader (GDV DV 990 B/V, Roma, Italy). Cytotoxicity was assessed using the MTT assay.

SOS-chromotest. Genotoxicity and antigenotoxicity assays were performed in accordance with Quillardet and Hofnung (1985). Exponential-phase culture of *Escherichia coli* PQ37 was obtained as follows: an aliquot of bacterial culture was inoculated to 5 mL of fresh LA medium (LB, lysogeny broth, plus 20 µg/mL ampicillin) and left to grow overnight and shaken constantly at 37 °C. Of the precedent culture, 1 mL was then added to 5 mL of fresh LA medium and was grown at 37 °C for 3.5 h. At this point, the bacterial concentration was 2×10^8 UFC/mL; the solution had an optical density closed to $\lambda = 0.6$.

This solution was diluted 1:10 with fresh LB medium, and 0.6 ml was distributed into test tubes containing 20 µL of genotoxic agent, 4-nitroquinoline *N*-oxide, and 20 µL of a solution of tested material (*H. indicus* decoction and hydroalcoholic extract, and pure molecules) in several concentrations. Every sample was dissolved in dimethyl sulfoxide and tested in triplicate. After 2 h of incubation at 37 °C, the evaluation of the genotoxic/antigenotoxic activity (β-galactosidase) and the cell viability (alkaline phosphatase) started.

To perform antigenotoxic assay (evaluation of the β-galactosidase expression), the method was the following: 0.3 mL of the last obtained bacterial solution was added to 2.7 mL of B buffer. After a period of incubation of 10 min at 37 °C, 0.6 mL of a 0.4% solution of 2-nitrophenyl β-D-galactopyranoside was added. After another 60 min of incubation, the addition of 2 mL of Na_2CO_3 1M solution stopped the reaction. The color of the mixture was read with a ThermoSpectronic Helios-γ spectrophotometer at wavelength of 420 nm.

For viability assay (evaluation of the alkaline phosphatase expression), the procedure stated that at the same time of the β-galactosidase assay, 0.3 ml of bacterial solution was added to 7 mL of P buffer. In this case, after a period of incubation of 10 min at 37 °C, 0.6 mL of a 0.4% solution of 4-nitrophenyl phosphate disodium salt hexahydrate was added. After another 60 min of incubation, the addition of 1 mL of HCl 2.5M stopped the reaction and caused the color disappearance. Five minutes later, the addition of 1 mL of tris(hydroxymethyl) aminomethane 2M changed the pH restoring the color. The mixture was read with a ThermoSpectronic Helios-γ spectrophotometer at wavelength of 420 nm.

Statistical analysis. All experiments were carried out in triplicate. Data were expressed as means ± standard error of mean. The concentration yielding 50% inhibition (IC_{50}) was calculated by nonlinear regression with the use of Prism Graphpad version 4.0 for Windows (GraphPad Software, San Diego, CA, USA). Statistical significance was assessed with one-way analysis of variance using SigmaStat software (Jantel scientific software, San Rafael, CA, USA). Significant differences among means were analyzed using Tukey's multiple comparisons test. Differences at $p < 0.05$ were considered significant.

RESULTS AND DISCUSSION

The yield of decoction was of 25.6%, analogously to what was recently reported (Guerrini *et al.*, 2014), while

Table 1. Yields, total polyphenols, procyanidins, and flavonoids of *Hemidesmus indicus* extracts

	Decoction	Hydroalcoholic extract
Yield (g/100 g of dried drug)	25.6 ± 0.4	17.1 ± 0.1
Total polyphenols[1]	11.60 ± 0.60	12.34 ± 0.48
Total procyanidins[2]	0.62 ± 0.04	0.37 ± 0.02
Total flavonoids[3]	2.19 ± 0.20	0.89 ± 0.05

[1]Per 100 g of lyophilized decoction, expressed as gallic acid.
[2]Per 100 g of lyophilized decoction, expressed as cyanidin chloride.
[3]Per 100 g of lyophilized decoction, expressed as hyperoside.

