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# Antioxidant and Antifungal activities of marrubiin, extracts and essential oil from *Marrubium vulgare* L. against pathogenic dermatophyte strains

M. Rezgui[a*], N. Majdoub[a], B. Mabrouk[a], A. Baldisserotto[b], A. Bino[b], L. Bettaieb Ben Kaab[a], S. Manfredini[b]

a : Faculty of Sciences of Tunisia, University Tunis El Manar, 2092 Tunis, Tunisia.

b: School of Pharmacy and Health Products, Department of Life Sciences and Biotechnology, University of Ferrara, Master Course in Cosmetic Sciences and Technology, Laboratory of Health Products, Via Fossato di Mortara 17/19, 44121 Ferrara, Italy.

∗: Corresponding author. Tel.: +21652254735; E-mail address: mar_warezgui@hotmail.fr (M. Rezgui).

## Introduction

Infectious diseases is considered as a worldwide problem still appearing in the modern life particularly in developing countries which can affect specially individuals with compromised immune systems [1,2]. The identification and the development of novel antifungal agents is going to be an obsession of scientists owing to the increase of fungal diseases in humans, animals caused by pathogens that are becoming more resistant to currently available drugs. In traditional medicine, many plants are used in the form of oils and crude extracts, infusion or plaster to treat common infections without any scientific evidence of their efficacies. In fact, the big variability of plants increases the possibility of finding novel structures. *Marrubium* genus, commonly known as horehound, belongs to the Lamiaceae family. It is used as a source for food flavoring and for medicinal purposes [3]. So far, several pharmacological properties of this plant have been confirmed in laboratory studies such as: hypoglycaemic, antioxidant, antibacterial, hypotensive effects [4,5,6,7]. Marrubiin is a widely known furane labdane diterpene that constitutes the bitter principle compound of *Marrubium vulgare*, it is reported to stimulate secretions of the bronchial mucosa and to possess antiarhythmic effects [8] and exhibits potent antioxidant, vasorelaxant, antinociceptive, gastroprotective, antispasmodic, immunomodulating, antioedematogenic, analgesic activities [9,10,11,12,13,14,15,16] but to the best of our knowledge, there are no reports which focused on the activity of *M.vulgare* and marrubiin against dermatophytes. Therefore, Therefore, this work was conducted to investigate for the first time the
antidermatophytic, antipathogenic and antioxidant activities of methanol, acetone extracts, and essential oil of *Marrubium vulgare* grown in Tunisia and its active principle marrubiin with consideration to their chemical compositions.

## Materials and methods

### *Reactifs and reagents*

Caffeic acid, p-coumaric acid, sinapic acid, ferulic acid, Quercetin, and Verbascoside were purchased from Sigma-Aldrich srl (Milan, Italy). Apigenin, and luteolin were from Alfa Aesar, Karlsruhe, Germany. HPLC grade solvent were from Sigma Aldrich, Milan, Italy.

### *Plant sampling*

*Marrubium vulgare* L. plants were collected in March 2013 from the region of Boussalem in Northwestern Tunisia (Latitude 36° 36’ 40” (N); Longitude 8° 58’ 11” (E); Altitude 141 m above sea). The climate of the sampling site is mild and continental with hot and dry summer, while it is cold and humid in winter. It is also characterized by an average annual precipitation of 700

mm and a mean temperature of 16.8 °C. Botanical identification of tbe plant was made by Dr Mouhiba Ben Nasri-Ayachi, a member of the Botanical laboratory, Faculty of Sciences of Tunis, according to the Tunisian flora [17].

***Extraction, clean up and fractionation***

Many steps are necessary to obtain phytochemicals. The method of extraction is the most important step for recovering and isolating compounds from plant materials.The aerial parts of *M.vulgare* were dried at room temperature for two weeks, the methanol and acetone extracts were obtained separately by mixing 2.5 g of the plant sample with 25 ml of solvent. Each extraction was carried out by incubation of the mixture for 24 hr. at room temperature and the resulting mixture was then filtered with Whatman filter paper N0. 4. The solvent was removed by vacuum distillation. The extracts were then conserved in the dark at 4°C until analysis. Three replications were carried out.

***Isolation and quantification of marrubiin***

50 g of dried plant material were extracted with acetone by percolation to obtain 18 fractions which were analyzed by TLC in silica gel plates using cyclohexane-ethyl acetate (6:4). The fractions having the same chromatographic profile were combined to obtain 5 fractions. The active fraction (fraction 2) was washed with petroleum ether to remove lipids and coloring matter after being fractionated by flash chromatography with the use of increased proportion of ethyl acetate in cyclohexane. After TLC, the fraction containing marrubiin were pooled and crystallized from methanol. Marrubiin was identified on the basis of its spectroscopic data (1H and 13C NMR, IR) in the literature [18].

***Extraction and characterization of essential oil***

The essential oil was obtained by hydrodistillation for 3 h by using a Clevenger-type apparatus (European Pharmacopoeia, 1996). Oil samples were dried over anhydrous sodium sulphate and kept at 4 °C until analysis. The volatile oil analysis was carried out using gas chromatography-mass spectrometry (GC-MS) performed on an Agilent 6890 Series gas chromatograph interfaced to an Agilent 5973N mass selective detector (Agilent Technologies, Little Falls, DE, USA). A vaporisation injector operating at 250 °C in the split mode (1: 100) an a fused silica capillary column of 30 m × 0.25 mm ID × 0.25 µm film thickness (TRB-5MS; (95%) Dimethyl-(5%) diphenyl polysiloxane,Teknokroma, Spain) were used.

The oven temperature was programmed from 45°C to 240 °C, and held isothermally for 5 min. High purity helium was used as carrier gas at 1ml/min. Electron ionisation–mass spectra were recorded at 70 eV electron energy with an ionisation current of 39.6 mA. The quadrupole, source and transfer line temperatures were maintained at 150 °C, 230 °C and 280 °C respectively. All data were recorded using a MS ChemStation (G1701CA; Rev C.00.00; Agilent Technologies). The identification of each compound was determined by comparison of its retention index (RI) relative to C10–C24 n-alkanes [19], as well as of its spectra with the Wiley's library spectral data bank (G1035B; Rev D.02.00; Agilent Technologies). The normalised peak area of each compound was used without any correction factors to establish abundances.

***Identification and quantification of phenolic compounds***

The HPLC system consisted of a RP-HPLC (series 1100, AGILENT TECHNOLOGIES) series instrument coupled to a quaternary pump, a diode array detector (DAD), an autosampler and a column compartment. UV–visible spectra were recorded from 210 to 520 nm and chromatograms were monitored at 280 nm, 320 nm and 360 nm. Samples were separated on a Hydro RP18 Sinergi 80A column (4.6 × 150 mm, 4 µm) from Phenomenex with a sample injection volume of 10 µl. The mobile phase consisted of water (0.01M $H_3PO_4$) (A) and acetonitrile (0,01M $H_3PO_4$) (B). The eluting conditions applied were: 0–5 min, linear gradient from 10 % to 20 % B; 5–10 min, isocratic gradient 20 % B; 10–35 min, linear gradient

from 20 % to 100 % B; 35–45 min, isocratic gradient 100 % B, for washing return to 10 % B at 50 min, and finally 5 min isocratic to re-equilibrate the column. The mobile phase flow rate was 1.2 ml/min. The identification and quantification of phenolic acids and flavonoids on the samples were carried out by comparing their retention times and UV spectra (at 280, 320, and 360 nm) with reference compounds.

***Antioxidant Activity Assays***

***Photochemiluminescence (PCL) Method***

The antioxidant activity of the extracts was measured against superoxide anion radicals by PCL assay method [20] with a Photochem® apparatus (Analytik Jena,Leipzig, Germany). Radicals are generated by UV irradiation (Double Bore® phosphorus lamp, output 351 nm, 3 mWatt/cm$^2$) of a photosensitizer compound (Luminol).

The manufacturer provide two different kits (ACW and ACL) to measure both hydrophilic than lipophilic compounds [21] with two different protocols that use the same photosensitizer agent but different calibration standard: Ascorbic acid and Trolox, respectively.

In particular, for ACL method, the signal caused by detection of luminescence is monitored for 180 s and the integral of the curve is calculated.

