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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

streptomycin (100 μg/mL), subsequently incubated at 37 °C under 5% CO_2 atmosphere until exponential growth phase.

Stock solutions of plant extracts were prepared in DMSO, diluted with the culture medium 1:10 (v/v) and applied to the 96 wells cell culture plate, proceeding to serial dilution in a 1:1 ratio. The concentration range test varied from 15.625 mg/mL to 2000 mg/mL. The cytotoxicity of the samples was determined by adding resazurin (0.1 mg/mL). The reading was performed on luminescence microplate reader Spectra Fluor Plus at 530 and 590 nm [33].

2.8. Statistical analysis

Analyses of variance (ANOVA) were performed, and the averages were compared by parametric test of Tukey ($p < 0.05$), using the software StatPlus 2009 (Soft Analyst®). Pearson linear correlation test ($p < 0.05$) was applied to indicate the correlation coefficient between total flavonoids content and the antioxidant activity of the extracts. All the tests were executed in triplicate.

3. Results

Yields of 44.9% for the $AP_{EtOH70\%}$, 31.9% for the $APWF_{EtOH70\%}$ and 41.2% for the $Fl_{EtOH70\%}$ were obtained in the extracts preparation. The average content of total flavonoids in the extract $AP_{EtOH70\%}$ and $Fl_{EtOH70\%}$ was approximately 7.27% and 12.45%, respectively (Table 1). The analysis of variance (ANOVA) revealed that the average of total flavonoid content of aerial parts extracts and flowers extract differ significantly ($p < 0.05$). The content of flavonoids (quercetin equivalents) obtained from the extract of flowers of *T. patula* is almost double the flavonoid content of the extract of the aerial parts.

The $Fl_{EtOH70\%}$ presented the best antiradicalar activity (Table 2), higher than the gallic acid and quercetin standards. There were no statistically significant differences ($p < 0.05$) by analysis of variance in mean values of antioxidant activity between the samples and the standards. This results show that the hydroalcoholic extracts of *T. patula* have a high antiradical potential. The analysis of Pearson linear correlation ($p < 0.05$) indicated a strong correlation between total flavonoids content and the respective antioxidant activity of $AP_{EtOH70\%}$ and $Fl_{EtOH70\%}$ ($\rho = 0.98$ and $\rho = 0.99$, respectively).

All extracts showed good results against the *T. rubrum*, highlighting the best antifungal action of $Fl_{EtOH70\%}$ against OCF strain (Table 3). Against *T. mentagrophytes*, the most effective extracts were $Fl_{EtOH70\%}$ and $AP_{EtOH70\%}$, with no statistically significant difference between them ($p < 0.05$). Significant results against *M. canis* were exhibited by all extracts, emphasizing the action of $Fl_{EtOH70\%}$, the lowest inhibitory concentration of all extract tested. The entomopathogenic fungi, was not sensitive to extracts showing MIC values only at the highest inhibitory concentration applied (Table 3). Considering that these microorganisms represent an efficient biological control of ticks, it was satisfactory that the extracts tested presented a low activity against them, thus preserving its acaricidal potential.

All extracts were highly cytotoxic, eliminating almost 100% of the macrophage cells, especially the $AP_{EtOH70\%}$ (96.24% cell lysis) (Fig. 1). The concentrations that destroy 50% of macrophages (IC_{50}) varied from $IC_{50} = 210.93$ μg/mL, for the $AP_{EtOH70\%}$, to $IC_{50} = 468.75$ μg/mL, for $Fl_{EtOH70\%}$ (Table 4). The IC_{50} values of the $AP_{EtOH70\%}$ and $APWF_{EtOH70\%}$ showed no statistically significant differences between them ($p < 0.05$).

Table 1
Spectrophotometric quantification (430 nm) of the total flavonoids (equivalent in quercetin) present in the dried extract of *T. patula*.

