

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

Giancarlo Statti,¹ Mariangela Marrelli,¹ Filomena Conforti,^{1*} Antonella Spagnoletti,² Massimo Tacchini,² Carmela Fimognari,³ Eleonora Brognara,² Roberto Gambari,² Gianni Sacchetti² and Alessandra Guerrini²

¹Department of Pharmacy, Health and Nutrition Sciences, University of Calabria, Italy

²Department of Life Sciences and Biotechnologies, University of Ferrara, Italy

³Department for Life Quality Studies, Alma Mater Studiorum-University of Bologna, Rimini, Italy

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 effects, 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 carcinoma 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 gonorrhoea and syphilis, impotence, and as a blood purifier, cooling tonic and appetite stimulant, and to promote health and vitality

* Correspondence to: Filomena Conforti, Department of Pharmacy, Health and Nutrition Sciences, University of Calabria, Italy.
E-mail: filomena.conforti@unical.it

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 Pharmacopoeia and Ayurvedic medicine. In this paper, we have compared the decoction with a hydroalcoholic extract, a traditional product used in western medicine.

Received 02 December 2014

Revised 03 February 2015

Accepted 07 February 2015

The major shortcoming in a large number of experimental and clinical studies is the absence of phytochemical standardization of the administered 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–Ciocalteu reagent, RPMI 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-naphthyl) ethylenediaminedihydrochloride in 2.5% H₃PO₄), and 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. Reference 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), Pharmacopée française, 11e édition). 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 phytochemicals 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 ThermoSpectronic Helios-γ spectrophotometer, 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 IC₅₀ 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 phytochemicals: 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 µg/mL streptomycin. Cells were maintained in a humidified atmosphere of 5% CO₂ 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 ZF2 Coulter Counter 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 Z2 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 μ L of medium were removed from each well. Subsequently, 100 μ L 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 μ L 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 μ g/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-naphthyl)ethylenediaminedihydrochloride in 2.5% H_3PO_4) as previously described (Conforti *et al.*, 2012). About 100 μ L of cell culture supernatant was combined with 100 μ L 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 (lysogeny broth plus 20 μ g/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 μ L of genotoxic agent, 4-nitroquinoline *N*-oxide, and 20 μ L 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 \pm 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

¹Per 100 g of lyophilized decoction, expressed as gallic acid.

²Per 100 g of lyophilized decoction, expressed as cyanidin chloride.

³Per 100 g of lyophilized decoction, expressed as hyperoside.

T1 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 **F1 T2** of main *H. indicus* phytochemicals (Table 2 and Fig. 1), 2-hydroxy-4-methoxybenzaldehyde, 3-hydroxy-4-methoxybenzaldehyde, 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 phytochemicals 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 phytochemical **F2** 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* **T3 Q10** 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 (IC₅₀ > 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 (IC₅₀ values of 34.50 µg/mL) similar to that of the decoction (IC₅₀ 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 **T4** 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 (IC₅₀ values of 177.11 µg/mL) and Jurkat (IC₅₀ values of 63.79 µg/mL) cells.

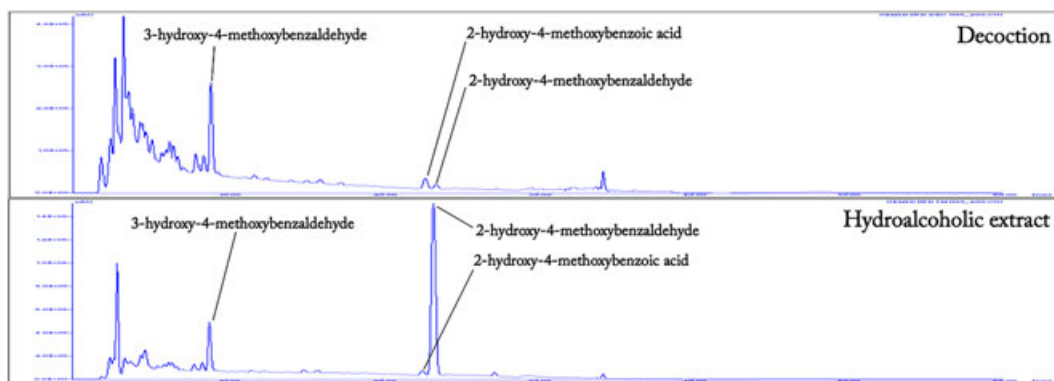
These data are of interest when compared with a previous study (Thabrew *et al.*, 2005) showing that the decoction prepared with *Nigella sativa* seeds, *H. indicus* (roots), and *Smilax glabra* (rhizome), used by traditional medical practitioners in Sri Lanka to treat cancer, has a dose-dependent inhibition activity with the maximum effect at concentrations higher than 40 mg/mL (dose causing 50% inhibition, ED₅₀ = 17 mg/mL). All three individual plant extracts demonstrated inhibitory activity with interesting *H. indicus* values for ED₅₀ (32 mg/mL). Our study showed instead the strongly different results for the *H. indicus* decoction evidencing IC₅₀ 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