for hydroalcoholic extract, it was 17.06% (Table 1), but for this latter, no data were shown in related literature for a comparison. Both extracts were chemically characterized to highlight their differences and performed their standardization and evidence of possible correlations and exploitations with biological activities. As previously suggested in literature (Ferruzzi *et al.*, 2013; Das and Bisht, 2013), we have determined the amount of main *H. indicus* phytomarkers (Table 2 and Fig. 1), 2-hydroxy-4-methoxybenzaldehyde, 3-hydroxy-4-meth. oxybenzaldehyde, and 2-hydroxy-4-methoxybenzoic acid, the content of all three compounds was higher in hydroalcoholic solution with 2-hydroxy-4-methoxybenzaldehyde as the most abundant (214.5 μg/mL and 0.879 g/100 g). If compared with total polyphenols data (Table 1), the amount due to the phytomarkers represented about 10% of the whole content of polyphenols, suggesting that other possible molecules, such as hemidesmins (Das *et al.*, 1992) and derivatives of vanillin isomers (Zhao *et al.*, 2014) could also be investigated. From 6 to 10 ppm, the nuclear magnetic resonance fingerprinting of hydroalcoholic extract highlighted the typical chemical shifts of 2-hydroxy-4-methoxybenzaldehyde, the most abundant phytomarker among those quantified by HPLC (Fig. 2).

The two *H. indicus* preparations were first tested for their cytotoxic effects using a panel of cancer cell lines commonly used for these assays, such as colorectal adenocarcinoma CaCo2, lung carcinoma A549, hepatocellular carcinoma HepG2, and colon carcinoma LoVo cells. Cytotoxicity was determined after 24 h of treatment. The results on the cytotoxic effects of *H. indicus* preparations are depicted in Table 3. Both preparations exhibited weak cytotoxicity on A549, CaCo2, and LoVo cell lines to the highest tested concentration of 100 μg/mL ($IC_{50} > 100$ μg/mL). Cytotoxicity of *H. indicus* preparations was, on the contrary, found against the HepG2 cell line. These results are in line with previous studies showing that *H. indicus* decoction is cytotoxic on HepG2 cells (Thabrew *et al.*, 2005). In the present research, we showed that hydroalcoholic extracts can also be responsible for cytotoxic activity. In fact, the *H. indicus* hydroalcoholic preparations displayed a cytotoxic activity (IC_{50} values of 34.50 μg/mL) similar to that of the decoction (IC_{50} values of 33.52 μg/mL). As for the decoction, the hydroalcoholic extract was not cytotoxic against the A549 and CaCo2 cell lines. On the contrary, cytotoxicity was found when treatment was performed on LoVo cells.

After these preliminary assays, we determined the *in vitro* antiproliferative activity selecting the breast cancer MCF-7 cells in comparison with the more aggressive MDA-MB-231 cell line. In addition to these experimental model systems for solid tumors, we determined the antiproliferative activity on the erythroleukemia K562 and T-lymphoid Jurkat cell lines. In these assays, the cells were cultured in the absence or presence of the tested agents and the cell number per mL determine after 3 and 4 days, when the untreated cells are in the log phase of *in vitro* cell growth. The results of these experiments are shown in Table 4. We found that MDA-MB-231 is resistant to all the treatments (inhibition of cell proliferation is obtained only at concentrations greater than 500 μg/mL). On the contrary, the hydroalcoholic preparation displayed activity of MCF-7 cells at about 200 μg/mL. K562 and Jurkat cells were differently sensitive to the treatments, because the decoction was only active on Jurkat cells, while the hydroalcoholic preparation was active on both K562 (IC_{50} values of 177.11 μg/mL) and Jurkat (IC_{50} values of 63.79 μg/mL) cells.