C (μg/mL)	FC (mgEQ/g)	
	$AP_{EtOH70\%}$ (±SD)	$Fl_{EtOH70\%}$ (±SD)
125.0	73.06[a] (±0.5351)	123.95[b] (±1.0480)
156.25	71.98[a] (±0.9612)	121.90[b] (±2.4881)
187.5	72.88[a] (±1.6569)	126.78[b] (±0.7272)
250.0	73.03[a] (±0.6638)	125.75[b] (±2.6054)
Mean	72.74[a] (±0.5012)	124.59[b] (±0.9680)

C: sample concentration; $AP_{EtOH70\%}$: 70% ethanolic extract from aerial parts of *T. patula*; $Fl_{EtOH70\%}$: 70% ethanolic extract from flowers of *T. patula*; FC: flavonoids content, calculated as milligrams of quercetin equivalents per gram of dry extract (mgEQ/g); SD: standard deviation. Values with the same superscript letters within a column do not showed statistically significant differences by Tukey test ($p < 0.05$).

Table 2
Antiradicalar activity of patterns and ethanolic extracts of *T. patula* by DPPH method.

Sample	Absorbance (±SD)	Antiradicalar Activity % (±SD)
Gallic Acid	0.0408 (±0.0023)	93.154[a] (±0.386)
Rutin	0.0532 (±0.0009)	91.075[a] (±0.157)
Quercetin	0.0457 (±0.0020)	92.327[a] (±0.339)
Vitamin C	0.0232 (±0.0010)	96.105[a] (±0.173)
$AP_{EtOH70\%}$	0.0563 (±0.0014)	90.556[a] (±0.243)
$APWF_{EtOH70\%}$	0.0593 (±0.0010)	90.047[a] (±0.171)
$Fl_{EtOH70\%}$	0.0396 (±0.0017)	93.350[a] (±0.285)

DPPH: 2,2-diphenyl-1-picrylhydrazyl; $AP_{EtOH70\%}$: 70% ethanolic extract from aerial parts of *T. patula*; $APWF_{EtOH70\%}$: 70% ethanolic extract from aerial parts without flowers of *T. patula*; $Fl_{EtOH70\%}$: 70% ethanolic extract from flowers of *T. patula*. Values with the same superscript letters within a column do not showed statistically significant differences by Tukey test ($p < 0.05$).

Table 3
Minimum inhibitory concentration (MIC) values of different extracts of *T. patula* against dermatophytes and entomopathogenic fungi.

Microorganisms	MIC (μg/mL)			
	$AP_{EtOH70\%}$	$APWF_{EtOH70\%}$	$Fl_{EtOH70\%}$	Ampho B
T. rubrum (40004)	$312.50^{A,a}$	$625.00^{A,b}$	$410.15^{A,a}$	$2.0^{A,c}$
T. rubrum (OCF)	$468.75^{B,a}$	$937.50^{B,b}$	$253.90^{B,a}$	$2.0^{A,c}$
T. mentagrophytes (40051)	$625.0^{C,a}$	$1041.66^{B,b}$	$572.91^{A,a}$	$4.8^{B,c}$
M. canis (CI)	$703.13^{C,a}$	$703.13^{A,a}$	$195.31^{B,b}$	$4.0^{B,c}$
M. anisopliae (343)	$1250.0^{D,a}$	$1250.0^{C,a}$	$1250.0^{C,a}$	$16.0^{C,b}$
M. anisopliae (OCF)	$1250.0^{D,a}$	$625.0^{A,b}$	$1250.0^{C,a}$	$16.0^{C,c}$
B. bassiana (507)	$1250.0^{D,a}$	$1250.0^{C,a}$	$1041.66^{C,a}$	$8.0^{D,b}$
B. bassiana (4531)	$1250.0^{D,a}$	$625.0^{A,b}$	$1250.0^{C,a}$	$8.0^{D,c}$

CI: clinical isolate; $AP_{EtOH70\%}$: 70% ethanolic extract from aerial parts of *T. patula*; $APWF_{EtOH70\%}$: 70% ethanolic extract from aerial parts without flowers of *T. patula*; $Fl_{EtOH70\%}$: 70% ethanolic extract from flowers of *T. patula*; Ampho B: amphotericin B. Superscript case letters refer to comparisons within the same column; Superscript lower case letters refer to comparisons within the same row. Values followed by the same letter do not show statistically significant differences by Tukey test ($p < 0.05$).