Q14 Figure 1. HPLC chromatograms of the two extracts.

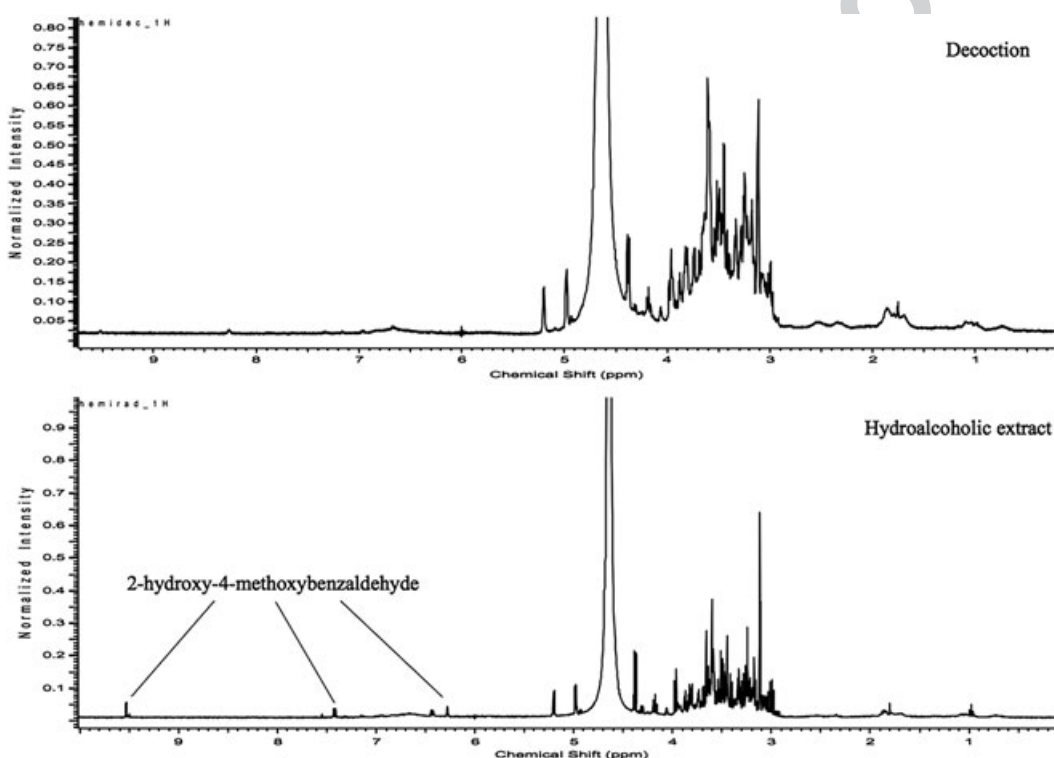


Figure 2. Proton nuclear magnetic resonance fingerprinting of the two extracts.