These data are of interest when compared with a previous study (Thabrew *et al.*, 2005) showing that the decoction prepared with *Nigella sativa* seeds, *H. indicus* (roots), and *Smilax glabra* (rhizome), used by traditional medical practitioners in Sri Lanka to treat cancer, has a dose-dependent inhibition activity with the maximum effect at concentrations higher than 40 mg/mL (dose causing 50% inhibition, $ED_{50} = 17$ mg/mL). All three individual plant extracts demonstrated inhibitory activity with interesting *H. indicus* values for ED_{50} (32 mg/mL). Our study showed instead the strongly different results for the *H. indicus* decoction evidencing IC_{50} values almost 1000-fold lower (33.52 μg/mL) than those reported by related paper (32 mg/mL), at least in some of the tumor cell lines used.

Moreover, the study of Samarakoon *et al.* (2012) demonstrated that the decoction of *N. sativa* seeds, *H. indicus* roots, and *S. glabra* rhizomes can induce apoptosis in human hepatocellular carcinoma HepG2 cell, in a dose and time-dependent manner through the activation of caspase-3 and caspase-9, and upregulation of pro-apoptotic Bax and downregulation of anti-apoptotic Bcl-2 genes, which are involved in intrinsic or mitochondrial pathway of apoptosis.

No previous studies were conducted against human colon carcinoma cell line (LoVo) for which the hydroalcoholic extract showed the best antiproliferative activity.

Table 2. HPLC quantification of chemical compounds in *Hemidesmus indicus* extracts

	Decoction		Hydroalcoholic extract	
	(μg/mL)	(g/100 g)	(μg/mL)	(g/100 g)
2-Hydroxy-4-methoxy-benzaldehyde	1.72 ± 0.09	0.050 ± 0.003	214.54 ± 5.17	0.879 ± 0.021
3-Hydroxy-4-methoxy-benzaldehyde	26.85 ± 0.92	0.788 ± 0.027	57.10 ± 1.39	0.234 ± 0.006
2-Hydroxy-4-methoxy-benzoic acid	23.54 ± 0.23	0.691 ± 0.007	32.51 ± 1.23	0.133 ± 0.005

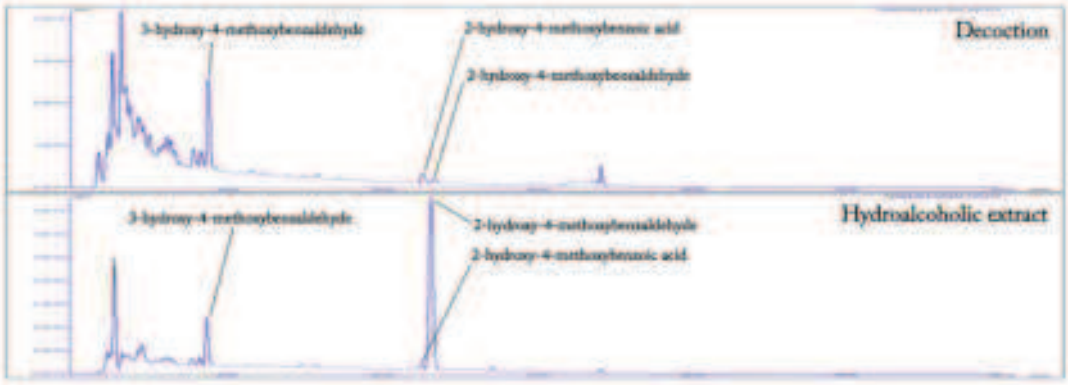

Figure 1. HPLC chromatograms of the two extracts.

