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Antioxidant and anticandidal activities of the Tunisian *Haplophyllum tuberculatum* (Forssk.) A. Juss. essential oils

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ABSTRACT

Haplophyllum tuberculatum Forssk. is a medicinal plant growing in Tunisia. It is widely used in traditional medicine against gastro-intestinal problems, fevers, ear infections and rheumatisms. The present investigation evaluated the effects of leaves, stems and leaves + stems essential oils of *Haplophyllum tuberculatum* Forssk. and of their pure compounds on free radicals as well as their anticandidal activities. Screening for the antioxidant activity of the oils, *R*-(+)-limonene, *S*-(−)-limonene and 1-octanol was conducted by DPPH, ABTS and β-carotene/linoleic acid radical scavenging assays. The essentials oils and their compounds were screened for antifungal activity against four *Candida* species: *Candida albicans* ATCC 90028; *Candida glabrata* ATCC 90030; *Candida parapsilosis* ATCC 27853 and *Candida krusei* ATCC 6258. When compared with ascorbic acid as standard, it was found that the essential oils have a significant inhibition in scavenging free radicals, resulting in an important IC_{50}. The pure compounds were inactive against the free radicals. The anticandidal test results showed that leaves, stems and leaves + stems oils strongly inhibited the growth of *Candida krusei* at 30 μg/mL leaves oils and 70 μg/mL for other oils and that moderately of the 3 other *Candida* species. The pure compound, 1-octanol, was active one against the *candida* species, with MIC-values between 0.07 and 1.25 mg/mL.
In all *in vitro* assays, a significant correlation existed between the concentrations of the essential oils, the percentage inhibition of free radicals and of the growth inhibitory of tested *candida* species. The results indicate the essential oils may be applied for treating diseases related to free radicals, potentially to prevent cancer development and as an antifungal agent against *Candida*.

1. Introduction

Haplophyllum is a genus belonging to the Rutaceae family. It is distributed in different floristic regions: the Irano-Turanian, Mediterranean, Saharo-Arabian, and Sudano-Zambezian regions. It includes 68 species (Navarro et al., 2004; Soltani and Khosravi, 2005; Townsend, 1986). In Tunisia, it is represented by 3 species *Haplophyllum linifolium* (L.) A. Juss (=*Haplophyllum hispanicum* Spach), *Haplophyllum tuberculatum* (Forsk) A. Juss and *Haplophyllum buxbaumii* Poiret (Pottier-Alapetite, 1979).

H. tuberculatum stretches from Morocco to western Pakistan. It is an annual herbaceous plant. The whole plant is covered with large glands containing essentials oils. The leaves are variable in shape, from almost linear to short. The flowers are yellow and variable in size (Pottier-Alapetite, 1979). The plant is traditionally known for its different medicinal properties, for instance against gastro-intestinal affections, intermittent fevers, and rheumatisms. This plant is also an aphrodisiac and is administered against ear infections (Le Floc'h, 1983). It was recommended against nausea and vomiting by taking the leaves' infusion in the morning before breakfast. It is also used for the nervous system, infertility and fever (Said et al., 2002). In northern Oman, the juice is used as a remedy for headaches and arthritis (Al-Burtamani et al., 2005). In Saudi Arabia, *H. tuberculatum* is used to treat malaria, rheumatoid arthritis and gynecological disorders (Al-Yahya et al., 1992). In Southern Tunisia, the fresh plant is used as an antiparasitic against *Enterobius vermicularis* by drinking the infusion of some fresh leaves and it's also used as flavor in the tea.

Abbreviations: DPPH, 1,1-Diphenyl-2-picryl-hydrazyl; ABTS, 2,2′-Azinobis-(3-ethylbenzothiazoline-6-sulfonic acid); $K_2S_2O_8$, Potassium persulfate.

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The essential oil of *H. tuberculatum* is anti-bacterial and partially inhibits the growth of *Escherichia coli, Salmonella choleraesuis, Bacillus subtilis* and *Candida albicans* (Al-Burtamani et al., 2005). It also has anti-fungal properties. In fact, the essential oil inhibits the mycelial growth of *Curvularia lunata* and *Fusarium oxysporum* (Al-Burtamani et al., 2005).

Some biochemical studies of the essential oils (EO) of *H. tuberculatum* aerial part have been published (Al-Burtamani et al., 2005; Al Yousuf et al., 2005; Yari et al., 2000). No studies for the antioxidant activity of the EO were found in the literature. For this reason, in the present study, an attempt was made to explore the possible antiradical properties of the aerial parts and of the individual stems and leaves, by different techniques, which may provide more comprehensive information on these properties.

In our previous work, we analyzed the chemical composition of the essential oils of the separated leaves, stems and leaves + stems from the Tunisian *H. tuberculatum* and we assessed their phytotoxic activities (Hamdi et al., 2017). The present investigation aimed evaluating the antioxidant of leaves (L), stems (S) and the aerial part (leaves + stems; LS) of this species essential oils (LEO, SEO and LSEO, respectively) and assessing their anticandidal activities. Limonene, as a major compound, its isomers and octanol, as a minor compound, were also tested.

2. Material and methods

2.1. Plant material

Plant material from the *H. tuberculatum* Forssk. species was collected at the flowering stage from Medenine in Tunisia. The leaves, stems, leaves + stems and roots were cut into small pieces and weighed before extraction of the volatile compounds (Hamdi et al., 2017).