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

Cell line	IC ₅₀ (µg/mL)					Doxorubicin (µM)
	Decoction (µg/mL)	Hydroalcoholic extract (µg/mL)	2-OH-4-OMeald (µM)	3-OH-4-OMeald (µM)	2-OH-4-OMeac (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 (IC₅₀ 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*

Cell line	IC ₅₀ (µg/mL)				
	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

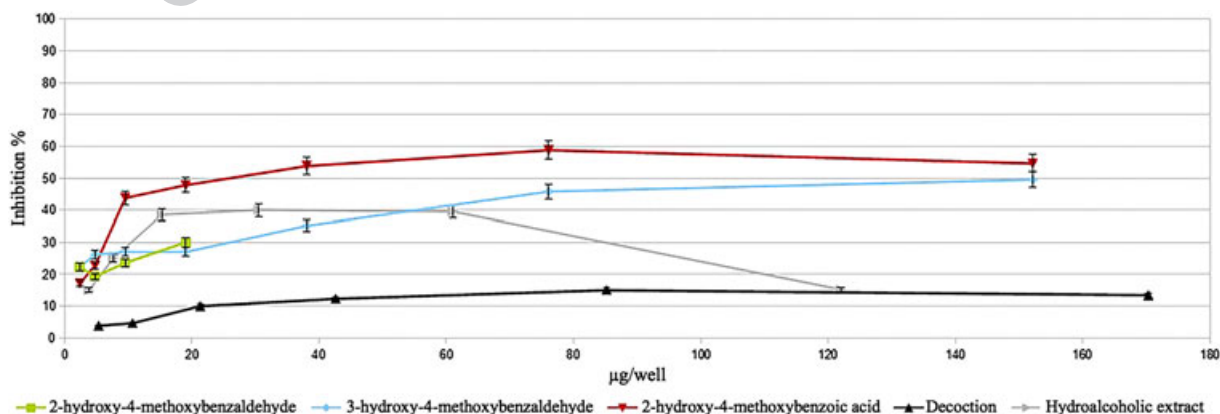
ABTS, 2,2'-azinobis(3-ethylbenzothiazoline-6-sulfonic acid)diammonium salt; DPPH, 2,2-diphenyl-1-picrylhydrazyl.

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 (IC₅₀ = 9.44 µg/mL) with respect to Trolox (IC₅₀ = 2.40 µg/mL) taken as the positive control T5 (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 (IC₅₀ = 8.17 µg/mL) and 3-hydroxy-4-methoxy-benzaldehyde (IC₅₀ = 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.

Acknowledgements

This research was supported by a PRIN grant (Progetti di Ricerca di Interesse Nazionale, MIUR of Italy) coded 2009LR9YLF_001.