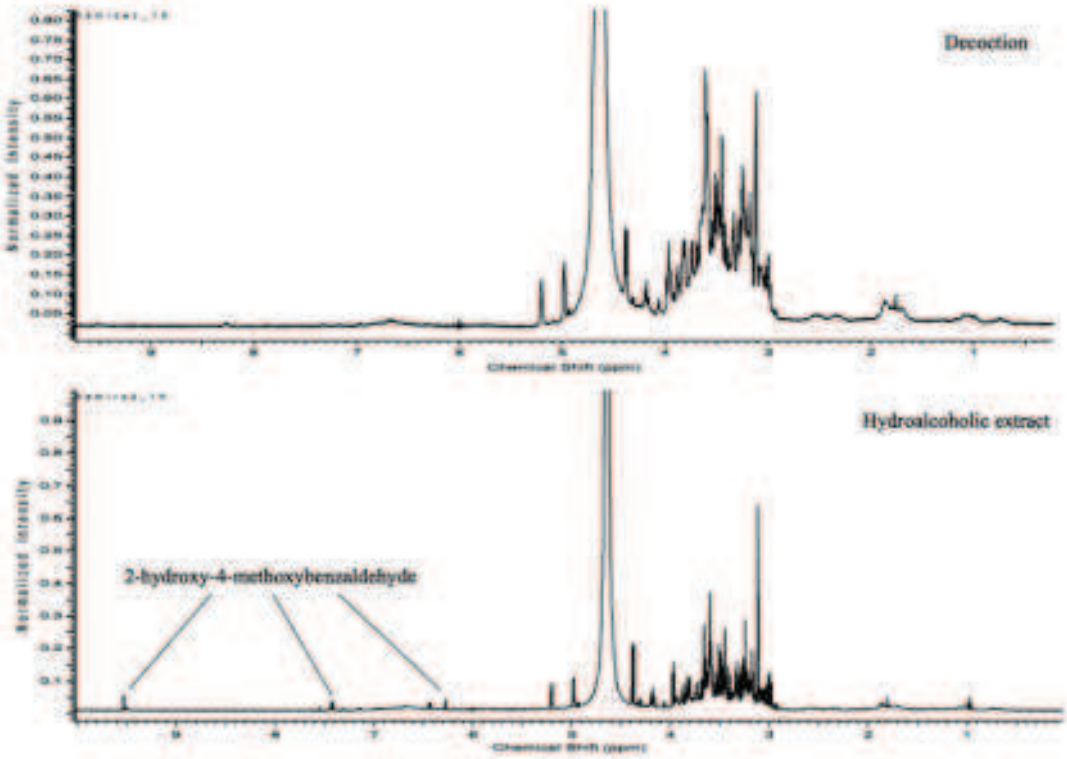

Figure 2. Proton nuclear magnetic resonance fingerprinting of the two extracts.

Table 3. Cytotoxic activities of preparations and pure molecules from *Hemidesmus indicus*

	IC_{50} (µg/mL)					
Cell line	Decoction (µg/mL)	Hydroalcoholic extract (µg/mL)	2-OH-4-OMeald (µM)	3-OH-4-OMeald (µM)	2-OH-4-OMeac (µM)	Doxorubicin (µM)
A549	>500	>500	>200	>200	>200	—
CaCo2	>500	>500	>200	>200	>200	—
HepG2	33.52 (±0.13)	34.50 (±0.14)	>200	>200	>200	0.39 (±0.02)
LoVo	>500	29.84 (±0.24)	>200	>200	>200	0.58 (±0.04)

In addition, with respect to isolated compounds, while 3-hydroxy-4-methoxy-benzaldehyde and 2-hydroxy-4-methoxy-benzoic acid were not or barely active on all the cell lines employed (IC_{50} values > 200 µg/mL), 2-hydroxy-4-methoxy-benzaldehyde inhibited the *in vitro* proliferation of K562 and Jurkat cells, displaying low activity on MCF-7 and MDA-MB-231 cells.