A reduction of the integral correspond to antioxidant capacity of a sample relative to the blank. This inhibition is used for quantification and it is also related to the calibration curve to different concentration of Trolox. Results are expressed as micromoles of Trolox per gram of dry matter. For each sample the analysis was carried out in triplicate: 20 µL of the diluted extract in HPLC-grade methanol were sufficient to correspond to the standard curve.

***Antifungal Activity***

***Microorganisms***

Extracts, essential oil and isolated compounds were tested on fungal species, pathogenic for animals and humans, such as some dermatophytes are pathogenic for plants, such as some phytopatogen. Eight of the specific fungi strains investigated in this study were purchased from the Centraalbureau voor Schimmelcultures (CBS), Baarn, The Netherlands: *Arthroderma cajetani* Ajello, strain CBS 495.70; *Epidermophyton floccosum* (Hartz) Langeron and Milochevitch, strain CBS 358.93; *Trichophyton violaceum* Malmsten, strain CBS 459.61; *Trichophyton tonsurans* Malmsten, strain CBS 483.76, *Trichophyton mentagrophytes* (Robin) Blanchard, strain CBS 160.66; *Microsporum canis* Bodin, strain CBS 4727; *Microsporum gypseum* (Bodin) Guiart e Grigorakis, strain IHME 3999. *Botrytis cinerea* CBS 179.71, obtained from CBS-KNAW Fungal Biodiversity Centre, an Institute of the Royal Netherlands Academy of Arts and Sciences, Uthrecht, Netherlands. *Pythium ultimum Trow* ATCC no. 58812, obtained from American Type Culture Collection (ATCC), Manassas, USA. All dermatophytes and phytopatogens were maintained at 4 °C as agar slants on Sabouraud dextrose agar (SDA; Difco Laboratories, Inc.).

***Antifungal Activity***

To evaluate antifungal activity, cultures of each fungus were obtained by transplanting mycelium disks, 10 mm in diameter, from a single culture in stationary phase. These were incubated at 26 ± 1 °C on the medium suitable for each organism (SDA or PDA), on thin sterile sheets of cellophane, until the logarithmic phase of growth was reached. Then the fungi were transferred to Petri dishes containing the medium supplemented with the compound to be tested. Each compound was dissolved into dimethyl sulfoxide (DMSO), and a proper dilution was aseptically added to the medium at 45 °C to obtain a final concentration of 20, or 100 µg/ml. The DMSO concentration in the final solution was adjusted to 0.1%. Controls were set up with equivalent quantities (0.1%) of DMSO. The growth rate was determined by
measuring daily colony diameter for 7 days after the transport of the fungus onto dishes containing the substance to be tested. At this time the percentage growth inhibition in comparison with the control was evaluated for each fungus. Three replicates were used for each

concentration. The percentage of growth inhibition was expressed as the mean of values obtained in three independent experiments.

The relative inhibition rate of the circle mycelium compared to blank assay was calculated via the following equation:

Relative inhibition rate (%) = [(dex − dex')/dex] × 100%

where dex is the extended diameter of the circle mycelium during the blank assay; and dex' is the extended diameter of the circle mycelium during testing.

***Statistical Analysis***

Relative standard deviations and statistical significance (Student's t-test; $p < 0.05$) were given where appropriate for all data collected. One-way ANOVA and Least Significant Difference (LSD) posthoc Tukey's honest significant difference test were used for comparing the bioactive effects of different samples. All computations were made using the statistical software STATISTICA 6.0 (StatSoft Italia s.r.l., Padova, Italy).

**Results and Discussion**

***Chemical composition of the essential oil of M. vulgare***

The percentages and the retention indices of the identified components are presented in Table 1. GC-MS analysis led to the identification of fourteen (14) compounds. The essential oil was characterized by its richness in Eugenol (15.29 %). These results confirmed that *M. vulgare* is a lamiaceae oil poor specie. Additionally, some analysis on the material of Tunisian origin differ largely, supporting the hypothesis of the great chemical variability present in this species [22]. The work concluded that the oil from Zaghouan region was characterized by the high content of β- caryophyllene (7.8 %) followed by (E)-β-farnesene (7.4 %) and 1,8-cineole (4.8 %). Some other studies about the same specie grown in different countries revealed that the essential oil constituents are rather different. More recently, eighty compounds were detected in the essential oil of Egyptian *M. vulgare*. Otherwise, Thymol (29.6–60.7%), carvacrol (0.5–19.3%), m-cymene (1.0–14.2%), γ-terpinene (1.1–12.1%), thymol methyl ether (0.4–10.4%) and α-himachalene (0.0–10.3%) were the major identified compounds. The literature data clearly indicate the presence of a remarkable chemical polymorphism and great interspecific variability. The observed variation among populations depended on the environmental factors, stress conditions and heritance [22].