Conflict of Interest

The authors are no conflict of interest

REFERENCES

- Aneja V, Suthar A, Verma S, Kalkunte S. 2008. Phyto-pharmacology of *Hemidesmus indicus*. *Pharmacogn Rev* **2**: 143–150.
- Austin A. 2008. A review on Indian Sarsaparilla, *Hemidesmus indicus* (L.) R. Br. *J Biol Sci* **8**: 1–12.
- Ayurvedic Pharmacopoeia Committee 1989. *Hemidesmus indicus*. Government of India, Ministry of Health and Family Welfare, Department of Ayurveda, Yoga and Naturopathy, Unani, Siddha and Homoeopathy (AYUSH). The Ayurvedic Pharmacopoeia of India, New Delhi, 107.
- Balkwill F, Charles KA, Mantovani A. 2005. Smoldering and polarized inflammation in the initiation and promotion of malignant disease. *Cancer Cell* **7**: 211–217.
- Q11** Bendicho S, Trigueros MC, Hernández T, Martín O. 2001. Validation and comparison of analytical methods based on the release of p-nitrophenol to determine lipase activity in milk. *J Dairy Sci* **84**: 1590–1596.
- Bianchi N, Ongaro F, Chiarabelli C, et al. 2000. Induction of erythroid differentiation of human K562 cells by cisplatin analogs. *Biochem Pharmacol* **31**: 31–40.
- Q12** Conforti F, Menichini F, Formisano C, et al. 2012. *Anthemis wiedemanniana* essential oil prevents LPS-induced production of NO in RAW 264.7 macrophages and exerts antiproliferative and antibacterial activity. *Nat Prod Res* **26**: 1594–1601.
- Das PC, Joshi PC, Mandal S, Das A, Chatterjee A. 1992. New coumarinolignoids from *Hemidesmus indicus* R. Br. *Indian J Chem* **31**: 342–345.
- Das S, Dash SK, Padhy SN. 2003. Ethnomedicinal information from the tribals of Orissa state-review. *J Hum Ecol* **14**: 165–227.
- Das S, Bisht SS. 2013. The bioactive and therapeutic potential of *Hemidesmus indicus* R. Br. (Indian Sarsaparilla) root. *Phytother Res* **27**: 791–801.
- Ferruzzi L, Turrini E, Burattini S, et al. 2013. *Hemidesmus indicus* induces apoptosis as well as differentiation in a human promyelocytic leukemic cell line. *J Ethnopharmacol* **147**: 84–91.
- George S, Tushar KV, Unnikrishnan KP, Hasim KM, Balachandran I. 2008. *Hemidesmus indicus* (L.) R. Br. A review. *J Plant Sci* **3**: 146–156.
- Guerrini A, Mancini I, Maietti S, et al. 2014. Expression of pro-inflammatory interleukin-8 is reduced by ayurvedic decoctions. *Phytother Res* **28**: 1173–1181.
- Kröncke KD, Fehsel K, Kolb-bachofen V. 1998. Inducible nitric oxide synthase in human diseases. *Clin Exp Immunol* **113**: 147–156.
- Marrelli M, Conforti F, Rigano D, et al. 2013. Cytotoxic properties of *Marrubium globosum* ssp. *libanoticum* and its bioactive components. *Nat Prod Commun* **8**: 567–569.
- Préparations homéopathiques. 1038. Pharmacopée française, 11e édition. **Q13**
- Paixão N, Perestrelo R, Marques JC, Câmara JS. 2007. Relationship between antioxidant capacity and total phenolic content of red, rosé and white wines. *Food Chem* **105**: 204–214.
- Quillardet P, Hofnung M. 1985. The SOS chromotest, a colorimetric bacterial assay for genotoxins: procedures. *Mutat Res* **147**: 65–78.
- Rossi D, Guerrini A, Bruni R, et al. 2012. Trans-resveratrol in nutraceuticals: issues in retail quality and effectiveness. *Molecules* **17**: 12393–12405.
- Samarakoon SR, Thabrew I, Galhena PB, Tennekoon KH. 2012. Modulation of apoptosis in human hepatocellular carcinoma (HepG2 cells) by a standardized herbal decoction of *Nigella sativa* seeds, *Hemidesmus indicus* roots and *Smilax glabra* rhizomes with anti-hepatocarcinogenic effects. *BMC Complement Altern Med* **12**: 25.
- Satoskar RS, Shah LG, Bhatt K, Sheth UK. 1962. Preliminary study of pharmacologic properties of Anantmul (*Hemidesmus indicus*). *Indian J Physiol Pharmacol* **6**: 68–76.
- Thabrew MI, Mistry RR, Morsy MA, Hughes RD. 2005. Cytotoxic effects of a decoction of *Nigella sativa*, *Hemidesmus indicus* and *Smilax glabra* on human hepatoma HepG2 cells. *Life Sci* **77**: 1319–1330.
- Wang J, Liu H, Zhao J, et al. 2010. Antimicrobial and antioxidant activities of the root bark essential oil of *Periploca sepium* and its main component 2-hydroxy-4-methoxybenzaldehyde. *Molecules* **15**: 5807–5817.
- Zhao Z, Matsunami K, Otsuka H, Negi N, Kumar A, Negi DS. 2014. A condensed phenylpropanoid glucoside and pregnane saponins from the roots of *Hemidesmus indicus*. *J Nat Med* **67**: 137–142.

Author Query Form

Journal: Phytotherapy Research

Article: ptr_5322

Dear Author,

During the copyediting of your paper, the following queries arose. Please respond to these by annotating your proofs with the necessary changes/additions.

- If you intend to annotate your proof electronically, please refer to the E-annotation guidelines.
- If you intend to annotate your proof by means of hard-copy mark-up, please use the standard proofing marks. If manually writing corrections on your proof and returning it by fax, do not write too close to the edge of the paper. Please remember that illegible mark-ups may delay publication.