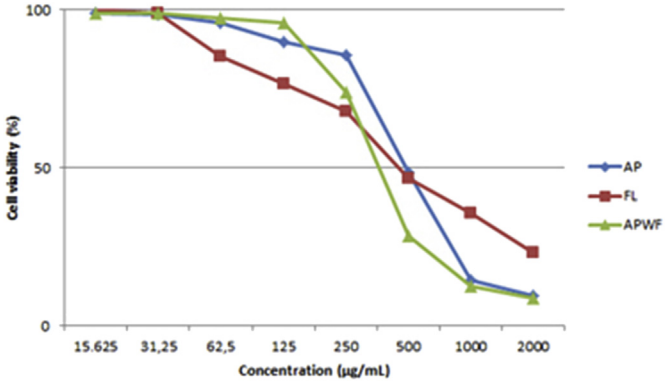

Fig. 1. Cell viability of murine macrophage front of ethanolic extracts of *T. patula*.

Table 4
IC_{50} (μg/mL) of 70% ethanolic extracts of *T. patula* front of J774 macrophage cells.

Sample	IC_{50} (μg/mL) ± SD
AP_{EtOH}	210.93[a] (±11.04)
$APWF_{EtOH}$	375.0[a] (±35.35)
Fl_{EtOH}	468.75[b] (±44.19)

SD: standard deviation; AP_{EtOH}: 70% ethanolic extract from aerial parts of *T. patula*; Fl_{EtOH}: 70% ethanolic extract from aerial parts of *T. patula*; $APWF_{EtOH}$: 70% ethanolic extract from aerial parts without flowers of *T. patula*. Values followed by the same letter do not show statistically significant differences by Tukey test ($p < 0.05$).

4. Discussion

During the last decades, the health professionals interest have been directed to natural therapies based on phytotherapics, not only in developing countries but also in global economic powers, which has attracted the attention of multinational pharmaceutical companies, encouraging the investment of millions of dollars in this research area. However, despite this expansion, there is still much to be done regarding to standardization, regularization and confirmation of the pharmacological activities of the great majority of natural products marketed.

Because the preponderant presence of flavonoids in the extract of *T. patula*, the quantifying of this important class of secondary metabolites was performed in this study, using a colorimetric assay of complexation of aluminum chloride with flavonoid nucleus of the compounds present in the sample [34]. This assay is accurate and reproducible method, providing very small or no deviations between a test and another with the same samples. In our work, the results obtained with the 70% ethanolic extract of *T. patula* are similar that found in other studies with plants of the same genus. Our results are quite superior of other plant species, for example, the value of total flavonoid content of 31.15 mg C/g (milligrams of catechin per gram of dry extract) provided by Hajimahmoodi et al. (2008) [35] for *Punica granatum* (Punicaceae). The search for the discovery of novel antioxidants compounds with natural origin to replace synthetics such as butylhydroxyanisole (BHA) and butylated hydroxytoluene (BHT) — used for preserving the quality and safety of food, drugs and cosmetics — is a scientific hotspot [36]. This demand is due to the fact that these synthetic antioxidants have a high volatility and instability at high temperatures, which present some toxicity and because they are less potent than the natural antioxidant agents [37,38].

In the present study, analysis of variance showed that the extracts tested presented similar values of antiradical activity when compared with each other and compared to pattern compounds (gallic acid, vitamin C, quercetin and rutin), highlighting the $Fl_{EtOH70\%}$, which showed antiradical activity higher than gallic acid, quercetin and rutin. For comparison, Li et al. (2007) [39] obtained with ethanolic extract of *Tagetes erecta* 93% of antiradical activity for Xinhong cultivar, however, for other cultivars the average activity was only 75.59%. The great antiradical potential presented by the extracts is related to its high content of flavonoids. It is generally assumed that the antioxidant or antiradical maximum activity of flavonoids is mainly due to the occurrence of the 2, 3 double bond in combination with the 4-keto group in the C ring, and the additional presence of hydroxyl groups at positions 3′, 4′ on B ring and hydroxy group in position 7 of the B ring [40]. Such interactions were observed in the compounds identified by Politi et al. (2012) [16] in ethanolic extracts of *T. patula*.

Considering the interest in use of *T. patula* extracts for preparation of acaricide topical formulations, it became imperative to determine the cytotoxicity. The results presented here indicate that the $Fl_{EtOH70\%}$ had the highest IC_{50}, however, at the highest concentration tested (2000 μg/mL) was as cytotoxic as the others. On average, up to 250 μg/mL, the only extract that showed cytotoxicity index higher than 50% was $AP_{EtOH70\%}$, showing 58% of cell lysis, with the lowest IC_{50}, characterizing it as the most cytotoxic sample. If we imagine the use of a product based on the *T. patula* extractives as an insecticide, to be sprinkled on the environment, significant results, as those mentioned by Dharmagadda et al. (2005) [41], could be achieved. However, if we develop a topical formulation to use in domestic animals, further studies with other cell strains would be necessary in order to ensure the security of your application.