2.2. Extraction of the essential oils

About 100 g fresh plant parts (leaves, stems, roots and leaves + stems) was subjected to a hydro-distillation for 3 h with 500 mL distilled water using a Clevenger-type apparatus. The LEO, SEO and LSEO obtained were dehydrated by passing through anhydrous sodium sulfate. No volatile oils were obtained from the roots. Then, the essential oils were stored in sealed glass vials in a refrigerator at 4–5 °C, until use (Hamdi et al., 2017).

2.3. Standard compounds

Octanol (for synthesis, Merck), *R*-(+)-limonene (97%, Aldrich) and *S*-(−)-limonene (96%, Aldrich) were provided from the commercial suppliers and dissolved in methanol (Merck). DPPH, ABTS, β-carotene and linoleic acid are provided from Sigma Aldrich.

2.4. Determination of antioxidant activity

Antioxidants are compounds capable to either delay or inhibit free radicals. They are involved in the defense mechanisms of the organism against pathologies related to free radicals. Amongst the most important natural or synthetic antioxidants, vitamin A, vitamin E, vitamin C, vitamin D, β-carotene and flavonoids allow free radicals inhibition because of their molecular structure.

Various methods to evaluate the antioxidant activity have been described. Antioxidant assays can be classified into two main groups, hydrogen atom transfer (HAT) and single electron transfer (ET) assays. Antioxidants are measured by their HAT or ET to probe molecules. In this study, the DPPH and ABTS assays were used. These assays usually are classified as ET reactions, but in fact, both radicals may be deactivated either by radical quashing HAT or by direct reduction through ET mechanisms (Apak et al., 2016; Jiménez et al., 2004).

Lipid peroxidation is the oxidation of lipids, especially of unsaturated fatty acids in cellular membranes mediated by oxidative stress in the cells. The β-carotene bleaching assay is an approach to directly assess the antioxidant activity of compounds toward a lipid substrate (Apak et al., 2016; Laguerre et al., 2007).

These, three above-mentioned methods were applied in this study.

Bellow the protocols of these methods, used to test the antioxidant activity of the essential oils are described. Ascorbic acid (vitamin C) was used as reference compound under the same conditions and with the same concentrations as the tested samples. The inhibition concentration (IC_{50}) value, which is the sample concentration providing 50% inhibition was determined.

2.4.1. DPPH radical-scavenging activity assay

The DPPH assay is known to provide reliable information concerning the antioxidant capacity of specific compounds or extracts. The hydrogen atoms accepting or electron donating ability of the corresponding samples were measured from decolorizing a purple colored methanolic DPPH solution. The effect of the essential oils and their pure components on the DPPH radical was estimated (Shimada et al., 1992). 0.5 mL of each sample was mixed with the same volume DPPH• ethanolic solution (60 μM). The mixture was shaken vigorously and allowed standing for 30 min in the dark at a temperature of 25 °C. The absorbance of the resulting solution was measured at 520 nm with a spectrophotometer. The analyses of the samples were performed in triplicate. A mixture of 0.5 mL DPPH• solution and 0.5 mL methanol was taken as a control. Methanol was used as a blank. The Inhibition of the free radical DPPH in percent (IP%) was calculated as follows:

$$IP\% = 100 \times (A_{Control} - A_{Sample}) / A_{Control}$$

where $A_{Control}$ is the absorbance of the control, and A_{Sample} of the tested sample.

2.4.2. ABTS radical scavenging activity assay

Antiradical activity was also determined using the $ABTS^{+}$• free radical decolorization assay (Re et al., 1999). Briefly, the preformed radical ABTS monocation was generated by reacting aqueous ABTS solution (7 mM) with 2.45 mM ethanolic $K_2S_2O_8$. The mixture was allowed standing for 15 h in the dark at room temperature. The solution was diluted with ethanol to obtain an absorbance of 0.7 ± 0.2 at 734 nm and used as control. Extracts (0.0312, 0.0625, 0.125, 0.25, 0.5 and 1 mg/mL) and pure compounds (1 mg/mL) were separately dissolved in ethanol. To estimate the antioxidant activity of the samples, 10 μL was added to 990 μL of diluted $ABTS^{\bullet+}$.

The absorbance was measured spectrophotometrically at 734 nm after 5, 10, 15 and 20 min. The samples/compounds were analyzed in triplicate. Distilled water was used as a blank. The percentage inhibition (PI) of $ABTS^{\bullet+}$ was calculated by the following equation:

$$IP\% = 100 \times (A_{Control} - A_{Sample}) / A_{Control}$$

where $A_{Control}$ is the absorbance of the control, and A_{Sample} of the tested sample.

2.4.3. β-Carotene/linoleic acid method

The β-carotene bleaching inhibition activity of *H. tuberculatum* Forssk EO was determined (Ikram et al., 2009). Briefly, 2 mL β-carotene solution (1.5 mg β-carotene/2.5 mL chloroform) was added to 20 μL linoleic acid and 200 μL Tween-20 and mixed. The chloroform was removed at 40 °C under vacuum using a rotary evaporator.

Immediately, 50 mL distilled water was added to the dried mixture to form a β-carotene-linoleic acid emulsion. In order to determine the β-carotene bleaching activity of the extract, 5 mL emulsion was added to 500 μL EO (0.0312, 0.0625, 0.125, 0.25, 0.5 and 1 mg/mL) or pure compound (1 mg/mL). The mixtures were incubated in a water bath