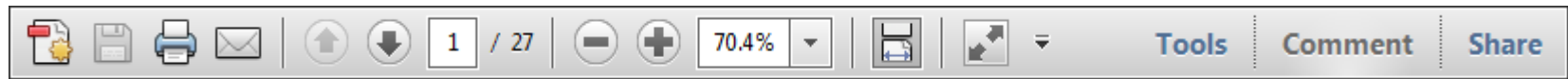
Whether you opt for hard-copy or electronic annotation of your proofs, we recommend that you provide additional clarification of answers to queries by entering your answers on the query sheet, in addition to the text mark-up.

Query No.	Query	Remark
Q1	AUTHOR: Please confirm that given names (red) and surnames/family names (green) have been identified correctly.	
Q2	AUTHOR: Please define RPMI on first mention.	
Q3	AUTHOR: Please define L-NAME on first mention.	
Q4	AUTHOR: Please give address information for American Type Culture Collection: town, state (if applicable), and country.	
Q5	AUTHOR: Please give manufacturer information for ThermoSpectronic Helios- γ spectrophotometer: company name, town, state (if USA), and country.	
Q6	AUTHOR: Do you mean to say "Z2 Coulter Counter"? Please provide its manufacturer information: company name, town, state (if USA), and country if this is not the same as "Z2 Coulter Counter" found in the next paragraph.	
Q7	AUTHOR: 'phosphate-buffered saline'. Is this the correct definition for PBS? Please change if this is incorrect.	
Q8	AUTHOR: Please define SOS on first mention.	
Q9	AUTHOR: Please define LA medium and LB medium on first mention.	
Q10	AUTHOR: Tables have been renumbered according to citation order. Please check.	
Q11	AUTHOR: Reference "Bendicho et al. (2001)" is not cited in the text. Please indicate where it should be cited; or delete from the reference list.	
Q12	AUTHOR: Please provide the volume number for reference <i>Bianchi et al.</i> [2000].	
Q13	AUTHOR: Please check and provide complete details for this reference if necessary.	
Q14	AUTHOR: Revised figures 1,2 and 3 still contains small and poor quality of text. Please check and resupply if necessary.	

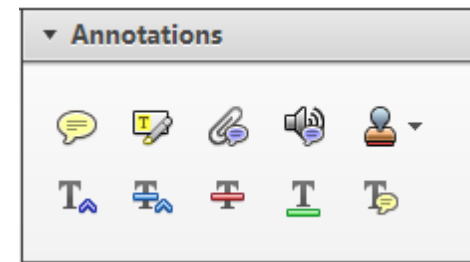
Required software to e-annotate PDFs: **Adobe Acrobat Professional** or **Adobe Reader** (version 7.0 or above). (Note that this document uses screenshots from **Adobe Reader X**)

The latest version of Acrobat Reader can be downloaded for free at: <http://get.adobe.com/uk/reader/>

Once you have Acrobat Reader open on your computer, click on the **Comment** tab at the right of the toolbar:



This will open up a panel down the right side of the document. The majority of tools you will use for annotating your proof will be in the **Annotations** section, pictured opposite. We've picked out some of these tools below:



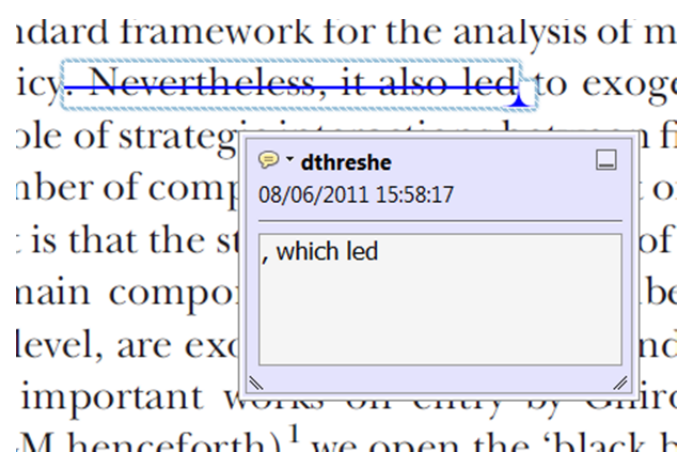
1. Replace (Ins) Tool – for replacing text.



Strikes a line through text and opens up a text box where replacement text can be entered.