For centuries, formulations containing flavonoids as the main pharmacological active constituents have been used to treat diseases. Antibacterial activity of flavonoids has been reported in several studies [42]. In the present work, the best results against the filamentous fungi were obtained with the $Fl_{EtOH70\%}$, probably due to its higher concentration of flavonoids, with MIC values ranging from 195.3 μg/mL to 572.9 μg/mL against *M. canis* and *T. mentagrophytes*, respectively. The $AP_{EtOH70\%}$ showed good antifungal activity against *T. rubrum* (40004), whereas Lima et al. (2009) [43], using methanolic extract of aerial parts of *Tagetes mendocina* found MIC values greater than 1000 μg/mL against *Mycrosporum gypseum*, *T. rubrum* and *T. mentagrophytes* strains. According to Rios and Recio (2005) [44], the presence of activity is very interesting in the case of concentrations below 100 μg/mL for extracts and 10 μg/mL for isolated compounds.

In addition, the antimicrobial activity of 70% ethanolic extracts of *T. patula* was evaluated against two strains of *B. bassiana* and two strains of *M. anisopliae*, with the objective of obtaining high MIC values, enabling, in further studies, a possible synergistic action of these samples with fungal suspensions. The pathogenicity of *M. anisopliae* fungus was observed in eggs and larvae of *Rhipicephalus microplus* [45], which was found high mortality rates. The *in vitro* action of the fungus *B. bassiana* for eggs of the same species of tick was also evaluated by Bittencourt et al. (1996) [46]. The authors observed that the percentage of hatching observed in the treated groups was lower than that observed in the control group. Garcia et al. (2004) [47] described the events involved in the mechanism of adhesion, penetration and colonization of adult engorged females of *R. sanguineus* by *M. anisopliae*. Prette et al. (2005) [24] found that *B. bassiana* were pathogenic for eggs, larvae and engorged nymphs of *R. sanguineus*, with higher efficiency pathogenic action as the concentration of spore suspension used to infect the different stages of the life cycle of the tick was increased. The results of our study are very promising, since, with the exception of the $APWF_{EtOH70\%}$ that showed MIC value of 625 μg/mL against *M. anisopliae* (OCF) and *B. bassiana* (4531), all other extracts had MIC values higher than 1250 μg/mL.

5. Conclusions

The high antioxidant potential presented by the samples, with percentages of antiradical activity similar to analyzed patterns, may be explained due to the high concentration of total flavonoids present in *T. patula*, especially in the flowers. No significant results against dermatophytes or against entomopathogenic fungi were obtained. In the second case, the result is interesting from the point of view of seeking a synergy of strains of *B. bassiana* and *M. anisopliae* with compounds of *T. patula*, in order to intensify the anti-tick potential of both, constituting a first step on this way. The high cytotoxic activity observed for these extractives in assays with murine macrophages suggest the application of products developed from these samples against ticks only on the environment, until further studies would be performed with other cell lines

especially dermal strains.

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Glossary

Dermatophyte a fungus belonging to the genera *Epidermophyton*, *Microsporum*, or *Trichophyton* with the ability to utilize keratin to infect hair, nail and skin.

Entomopathogenic fungi a fungus able of causing disease or kill insects.

Flavonoids secondary metabolites found in plants, including many different compounds, such as flavonols, flavones, isoflavones, catechins and anthocyanidins, which has antioxidant potential.

Ixodid a tick belonging to the family Ixodidae, also known as hard tick.

Macrophage any mononuclear phagocytic cell arising from monocytic stem cells in the bone marrow.

Minimum inhibitory concentration the smallest concentration of an antibiotic or other product that regularly inhibits growth of a microorganism *in vitro*.

Pearson linear correlation is a measure of the strength of the linear relationship between two variables. It can range from −1 to 1, indicate by ρ coefficient.

Percolation procedure of extraction of a plant material using a special apparatus through which a solvent is gradually released.

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