When the effects of the *H. indicus* decoction and hydroalcoholic preparation are compared with those of the plant-derived products, we can conclude that the obtained results reflect the typical efficacy expression of plant-derived products where bioactivities do not complete because of a single compound but often to a synergic interaction among different molecules present

Table 4. Antiproliferative effects of preparations and pure molecules from *Hemidesmus indicus*

	IC_{50} (μg/mL)				
Cell line	Decoction (μg/mL)	Hydroalcoholic extract (μg/mL)	2-OH-4-OMeald (μM)	3-OH-4-OMeald (μM)	2-OH-4-OMeac. (μM)
MCF-7	829.32 (±74.14)	209.73 (±18.75)	498.67 (±44.58)	>1000	>1000
MDA-MB-231	732.40 (±65.48)	521.65 (±46.63)	448.32 (±40.08)	>1000	>1000
K562	585.95 (±99.0)	177.11 (±15.83)	79.52 (±18.27)	361.57 (±10.81)	658.41 (±10.79)
Jurkat	349.66 (±49.38)	63.79 (±7.97)	86.46 (±3.66)	329.96 (±17.20)	790.37 (±70.66)

Table 5. Determination of antioxidant activity with ABTS and DPPH tests

	IC_{50} (μg/mL)					
	Decoction	Hydroalcoholic extract	2-Hydroxy-4-OMeald	3-Hydroxy-4-OMeald	2-Hydroxy-4-OMeac	Trolox
ABTS test	29.4 ± 1.4	9.44 ± 0.47	8.17 ± 0.41	1.03 ± 0.06	23.6 ± 0.3	2.40 ± 0.12
DPPH test	82.3 ± 4.1	69.4 ± 3.5	>1000	>1000	>1000	4.95 ± 0.26

ABTS, 2,2'-azinobis(3-ethylbenzothiazoline-6-sulfonic acid)diammonium salt; DPPH, 2,2-diphenyl-1-picrylhydrazyl.

in different amounts. Epidemiological studies have established that many tumors occur in association with chronic infectious diseases, and it is also known that persistent inflammation in the absence of infections increases the risk and accelerates the development of cancer (Balkwill *et al.*, 2005). NO is known to play an important role in maintenance of tissue homeostasis; it is produced by NO synthase, whose inducible isoform (iNOS) is known to be implicated in several pathological conditions and inflammation. NO produced by iNOS kills infectious pathogens, but overproduction of NO results in damage to tissues and, eventually, destruction of tissue homeostasis (Kröncke *et al.*, 1998). Thus, iNOS expression and NO production might be a good target for research into disturbed inflammatory conditions. Macrophages can release inflammatory mediators, such as prostaglandins, cytokines, and NO in response to LPS stimulation, validating use of LPS-treated macrophages as a model of inflammation. Here, the hydroalcoholic extract caused inhibition of NO production in the murine monocytic macrophage cell line RAW 264.7, with inhibition of 32%.

For what concerns antioxidant capacity, the most interesting radical scavenging activity, in particular with ABTS test, has been shown by hydroalcoholic extract (IC_{50} = 9.44 μg/mL) with respect to Trolox (IC_{50} = 2.40 μg/mL) taken as the positive control (Table 5). Literature reported a good correlation between total phenolic content and antioxidant activity (Paixão *et al.*, 2007). The hydroalcoholic extract evidenced only a slightly higher amount of total polyphenols than that of decoction, but the relevant activities of 2-hydroxy-4-methoxy-benzaldehyde (IC_{50} = 8.17 μg/mL) and 3-hydroxy-4-methoxy-benzaldehyde (IC_{50} = 1.03 μg/mL), presenting in higher amount in alcoholic extract than in decoction, can explain the better antioxidant capacity of the first phytocomplex. On the other hand, the good antioxidant activity of 2-hydroxy-4-methoxybenzaldehyde has been yet reported in literature (Wang *et al.*, 2010). However, the DPPH test did not support this evidence for the aldehyde compounds; further investigations are required for better discuss these results.

Finally, in order to check possible genotoxic/genoprotective properties of *H. indicus* traditional preparations and single compounds, the SOS-chromotest was performed. The assay gave negative response, toward cytotoxicity and DNA damage, in presence of decoction, hydroalcoholic extract, and their phytomarkers, except for the 2-hydroxy-4-methoxybenzoic acid that showed cytotoxicity at concentrations equal and higher than 125 μg/mL. Therefore, it was impossible to assess the genotoxic potential after this value, but it exhibits a 30% inhibition at 75 μg/mL. The SOS induction was caused by a 2.5 μg/mL solution of 4-nitroquinoline *N*-oxide, and the inhibition of the system was registered in the tests conducted with hydroalcoholic extract (39.56%

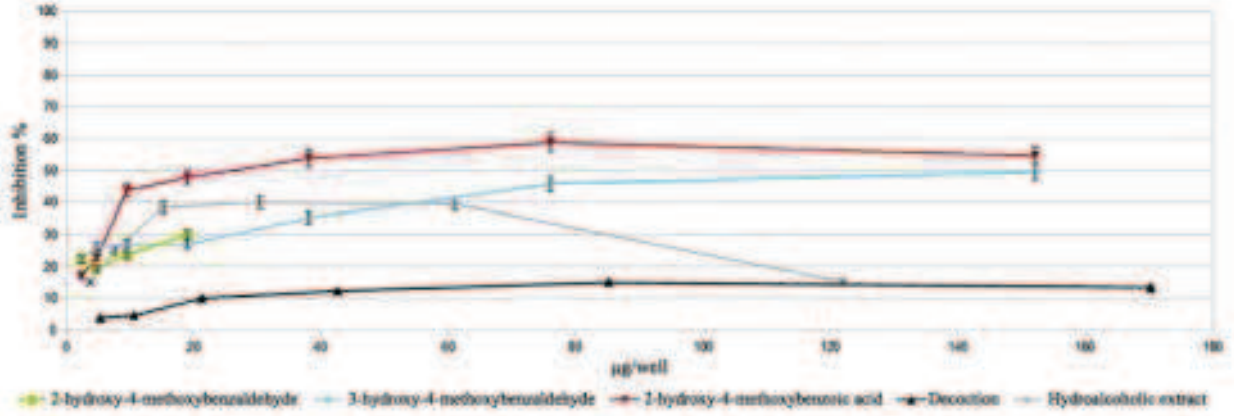

Figure 3. Genotoxic action of preparations and pure molecules.

of inhibition at 300 µg/mL), 3-hydroxy-4-methoxybenzaldehyde (45.79% of inhibition at 375 µg/mL), and 2-hydroxy-4-methoxybenzaldehyde that exhibit the highest genoprotective potential (58.79% of inhibition at 375 µg/mL) (Fig. 3). Decoction and pure compounds followed a dose-response correlation, while the last two concentrations of hydroalcoholic extract did not respect the same trend, this fact could be due to the dark color of phytocomplex solution. The activity of the this latter could be explained in light of the observations that 2-hydroxy-4-methoxybenzaldehyde was the most active compound present 200 times more concentrated in this phytocomplex than decoction. However, we cannot exclude the possibility that the inhibitory effect of this preparation could be ascribed to possible agonistic effect of other compounds.

CONCLUSIONS

The results of the study provide further supporting data for the reported anticancer potential of the decoction of *H. indicus* that will help to determine its selective cytotoxic effects on cancer cells. In particular, decoction showed promising effect on HepG2 cells, while the hydroalcoholic extract was active against HepG2, LoVo, MCF-7, K562, and Jurkat cell lines. An interesting antioxidant activity, particularly for hydroalcoholic extract in ABTS test, may be correlated to the higher amount of 2-hydroxy-4-methoxybenzaldehyde in this preparation. A relevant aspect of our research was also the standardization of the two preparations, in order to give more solid foundation to develop further investigations. Overall findings provide confirmatory evidence to demonstrate the activity of the decoction, traditionally used in Ayurveda, and its comparison with hydroalcoholic extract for new perspectives of uses as food supplement.

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Conflict of Interest

The authors are no conflict of interest

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CONGRESS ABSTRACTS

In vitro evaluation of anti-proliferative and geno-protective activity of *Hemidesmus indicus* crude drug extracts

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One aspect of modern research focused on prevention and cure of cancer is the investigation of new material of plant origin, with the objective of discovering new botanicals to use as active principle or to draw inspiration in the design of new molecular models[1].
For this aim, decoction (belonging to Ayurveda culture) and hydro-alcoholic extract (closer to occidental tradition) of the *Hemidesmus indicus* R.Br. (Asclepiadaceae) root was studied .
The phytochemical characterisation of the hydro-alcoholic extract, performed by RP-HPLC-DAD, showed an higher quantity of vanillin isomers and derivatives (2-hydroxy-4-methoxybenzaldehyde, 3-hydroxy-4-methoxybenzaldehyde and 2-hydroxy-4-methoxybenzoic acid) in comparison to decoction. In the aqueous preparation, in addition to these molecules, lupeol, lupeol acetate, β-amyrin acetate and β-sitosterol were identified by GC-MS.
Being aware that oxidative stress is a key factor in the cancer development, the study of bio-activities started from the evaluation of the antioxidant capacity through spectrophotometric methods (DPPH, ABTS and β-carotene bleaching test) and HPTLC-bioautographic assays[4] (DPPH, ABTS test). At a later stage, the evaluation of geno-protective (SOS-Chromotest) and anti-proliferative activity (using CCRF-CEM, CEM/ADR5000, MCF7, A549, MDA-MB-231, LoVo, HepG2, K562 and Jurkat cell lines) was carried out, focusing on the vanillin derivatives identified and quantified in *H. indicus*.
Plant decoction had already evidenced an important anti-leukemic effect through the modulation of different critical targets[2,3], and the vanillin derivative showed relevant data for the geno-protective and anti-proliferative activity. In particular, the most active vanillin isomer 2-hydroxy-4-methoxybenzaldehyde showed IC50 values of 79.52 ± 18.27 μM against K562, 85.39±1.7 μM against CCRF-CEM and 86.46 ± 3.66 μM against Jurkat.
Further in-depth analysis regarding anti-proliferative bioactivity of traditional preparations, identification and quantification of other characteristic compounds and more selective extractions are still in progress.
In conclusion: *H. indicus* evidenced promising data against Jurkat (63.79±7.97 μg/mL), CCRF-CEM (46.23±1.12 μg/mL), Hep-G2 (34.50±0.14 μg/mL) and LoVo (29.84±0.24 μg/mL); *H. indicus* hydro-alcoholic extract and decoction were more effective than 2-hydroxy-4-methoxybenzaldehyde against Hep-G2 cell, pointing out possible synergistic (agonistic) activity of minor compounds.

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In vitro evaluation of the anti-proliferative and geno-protective activity of traditional preparations of ayurvedic crude drugs

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The research of new botanicals is an important aspect of the modern research focused on prevention and cure of cancer. This project evaluated the *in vitro* geno-protective capacity (SOS-Chromotest) and anti-proliferative activity (MCF7, A549, MDA-MB-231, LoVo, HepG2, K562, Jurkat and IB3-1 cell lines) of two traditional preparations, decoction and mother tincture, of two ayurvedic crude drugs: *Hemidesmus indicus* roots and *Azadirachta indica* leaves. Our previous results demonstrated an interesting antileukemic effect[1,2] of *H. indicus* decoction. In present study both *H. indicus* preparations possess anti-proliferative activity against all the cell lines considered. Among phytochemical markers, the most active, 2-hydroxy-4-methoxybenzaldehyde, showed an IC_{50} of 12.09±2.78 πg/ml against K562 and an IC_{50} of 13.15±5.57 πg/ml against Jurkat cells. The only relevant data for *A. indica* were against: MDA-MB-231 cell (IC_{50}= 381.57±43.62 πg/ml) for decoction and K562 (IC_{50}= 276.05±26.60 πg/ml) and Jurkat cells (IC_{50}= 207.18±29.97 πg/ml) for mother tincture. Further analyses of anti-proliferative bioactivity for *A. indica* are still in progress.
In conclusion: *H. indicus* preparations were more effective than those of *A. indica*; mother tincture of both crude drugs showed a general wider anti-proliferative activity than decoction, in particular for H. indicus, it evidenced promising data against Jurkat (63.79±7.97 πg/ml), Hep-G2 (34.50±0.14) and LoVo (29.84±0.24); *H. indicus* mother tincture and decoction were more effective than 2-hydroxy-4-methoxybenzaldehyde against Hep-G2 cell, pointing out possible synergistic (agonistic) activity of minor compounds.

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CARATTERIZZAZIONE CHIMICA E BIOATTIVITÀ DI PREPARAZIONI TRADIZIONALI DI DROGHE AYURVEDICHE: *HEMIDESMUS INDICUS* E *AZADIRACHTA INDICA*

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L'Ayurveda, medicina tradizionale indiana, è oggi riconosciuta dall'UE tra le medicine non convenzionali e annovera più di 7000 piante utilizzate a scopo terapeutico in complesse formulazioni scarsamente indagate sotto il profilo chimico e di bioattività[1]. Su questi presupposti e nell'ambito di una progettualità di più ampio respiro (PRIN2009: 2009LR9YLF) che coinvolge Unità di Ricerca di Ferrara, Parma e Cosenza, è iniziato uno studio di approfondimento fitochimico e funzionale di droghe ayurvediche. In particolare, vengono qui riportati i primi riscontri riguardo alle preparazioni tradizionali di *Hemidesmus indicus* R.Br. (Ranunculaceae; radici) e *Azadirachta indica* A. (Meliaceae; foglie), i cui decotti da droghe polverizzate ottenute da un circuito commerciale qualificato sono stati valutati con analisi NMR, GC-MS, HPLC-DAD/ELSD, HPTLC e spettrofotometriche finalizzate sia ad un fingerprinting del fitocomplesso, sia alla caratterizzazione di singole frazioni e composti. L'analisi del decotto di *H. indicus* ha evidenziato una frazione zuccherina preponderante, la presenza di polifenoli totali (11.6±0.6%), proantocianidine totali (0.62±0.04%), flavonoidi totali (2.19±0.20%); una frazione organica ottenuta con soxhlet è risultata ricca in 3-idrossi-4-metossibenzaldeide e 2-idrossi-4-metossibenzaldeide. Le analisi su *A. indica* hanno evidenziato, oltre ad una importante frazione zuccherina, polifenoli totali (9.99±1.9%), proantocianidine totali (minori del 0.3%), flavonoidi totali (2.22±0.13%). Si sono inoltre svolti diversi saggi di bioattività (efficacia e sicurezza) seguendo, in parte, le numerose indicazioni etnomediche. Con *S. cerevisiae* D7 sembra emergere una interessante capacità mutageno protettiva da parte di *A. indica*; il saggio di screening Trombin Generation assay ha mostrato una sensibile attività sui processi coagulativi per *H. indicus*. L'attività antimicrobica (Disk diffusion assay; TLC-bioautografica) è risultata efficace per *A. indica* soprattutto verso Gram+. L'attività antiossidante (DPPH e ABTS) ha evidenziato la maggiore efficacia di *H. indicus* come fitocomplesso (IC_{50}-DPPH=82.26µg/ml; IC_{50}-ABTS=29.40µg/ml) rispetto ad *A. indica* (IC_{50}-DPPH=219.55µg/ml; IC_{50}-ABTS=36.03µg/ml). *A. indica*, infine, ha evidenziato una potenziale attività antinfiammatoria in modello cellulare IB31 (fibrosi cistica) riducendo del 50% i livelli di mRNA IL-8 già a concentrazioni pari all'IC_{25} rispetto a *H. indicus* (IC_{50}). Ulteriori approfondimenti sono in corso al fine di puntualizzare e completare i dati sin qui raccolti sia sul piano fitochimico, confrontando la composizione della preparazione rispetto ad estratti idroalcolici della droga secca, sia dell'efficacia funzionale puntualizzando i composti più attivi e ampliando l'orizzonte d'efficacia e sicurezza sin qui prodotto.

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