How to use it

- Highlight a word or sentence.
- Click on the **Replace (Ins)** icon in the Annotations section.
- Type the replacement text into the blue box that appears.



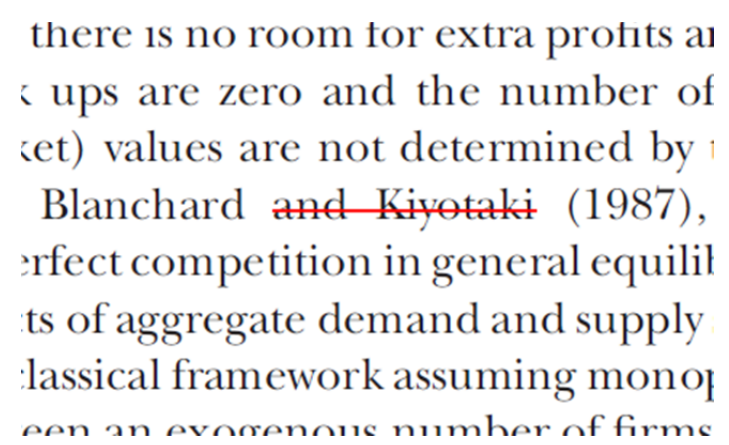
2. Strikethrough (Del) Tool – for deleting text.



Strikes a red line through text that is to be deleted.

How to use it

- Highlight a word or sentence.
- Click on the **Strikethrough (Del)** icon in the Annotations section.



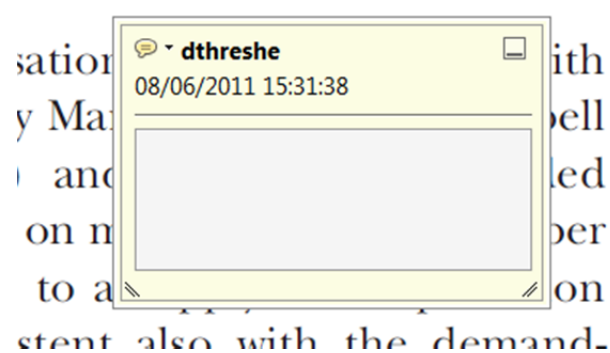
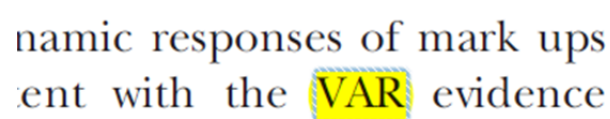
3. Add note to text Tool – for highlighting a section to be changed to bold or italic.



Highlights text in yellow and opens up a text box where comments can be entered.

How to use it

- Highlight the relevant section of text.
- Click on the **Add note to text** icon in the Annotations section.
- Type instruction on what should be changed regarding the text into the yellow box that appears.



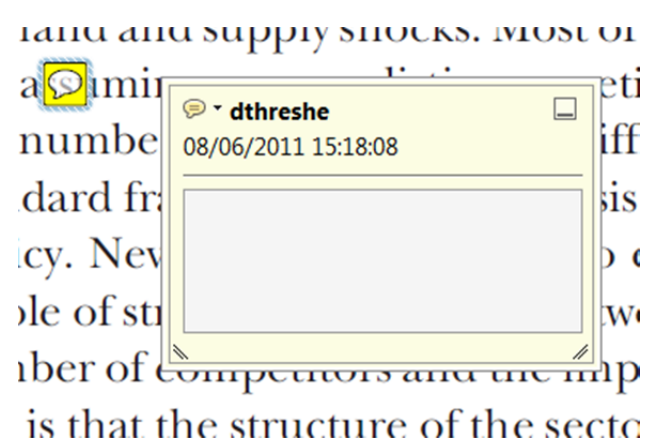
4. Add sticky note Tool – for making notes at specific points in the text.



Marks a point in the proof where a comment needs to be highlighted.

How to use it

- Click on the **Add sticky note** icon in the Annotations section.
- Click at the point in the proof where the comment should be inserted.
- Type the comment into the yellow box that appears.