***Chemical composition of methanol and acetone extracts***

The present study focused on the phenolic composition of *M.vulgare.* The methanol and acetone extracts of *M.vulgare* were analyzed by HPLC–DAD (at 280, 320, and 360 nm). Peaks were identified by comparing their retention time and UV spectra with chromatograms using reference standards in the same conditions. Since there is no standard of all *M. vulgare* phenolics and flavonoids, only individual components which were commercially available were quantified.The retention times (Rt) are listed in Table 2. We obtained good separation for most peaks. The HPLC–DAD analysis allowed identifying peaks corresponding to 3 flavonoids, 4 phenolic acids and one phenylethanoid derivative (verbascoside). However, further analytical investigations are required for the identification of remaining peaks present in the chromatograms. HPLC analysis showed that the main phenolic acids found are caffeic acid, sinapic acid, ferulic acid, and p-coumaric acid. Also, apigenin, luteolin, and quercetin were identified as the main flavonoid aglycones present in *M.vulgare*. These compounds are reputed for high antioxidant capacity and some of these are used as standards in various antioxidant tests. In the present study, the major component in both extracts is marrubiin, appearing predominant in the acetone extract. Similar study has been conducted to investigate the phenolic composition of *M.vulgare* in Tunisia and found that caffeic acid, syringic acid, sinapic acid, ferulic acid, 2-hydroxy cinnamic acid, rosmarinic acid, transcinnamic acid, and p-coumaric acid occurs as the main detected phenolic acids [6]. Comparing with the different investigation in

the current interest, the discrepancy between studies could be attributed to chemotype, environmental and climatic condition, extraction and quantification method plant populations, genetic (genus, species and ecotype). Thus, it's quite interesting to observe such profil variability in the same country in order to justify the different uses of these plants. These findings could be supported by the phytochemical profiling of Algerian *M. vulgare* characterized by more sophisticated method *UHPLC–MS/MS* suggesting the detection of a wide array of phytochemical compounds in leaves [23].

***Antioxidant activity***

The PCL method is based on the photo-induced autoxidation inhibition of luminol by antioxidants mediated from the radical anion superoxide ($O^{-}_{2}$). Data obtained from PCL testing are summarized in table 2, methanol and acetone extracts showed an interesting antioxidant power (261.41 and 272.90 µmol TE/g respectively). Similarly to our work, another studies confirmed these good results using the total antioxidant capacity, iron-reducing power, and DPPH assays [6, 23]. These findings might be explained by the pronounced potentiality of antioxidant substances detected in the samples. In fact, crude extracts were known to contain different components and synergy between them may contribute to the overall observed antioxidant activity. Recently, in a similar study about Algerian *M.vulgare* leaves, the authors found that methanol and acetone extract contain a high amounts of phenolic compounds. In addition, a highly significant correlation between the amounts of flavonoids, tannins, phenolic compounds and the total antioxidant capacity of methanol and acetone extract was observed [23]. Indeed, it has been reported that the solubility of phenolic compounds is governed by the type of solvent used, the degree of polymerization of the phenolics, as well as by the interaction of the phenolics with other food constituents and the formation of insoluble complexes [25, 26].

***Antifungal Activity***

The present study was investigated to determine the antidermatophytic, antiphytopathogenic activity of various extracts and EO of *M.vulgare* and its active principle compound marrubiin against some pathogenic dermatophyte strains including *M. canis, A. cajetani, T. mentagrophytes, E. floccosum, T. tonsurans, T. mentagrophytes, M. canis, M. gypseum* and two phytopathogens, *P. ultimum and B. cinerea* (Table 4). After treatment with methanol and acetone extract of *M. vulgare*, increased fungal growth was observed for *M. gypseum* treated with methanol extract, and for *A. cajetani* treated with both extracts. Marrubiin was much more effective than various extracts and EO once it exhibited an inhibition value close to 50% at the highest dose (100 µg/ml) for *T. mentagrophytes* (48.78%), and *E. floccosum* (42.2 %).

Concerning the antiphytopathogenic activity, The results had not shown any inhibition zone against for *B. cinerea*, except marrubiin at the highest dose (32.40%), while for *P. ultimum,* it could be observed that methanol extract followed by marrubiin had more antiphytopathogenic effects than various extracts of *M.vulgare* with the higher percentage of inhibition of growth (45.15 and 40.30 % respectively). The solvent used in the extraction influenced the antifungal activities displayed by the plant, which is in accordance with the literature, and concerning essential oils when acting alone, their high doses are required to achieve the significant antifungal effect. Therefore, the development of new strategies that allow the increase of their efficacy at lower concentrations is needed. On the other hand, it was shown that the antifungal activity of different investigated extracts was dose-dependent, but also it depends across the studied pathogens. These findings were consistent with the previous studies on extracts and essential oil of *M.vulgare* [27, 28]. Despite the large data have now been existing about antibacterial and antimicrobial effects of medicinal plants, little had been conducted in the area of antifungal [29, 30]. Previous studies indicate that the effect of plant extracts on fungal pathogen may be attributed to their content on secondary metabolites (e.g., alkaloids, phenolic, flavonoids and terpenoids compounds) with known antifungal activity [31].

However the activity of marrubiin was the most relevant against tested dermatophytes strains,

we explain this by the fact, that pure component give a more potent antifungal activity when compared to a complex mixture of components such as extracts and essential oils which always represent a complex mixture of different chemical components and a few active principles. To the best of our knowledge, there are no reports on the antidermatophytic and antiphytopathogenic properties of marrubiin has been published but in the same line of research the bactericidal, fungicidal and antiviral properties of vegetal terpenes are widely documented [32, 33, 34], it increase the permeability of the plasma membrane and inhibit process of respiration on mitochondrial membrane of fungi [35, 36].

**Conclusions**

To conclude, the *M.vulgare* samples were found to have a diversity in their phytochemical compounds and chemical composition of essential oil (EO) resulting in an observed powerful antioxidant effects added to antidermatophytic and phytopahtogenic properties. These findings supported the use of *M.vulgare* and marrubiin in the traditional medicine for dermatophytic infections, particularly in the treatment of onychomycosis. Complementary investigations should be conducted to assess the effectiveness of this compound in the human health and phytopathology field.

Conflict of Interest

No potential conflict of interest was reported by the authors.

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**List of table captions:**

**Table 1.** Essential oil composition (%) of *M. vulgare.*

[a](RI): Values obtained by comparison of retention time and mass spectrum using the same column phase (5%-phenyl-methylpolysiloxane). [b]: Tentative using mass spectra data bases. [c]: See references. n.a: Not available.

**Table 2.** Quantitative analysis of major phenolic compounds identified in the different extract of *M. vulgare* (g/100 g DW) (- not detected).

**Table 3.** PCL analysis of marrubiin, extracts and essential oil of *M. vulgare*. Each value was obtained from three experiments (mean ± SE).

**Table 4.** Percent growth inhibition of dermatophytes and phytopathogens treated with extracts, essential oil and isolated marrubiin at 20 or 100 µg/ml. Each value is the mean of three measurements.

+ the compound stimulates the growth of the fungus.

| Compound | Retention index (RI)[a] | | % |
|---|---|---|---|
| | Observed[b] | Adams[c] | |
| 2-methoxy-4-vinylphenol | 1282 | 1309 | 1.72 |
| Indole | 1293 | 1290 | 1.17 |
| Eugenol | 1356 | 1355 | 15.29 |
| Geranyl acetone | 1429 | 1453 | 0.75 |
| B-ionone | 1482 | 1487 | 4.14 |
| B-bisabolene | 1510 | 1509 | 2.37 |
| Nerolidol | 1566 | 1561 | 1.02 |
| Megastigmatrienone 2 | 1579 | n.a | 2.45 |
| Caryophyllene oxide | 1581 | 1579 | 0.90 |
| Megastigmatrienone | 1591 | n.a | 1.68 |
| Hexahydrofarnesyl acetone | 1827 | 1838 | 1.40 |
| Phytol | 1921 | 1942 | 1.04 |
| Butyl palmitate | 2185 | n.a | 1.09 |

Table 1.

Table 2

| | | Phenolic acids | | | | Flavonoids | | | Phenylpropanoid glycoside |
|---|---|---|---|---|---|---|---|---|---|
| | Marrubiin | Ferulic acid | p-cumaric acid | Sinapic acid | Caffeic acid | Luteolin | Apigenin | Quercetin | Verbascoside |
| **Rt (min)** | 22.04 | 9.67 | 8.74 | 9.5 | 6.25 | 17.1 | 18.83 | 16.9 | 8.5 |
| **Methanol extract** | 34.68 ±0.92 | 0.033 ±0.0001 | 0.00093 ±0.000004 | - | 0.024 ±0.0008 | 0.066 ±0.0023 | 0.008 ±0.0005 | 0.006 ±0.0001 | 0.087 ±0.003 |

| | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| **Acetone extract** | 91.4 ±8.72 | 0.014 ±0.002 | - | - | 0.0051 ±0,0003 | 0.026 ±0.0011 | 0.013 ±0.002 | 0.0068 ±0.00021 | - |



Table 3

| | **µmol TE/g** |
|---|---|
| **Methanol extract of *M.vulgare*** | 261.41 ± 2.05 |

| | |
|---|---|
| **Acetone extract of *M.vulgare*** | 272.90 ± 3.1 |
| **Marrubiin** | 3.12 ± 0.06 |
| **Essential oil of *M.vulgare*** | 0.48 ± 0.02 |



Table 4

| | | | % Inhibition of growth | | |
|---|---|---|---|---|---|
| **Dermatophytes (7days)** | **Concentration** | **Methanol extract** | **Acetone extract** | **Marrubiin** | **Essential oil of *M. vulgare*** |
| *M. gypseum* | 20 µg/ml | + | 14.87 ± 0.95 | 17.76 ± 0.87 | 7.02 ± 0.55 |

| | | | | | |
|---|---|---|---|---|---|
| | 100 µg/ml | + | 22.30 ± 1.22 | 23.68 ± 1.42 | 21.05 ± 1.35 |
| *M. canis* | 20 µg/ml | 1.70 ± 0.01 | 1.50 ± 0.03 | 24.60 ± 1.29 | 1.71 ± 0.08 |
| | 100 µg/ml | 11.9 ± 0.5 | 15.67 ± 1.24 | 27.78 ± 0.97 | 11.93 ± 0.87 |
| *A. cajetani* | 20 µg/ml | + | + | 3.39 ± 0.04 | 8.33±0.62 |
| | 100 µg/ml | 0.00 | + | 5.08 ± 0.03 | 19.44± 0.81 |
| *T. mentagrophytes* | 20 µg/ml | 2.76 ± 0.08 | 6,25 ± 0.35 | 20.33 ± 0.94 | 22.52 ± 1.11 |
| | 100 µg/ml | 9.0 ± 0.6 | 11.72 ± 0.84 | 48.78 ± 1.85 | 25.83 ± 1.32 |
| *E. floccosum* | 20 µg/ml | 11.63 ± 0.94 | 16.67 ± 0.93 | 35.56 ± 1.47 | 10.42 ± 0.25 |
| | 100 µg/ml | 16.28 ± 1.1 | 29.63 ± 0.48 | 42.22 ± 2.1 | 27.08 ± 1.42 |
| *T. tonsurans* | 20 µg/ml | 9.38 ± 0.4 | 7.41 ± 0.52 | 0.00 | + |
| | 100 µg/ml | 17.19 ± 0.88 | 9.26 ± 0.38 | 8.70 ± 0.47 | 2.38 ± 0.06 |
| **Phytopathogens** | | | | | |
| *B. cinerea* | 20 µg/ml | 0.00 | 0.00 | 0.35 ± 0.02 | 0.00 |
| | 100 µg/ml | 0.00 | 0.00 | 32.40 ± 1.68 | 0.00 |
| *P. ultimum* | 20 µg/ml | 17.48 ± 1.2 | 12.82 ± 0.98 | 17.86 ± 0.93 | + |
| | 100 µg/ml | 45.15 ± 1.5 | 27.69 ± 1.24 | 40.31 ± 1.85 | 24.30 ± 0.99 |