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Antifungal, antiradical and cytotoxic activities of extractives obtained from *Tagetes patula* L. (Asteraceae), a potential acaricide plant species

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ABSTRACT

Tagetes patula L. shows a complex chemical composition, ranging from glycosylated flavonoids and thiophenes in extracts until terpenoids in the essential oil. In the present study, due to this rich flavonoidic constitution, its antioxidant potential was determined, having shown values of antiradical percentage superior to reference compounds, mainly the extracts prepared with flowers. Previous studies performed emphasized the acaricide potential of *T. patula* and thus, the present study aimed to verify the action of extractives obtained from aerial parts on growth of entomopathogenic fungi related to biological control of brown dog tick *Rhipicephalus sanguineus* and the action against pathogenic fungi closely associated with pets. None of the samples inhibited the growth of strains of *Beauveria bassiana* or *Metarhizium anisopliae*, enabling feasible future studies of synergism on acaricide activity of formulations containing fungi and extracts. The antimicrobial activity of ethanolic extract of flowers ($Fl_{EtOH70\%}$) against *Microsporum canis* and *Trichophyton rubrum* was significant (193.3 μg/mL and 253.9 μg/mL, respectively), as well as ethanolic extract from aerial parts ($AP_{EtOH70\%}$) against *T. rubrum* (312.5 μg/mL). In order to ensure the safety of a topical formulation containing the extractives of *T. patula*, the cytotoxic potential of these samples were tested in murine macrophages cells. At higher concentrations all extracts were quite lethal, with IC_{50} ranging from 210.96 μg/mL to 468.75 μg/mL for $AP_{EtOH70\%}$ and $Fl_{EtOH70\%}$, respectively. These results suggest that the application of a product containing *T. patula* extractives in the control of ticks could be used, at principle, only on the environment.

1. Introduction

Researches about the use of plant species in the control of animal parasites are scarce, with a lack of further information regarding the conditions of production, harvest time, plant parts and quantities used in the elaboration of products [1]. The use of plants, either as phytotherapics or a source of prototype substances, shows the importance of scientific research for the development of a new drug. Among the advantages of herbal medicines that justify their use are synergistic effects of its components, the combination of mechanisms for compounds acting on different molecular targets, lower risk of side effects and less costs in research [2].

Tagetes patula L. (Asteraceae), popularly known as dwarf marigold or French marigold is an annual plant, 20–30 cm height, native to North America and widely disseminated throughout the world. The genus *Tagetes* has many biological activities reported against many organisms such as fungi [3–5], Gram positive and Gram negative bacteria [6–8], virus [9], nematodes [10,11], insects [12–14], ticks [15–18] and others. The phytochemical investigation of different parts of *T. patula* has resulted in the isolation of several chemical constituents of different classes of secondary metabolites, such as benzofurans, carotenoids, flavonoids and thiophenes, the latter being responsible for a variety of biocides properties [19]. Bano et al. (2002) [20] isolated and characterized thiophenes, steroid and terpenoids from roots, leaves and flowers of *T. patula*. Flavonoids, such as quercetin and kaempferol were reported by Ivancheva and Zdravkova (1993) [21]. Politi et al. (2012) [16], using the same ethanolic extract applied in the tests of the present study, identified by HPLC-MS twelve *O*-glycosylated flavonoids:

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kaempferol, patuletin, quercetin-3-*O*-pentoside, quercetin-3-*O*-glucoside (isoquercitrin) and quercetin-3-*O*-galactoside (hyperoside), patuletin-7-*O*-glucoside (patulitrin) or 6-*O* -methyl-quercetin-3-*O*-glucoside, quercetin-3-*O*-rhamnosyl-*O*-xyloside, quercetin-3-*O*-di-rhamnoside, quercetin-3-*O*-glycosyl-7-*O*-rhamnosyl, quercetin-3-*O*-rhamnosyl-7-*O*-glycosyl, kaempferol-3-*O*-dihexoside and quercetin-3-*O*-galloyl-hexoside. In another study, by GC–MS, Politi et al. (2013) [17] identified in the essential oil of the aerial parts of *T. patula* 55 compounds, being the main, 4-vinyl guaiacol and gamma-terpinene, appearing also in good proportions limonene, (E)-tagetone and spathulenol.

Researches with entomopathogenic fungi as biological controllers have been made in order to assist the establishment of rational and effective strategies against arthropods of commercial interest or pathogenic [22]. *Metarhizium anisopliae* and *Beauveria bassiana* are the most well characterized entomopathogenic fungi. Hence, many studies describe its potential for controlling many plagues, including ticks [23–26]. The capacity of production and obtainment of formulations from the association of this fungus with different compounds makes it one of the most traded in the world [27].

Based on the phytochemistry previously described and the biocide potential of the *T. patula* reported on recent studies of anti-tick potential of the 70% ethanolic extract [16,18] and essential oil [17] of this species against the brown dog tick *Rhipicephalus sanguineus*, the present investigation aimed to verify the action of these plant extractives on growth of entomopathogenic fungi directly related to the biological control of such ixodids. Besides, the investigation of the activity of these plant extractives over dermatophytes, may also contribute to eliminate pathogenic fungi closely associated with the primary host of these ticks [28]. Furthermore, based on the rich constitution of flavonoids, was analyzed the antioxidant potential of the samples. In order to verify the safe use of these extractives like a possible acaricidal formulation, in a first moment, cytotoxicity assays were conducted in macrophages cells.

2. Material and methods

2.1. Plant material

Aerial parts of *T. patula* (stems, leaves and flowers) were obtained from the Collection of Medicinal and Aromatic Plants (CPMA) of the Multidisciplinary Center for Chemical, Biological and Agricultural Research (CPQBA), Universidade Estadual de Campinas (UNICAMP). The planting was done from seeds of Top Seed Garden line (Agristar®). A voucher specimen was deposited in the CPQBA Herbarium (process number 1421/2013).

2.2. Test microorganisms

The microbiological tests were conducted with the following strains: *Trichophyton rubrum* (INCQS 40004 and a clinical isolate of Oswaldo Cruz Foundation - OCF), *Trichophyton mentagrophytes* (INCQS 40051), *Microsporum canis* (clinical isolate, Laboratório de Micologia Clínica, FCFAR/UNESP), *M. anisopliae* (ATCC 343 and a clinical isolate of Oswaldo Cruz Foundation - OCF) and *B. bassiana* (ATCC 507 and 4531).

2.3. Extracts preparation

After the stabilization and drying, the aerial parts of the plant were triturated into cutting mill. The powdered drug was used for preparing the extracts by percolation using ethanol 70% (v/v) as solvent, with average flow rate of 40 drops/minute. After complete solvent evaporation, the dry extract was lyophilized and stored in a desiccator.

2.4. Determination of total flavonoids content

The total flavonoid content was estimated using a colorimetric method based on the formation of a flavonoid–aluminum complex [29]. The values were calculated from a calibration curve obtained with quercetin (95% purity, Merk) at concentrations of 0.25, 0.5, 1.0, 2.0, 4.0, 8.0 and 16.0 mg/mL. Final results were expressed in milligrams per gram of quercetin equivalent (QE), performed at 430 nm in a spectrophotometer Shimadzu UV-1603. The reading was done after 15 min of color reaction in the dark. An 80% methanolic solution (v/v) was used as blank. Samples of 70% ethanolic extract of the aerial parts ($AP_{EtOH70\%}$) and flowers ($Fl_{EtOH70\%}$) of *T. patula* were prepared to a stock concentration of 0.5 mg/mL in 80% MeOH solution (v/v). Aliquots of each stock solution were added to 2 mL of hexahydrate aluminum chloride solution $AlCl_3$ ($6H_2O$) in 2% MeOH (v/v), adjusting final volume to 4 mL in 80% MeOH solution (v/v).

2.5. Antiradical potential

The antioxidant activity assay of extractives was based on free radical scavenging activity of the 2,2-diphenyl-1-picrylhydrazyl (DPPH) solution [30]. Gallic acid, rutin, quercetin and vitamin C were used as standards; dilutions of $AP_{EtOH70\%}$, aerial parts without flowers ($APWF_{EtOH70\%}$) and $Fl_{EtOH70\%}$ were tested. Briefly, 2.5 mL of DPPH solution in 0.004% MeOH was added to 1 mL of different concentrations of plant extracts. A solution in methanol (2.5:1, v/v) was used as negative control and pure MeOH was the blank. The absorbance was read at 517 nm in spectrophotometer (Shimadzu-1603). The antiradical activity was calculated as the percentage of DPPH discoloration, according to the equation below:

$$\text{DPPH Scavenged } (\%) = \frac{Ac - At}{Ac} \times 100$$

Where: Ac = absorbance of DPPH solution (negative control); At = absorbance of test sample.

2.6. Antimicrobial activity

The antimicrobial activity was determined by minimum inhibitory concentration (MIC) according to adapted protocols from CLSI M38-A2 [31]. Extracts were prepared in dimethylsulfoxide (DMSO) and diluted in RPMI 1640 medium to obtain a 5 mg/mL solution. Amphotericin B (16 mg/mL) was the antibiotic used as positive control for fungal strains. The inoculum was obtained by resuspending fungal cells in 0.9% saline and adjusted to obtain approximately 5×10^3 CFU/mL. Briefly, 100 μl of this cell suspension were applied in 96 wells cell culture plates with 100 μl of medium and 100 μl of the plant extracts, performing serial dilutions. The plates were incubated in an orbital shaker at 100 rpm for 7 days at 28 °C and then the visual reading was done. There were a negative growth control constituted by only medium, a negative extract control containing extracts and medium and a growth control containing cells and medium. The MIC was considered as the lowest concentration that inhibited fungal growth.

2.7. Cytotoxicity assay

Cytotoxicity assay was adapted from Ahmed et al. (1994) [32]. The murine macrophage strain J774 was maintained in RPMI medium (pH 7) supplemented with 10% fetal bovine serum, 0.2% sodium bicarbonate, 10 mM Hepes, penicillin (100 U/mL) and