USING e-ANNOTATION TOOLS FOR ELECTRONIC PROOF CORRECTION

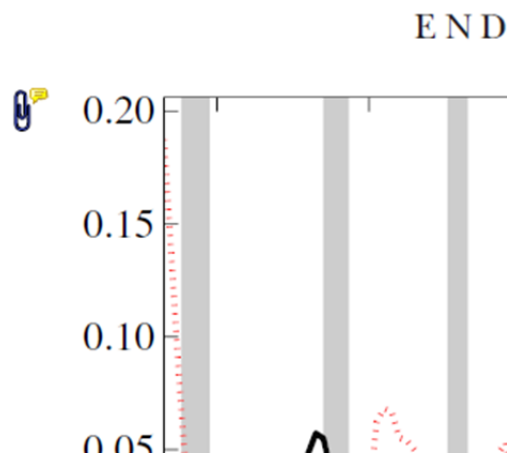
5. Attach File Tool – for inserting large amounts of text or replacement figures.



Inserts an icon linking to the attached file in the appropriate place in the text.

How to use it

- Click on the [Attach File](#) icon in the Annotations section.
- Click on the proof to where you'd like the attached file to be linked.
- Select the file to be attached from your computer or network.
- Select the colour and type of icon that will appear in the proof. Click OK.



6. Add stamp Tool – for approving a proof if no corrections are required.

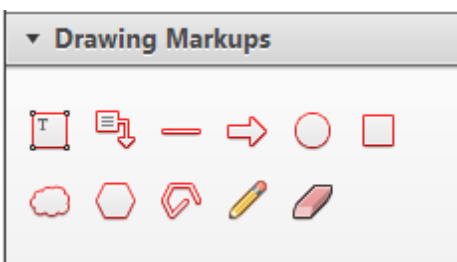


Inserts a selected stamp onto an appropriate place in the proof.

How to use it

- Click on the [Add stamp](#) icon in the Annotations section.
- Select the stamp you want to use. (The [Approved](#) stamp is usually available directly in the menu that appears).
- Click on the proof where you'd like the stamp to appear. (Where a proof is to be approved as it is, this would normally be on the first page).

of the business cycle, starting with the
 on perfect competition, constant return
 production. In this environment goods
 extra profits and the number of firms
 he number of firms is determined by
 determined by the model. The New-Key
 otaki (1987), has introduced produc
 general equilibrium models with nomin
 ed and supply shocks. Most of this literat

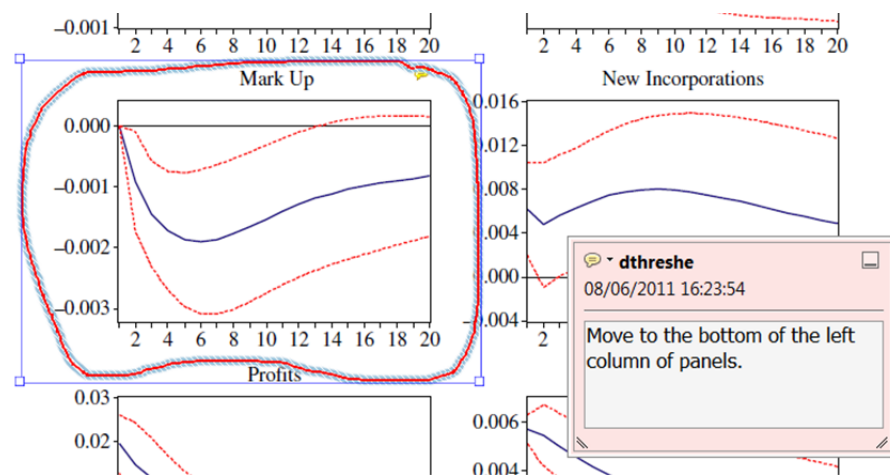


7. Drawing Markups Tools – for drawing shapes, lines and freeform annotations on proofs and commenting on these marks.

Allows shapes, lines and freeform annotations to be drawn on proofs and for comment to be made on these marks..

How to use it

- Click on one of the shapes in the [Drawing Markups](#) section.
- Click on the proof at the relevant point and draw the selected shape with the cursor.
- To add a comment to the drawn shape, move the cursor over the shape until an arrowhead appears.
- Double click on the shape and type any text in the red box that appears.



For further information on how to annotate proofs, click on the [Help](#) menu to reveal a list of further options:

