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Correlation of radical-scavenging capacity and amoebicidal activity of *Matricaria recutita* L. (Asteraceae)

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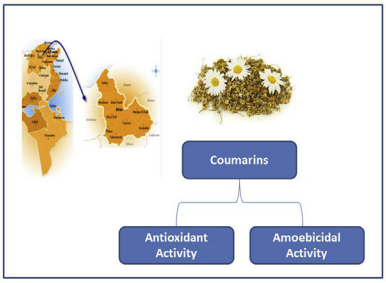
HIGHLIGHTS

- The evaluation of the antioxidant and anti-*Acanthamoeba* activity of chamomile extracts.
- Bio-guided fractionation was developed in order to identify and isolate the molecules responsible for the observed effects.
- Our results suggest coumarins from chamomile as a novel source of anti-*Acanthamoeba* compounds.

GRAPHICAL ABSTRACT



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ABSTRACT

Some Acanthamoeba strains are able to cause Granulomatous Amoebic Encephalitis (GAE) and *Acanthamoeba* keratitis (AK) worldwide because of their pathogenicity. The treatment of *Acanthamoeba* infections is complicated due to the existence of a highly resistant cyst stage in their life cycle. Therefore, the elucidation of novel sources of anti-*Acanthamoeba* agents is an urgent need. In the present study, an evaluation of the antioxidant and anti-*Acanthamoeba* activity of compounds in flower extracts of Tunisian chamomile (*Matricaria recutita* L.) was carried out. Chamomile methanol extract was the most active showing an IC_{50} of 66.235 ± 0.390 μg/ml, low toxicity levels when checked in murine macrophage toxicity model and presented also antioxidant properties. Moreover, a bio-guided fractionation of this extract was developed and led to the identification of a mixture of coumarins as the most active fraction. These results suggest a novel source of anti-*Acanthamoeba* compounds for the development of novel therapeutic agents against *Acanthamoeba* infections.

1. Introduction

Acanthamoeba is a widely distributed genus worldwide and it has been isolated from many environments such as water, soil, dust and many others (Lorenzo-Morales et al., 2015). To date, molecular

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classification of *Acanthamoeba* genus established 20 genotypes: T1-T20 that are based on the rRNA sequence of the isolates (Booton et al., 2005; Corsaro et al., 2015; Magnet et al., 2014; Nuprasert et al., 2010; Qvarnstrom et al., 2013). Of the 20 genotypes, T4 is the most abundant in the environment and includes many pathogenic strains that have been associated with lethal encephalitis and *Acanthamoeba* keratitis (AK) (Lorenzo-Morales et al.,. 2013; Siddiqui et al., 2012). Current therapeutic approaches against these infections are affected by drug resistance, variable efficacy between strains, toxic side effects and long course treatments. Therefore, there is a need to identify novel sources of drugs that are active against *Acanthamoeba*. Recently, agents with plant origins have been widely studied as a source of novel antiprotozoal drugs including *Acanthamoeba* (Derda and Hadaś, 2014; Lorenzo-Morales et al., 2015; Sifaoui et al., 2014).

Chamomile, (*Matricaria recutita* L.) has been included for centuries in the pharmacopoeia of several countries including Tunisia (Hajaji et al., 2017; Jabri et al., 2016; Sebai et al., 2014). Previous phytochemical screenings of this plant have discovered a high content of antioxidant and anti-inflammatory active molecules including phenolic compounds (McKay and Blumberg, 2006; Sebai et al., 2015). Many of these compounds are responsible for antimicrobial, larvicidal, anti-cancer and anti-inflammatory effects of chamomile reported previously (Miraj and Alesaeidi, 2016; Sakkas et al., 2016). Compounds showing these activities have been found to include coumarins, flavonoids, phenolic acids, and fatty acids among others (Morales-Yuste et al., 2010; Miraj and Alesaeidi, 2016; Sebai et al., 2015).

No previous studies have been carried out to evaluate the activity of this plant and its extracts against *Acanthamoeba*. Therefore, the aim of this study was to evaluate the antioxidant and anti-*Acanthamoeba* activities of flower extracts of Tunisian chamomile (*Matricaria recutita* L.), and to explore a possible correlation between these activities. After an initial screening of extracts, a bio-guided fractionation of the most active fraction was carried out to identify and isolate the major molecules responsible for these activities.

2. Materials and methods

2.1. Plant material

Chamomile flowers were collected in March 2013 from the region of Amdoun, Beja governorate (North-West of Tunisia, alt. 448 m; 36° 81′ N; 9° 05′E). Collected flowers were separated and thoroughly rinsed in running tap water and air dried for a period of 14 days. Finally, they were ground to a fine powder using a mill and stored at 4 °C until subsequent experiments were carried out.

2.2. Extract preparation

Chamomile extracts were prepared by maceration of 100 g of powdered plant material in 500 ml of extraction solvent (chloroform (99%), n-hexane (95%), water and methanol (99.9%). After 24 h of agitation at room temperature (20–25 °C, 3 × 500 ml) in the dark, the collected extracts were filtered using Whatman No 1 paper for three times. The solvents were removed by a rotary vacuum evaporator at 40 °C and finally the samples were weighed and stored at 4 °C until used.

The essential oil of *Matricaria recutita* L dried flowers was extracted by hydrodistillation using a Clevenger type apparatus. The volatile distillate was dried over anhydrous sodium sulfate and stored at 4 °C in a dark and sealed container prior to analysis.

2.3. Bioassay guided fractionation of chamomile

Matricaria recutita L. essential oil, aqueous and organic extracts were initially tested for their antioxidant and anti-*Acanthamoeba* activities using *Acanthamoeba castellanii* Neff (ATCC 30010), a type strain from the American Type Culture Collection (ATCC) was used in this study. The fractionation of the active extract was guided by the anti-amoebic activity.

Initially, 5 g of methanolic extract were diluted in water. The aqueous solution was fractionated by liquid-liquid bipartition using 450 ml of organic solvents (hexane, chloroform and ethyl acetate successively). The obtained organic phases were evaporated and weighed. The water phases were collected and lyophilized. The most active fractions were subjected to silica or Sephadex column and subanalysed by TLC and NMR (Fig. 1).

^{1}H (600 MHz) and ^{13}C (125 MHz) NMR spectra were recorded on a Bruker Avance 600 spectrometers; the chemical shifts are given in δ (ppm) with residual $CDCl_3$ (δ_H 7.26, δ_C 77.0) as internal reference and coupling constants in Hz; experiments were carried out with the pulse sequences given by Bruker. Silica gel 60 (particle size 15–40 and 63–200 μm, Macherey-Nagel) and Sephadex LH-20 (Pharmacia Biotech) were used for column chromatography, while silica gel 60 F_{254} (Macherey-Nagel) were used for analytical or preparative thin layer chromatography (TLC). Centrifugal preparative TLC was performed using a Chromatotron (Harrison Research Inc. model 7924T) on 4 mm or 1 mm silica gel 60 PF_{254} disks with flow rate 2–4 mL min^{-1}. The spots were visualized by UV light and heating silica gel plates sprayed with H_2O-H_2SO_4-$C_2H_4O_2$ (1:4:20). All solvents used were analytical grade from Panreac.

2.4. Antioxidant activities

2.4.1. $ABTS^{+}$ method

This assay is based on decoloration that occurs when the radical cation $ABTS^{+}$ is reduced to ABTS (2,20-azinobis-3-ethylbenzothiazoline-6-sulfonic acid). The radical was generated by reaction of a 14 mM solution of ABTS in water with 4.9 mM potassium persulphate (1:1). The mixture was then diluted with ethanol until an absorbance of 0.70 ± 0.02 at 734 nm. A volume of 20 μl of each sample solution at different concentrations was added to 180 μl of ABTS solution (Siddhuraju, 2006). The radical-scavenging activity is calculated as the inhibition percentage using the equation:

$$\%\text{inhibition} = ((A_C - A_S)/A_C)*100$$

A_C is the ABTS + solution absorbance at 734 nm and A_S is the sample absorbance at 734 nm.

2.4.2. DPPH- method

The DPPH assay (diphenyl-1-picrylhydrazyl) was performed according to the method of Brand-Williams et al. (1995). Briefly, 20 μL of various concentrations of extract or fractions were added to 180 ml of 6.10^{-5} mM methanol solution of DPPH and incubated at 25 °C during 60 min. DPPH radical scavenging activity (RSA), expressed as a percentage, was estimated using the same formula used in ABTS test.

2.4.3. FRAP method

The Ferric-reducing antioxidant power FRAP assay method measures the ability of antioxidants to reduce ferric-2,4,6- tripyridyl-s-triazine (Fe3+-TPTZ) to a ferrous form (Fe2+), which absorb at 593 nm. Briefly, a mixture of 0.3 mol/L acetate buffer (pH 3.6), 10 mmol/L TPTZ dissolved in 40 mmol/L hydrochloric acid, and 20 mmol/L ferric chloride (10:1:1 v:v:v) was prepared. 3.75 μl of

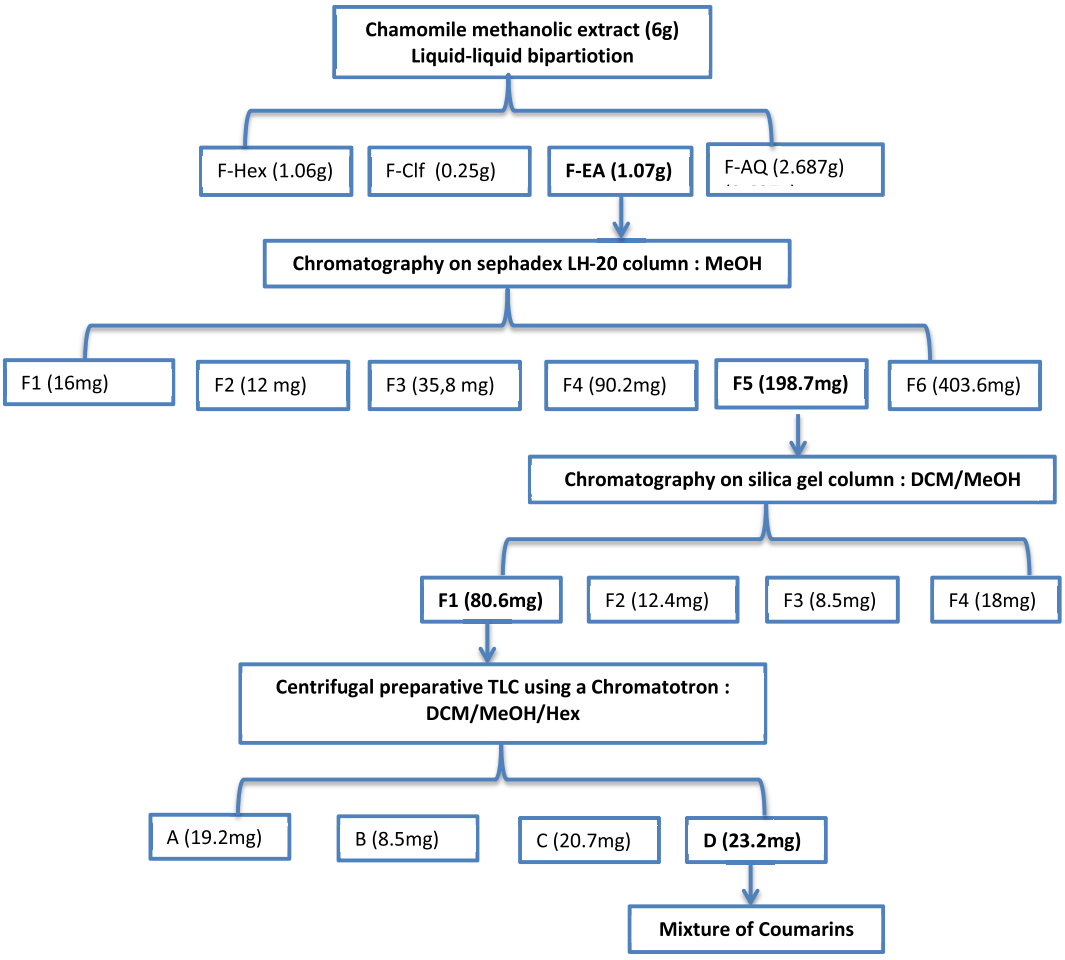

Fig. 1. Bio-guided assay performed for the evaluation of anti-Acanthamoeba compounds from the methanolic extract of chamomile.

samples at concentration of 25 μg/ml was added to 196.25 ml of FRAP reagent and incubated at 37 °C for 10 min (Benzie and Strain, 1996). The absorbance was measured at 593 nm and values were calculated based on a FeSO4 $7H_2O$ standard curve and were expressed as $FeSO_4$ equivalent (μM).

2.5. Assessment of anti-Acanthamoeba activity

2.5.1. Amoebic strain

Acanthamoeba castellanii Neff (ATCC 30010), a type strain from the American Type Culture Collection (ATCC) was used in this study. This strain was axenically grown in PYG medium (0.75% (w/v) proteose peptone, 0.75% (w/v) yeast extract and 1.5% (w/v) glucose) containing 40 μg gentamicin ml^{-1} (Biochrom AG, Cultek, Granollers, Barcelona, Spain) previous it use for the assays as previously described (Reyes-Batlle et al., 2014).

2.5.2. In vitro effect against the trophozoite stage of Acanthamoeba

The anti-*Acanthamoeba* activities of the molecules were determined using the Alamar Blue® assay as previously described (Martín-Navarro et al., 2008; Mc Bride et al., 2005). Briefly, *Acanthamoeba* strains were seeded in duplicate on a 96-well microtiter plate with 50 μl from a stock solution of 10^4 cells ml^{-1}. Amoebae were allowed to adhere for 15 min process which was checked using a Leika DMIL inverted microscope (Leika, Wetzlar, Germany). After that, 50 μl of serial dilutions of the tested extract was added to each well. Chlorhexidine was used as a positive control. Finally, the Alamar Blue Assay Reagent® (Life Technologies, Barcelona-Spain) was placed into each well at an amount equal to 10% of the medium volume. Test plates containing Alamar Blue were then incubated for 120 h at 28 °C with a slight agitation. Subsequently, plates were analyzed, during an interval of time between 72 and 144 h, with an Enspire microplate reader (PerkinElmer, Massachusetts, USA) using a test wavelength of 570 nm and a reference wavelength of 630 nm. Percentages of growth inhibition, 50% and 90% inhibitory concentrations (IC_{50} and IC_{90}) were calculated by linear regression analysis with 95% confidence limits. All experiments were performed three times each in duplicate, and the mean values were also calculated.

2.5.3. Cytotoxicity assay

For this test, the cell line J774A.1 (ATCC TIB-67) of murine macrophages was used. Initially, macrophages cultured in RPMI 1640 medium (Gibco/Life Technologies, Madrid, Spain) were counted and seeded in 96-well plates (10^5 cells/ml) and then the tested extract was diluted in the same medium and added in a total volume 100 μl in each well as previously described (Sifaoui et al., 2014). As a negative control, cells were incubated with medium alone. Finally, 10 μl of Alamar Blue was added into each well and the mixture was incubated for 24 h at 37 °C and 5% CO_2 atmosphere.

After that, the plates were analyzed using Enspire microplate reader (PerkinElmer, Massachusetts, USA) using a test wavelength of 570 nm and a reference wavelength of 630 nm. The toxicity values (calculated 50% cytotoxicity (CC_{50})) were calculated as using

Sigma Plot 12.0 statistical analysis software. The selectivity index was calculated using the IC_{50} for the trophozoite stage of *Acanthamoeba* and the calculated CC_{50}.

2.6. Statistical analysis

Obtained results were expressed as mean ± standard deviation. Analysis of variance was determined by one-way ANOVA using STAT- GRAPHICS Centurion XVI. Differences at $p < 0.05$ were considered statistically significant. The IC_s were calculated with Sigmaplot 12.0 software.

3. Results and discussion

3.1. In vitro activity of chamomile extracts against Acanthamoeba

Matricaria recutita L. extract, fractions and sub-fractions were screened for their activity against *Acanthamoeba castellanii* Neff. The IC_{50}/96 h was chosen as the appropriate and comparable data to give as previously described (Martín-Navarro et al., 2008; Sifaoui et al., 2013). These values are summarized in Table 1. The amoebicidal activity was observed in a concentration and dose dependent manner. The parasite was inhibited by the tested extracts with IC_{50s} ranging from 37.02 ± 0.64 μg/ml for the fraction D to 90.26 ± 0.05 μg/ml for the Chloroform extract. The Chlorhexidine was used as a positive control that has a good effect on *Acanthamoeba* with a low IC_{50} (2.643 ± 0.55 μg/ml). In comparison to other reports, *Matricaria recutita* L. extract exhibited a stronger activity against *Acanthamoeba*, For example, it has been reported that in the presence of 16 mg/ml of *Peucedanum longibracteolatum* methanol extract, no viable trophozoites were observed between 24 and 72 h (Malatyali et al., 2012). Some other authors observed the absence of viable trophozoites under 32 mg/ml of *Origanum syriacum* and *Origanum laevigatum* extract after 3 h of incubation (Degerli et al., 2012).

The bioassay guided fractionation of the ethyl acetate fraction of *Matricaria recutita* L yielded to an extract rich in coumarin type compounds which were identified by ^{1}H-NMR (Fig. 1). This phytochemical family has been already described in *Matricaria recutita* L. extract however their amoebicidal activity has never been reported before. Furthermore, toxicity assays revealed CC_{50} values of 127.01 μg/ml which is much higher than the concentration of the extract needed to eliminate *Acanthamoebae*. The selectivity index (SI) was calculated using the IC_{50} for the trophozoite stage of *Acanthamoeba* and the CC_{50} (SI = 3.43, see Table 1). Several reports have confirmed the antiprotozoal activity of the total coumarin fraction in plant extracts and several studies have demonstrated that coumarin (−) mammea A/BB isolated from *Calophyllum brasiliense* leaves inhibited the promastigotes of *Leishmania. (V.) braziliensis* via the mitochondrial pathway (Cardoso et al., 2016). Additionally, a coumarin (soulamarin) from the stem bark of *C. brasiliense* had reported activity against *Trypanosoma cruzi* by damaging the plasma membrane and mitochondrial function (Rea et al., 2013).

Using antiplasmodial activity bioguided fractionation Moon et al. (2011) could isolate two coumarin molecules (marmesinin and magnolioside) from the dried root of *Angelica gigas*. Both molecules were selectively active against *Plasmodium falciparum*. Beside the antiprotozoal activity, many studies have shown that coumarin possesses a wide range of pharmacologic and health promoting properties including anti-inflammatory, anti-coagulant, antiviral, antibacterial, antifungal, anticancer, antihypertensive, antitubercular and anticonvulsant (Venugopala et al., 2013).

Table 1
Anti-*Acanthamoeba* activity of chamomile extracts and fractions.

Extracts/Fractions	IC_{50} (μg/ml) at 96 h	CC50 (μg/ml)
Essential oil	123.274 ± 1.456	
Hexanic extract	88.674 ± 1.185	
Chloroformic extract	90.263 ± 0.048	
Methanolic extract	66.235 ± 0.390	
Aqueous extract	72.016 ± 0.233	
Fraction Hexane	137.316 ± 0.703	
Fraction Chloroforme	>100	
Fraction Ethyl Acetate	53.996 ± 0.780	
Fraction Aqueous	191.652 ± 1.209	
Fraction 1	123.325 ± 0.641	
Fraction 2	144.584 ± 0.540	
Fraction 3	156.809 ± 0.799	
Fraction 4	170.680 ± 0.669	
Fraction 5	80.708 ± 1.256	
Fraction 6	84.125 ± 0.976	
Fraction 5-F1	53.592 ± 0.668	
Fraction 5-F2	>100	
Fraction 5-F3	>100	
Fraction 5-F4	>100	
Fraction A	143.023 ± 2.002	
Fraction B	124.154 ± 1.458	
Fraction C	42.669 ± 0.683	
Fraction D	37.020 ± 0.636	127.002 **SI** = 3.430

3.2. Antioxidant activity

An antioxidant is known as a molecule that could stop or inhibit an oxidation process. This activity is in generally monitored by measuring the ability of an antioxidant to inhibit the oxidation process in a solution (Bardaweel et al., 2014).

The antioxidant capacities for the *Matricaria recutita* L., in this study were determined using three methods: DPPH, ABTS and FRAP. DPPH and ABTS are extensively used to determine the anti-radical activity of often samples. Those methods are based on the capability of an antioxidant to scavenge free radicals (Bardaweel et al., 2014). Table 1 illustrates the IC_{50} values. IC_{50} denotes the concentration of the sample required to inhibit 50% of free radicals. These values were obtained from the regression equations, plotting extract concentrations (μg/ml) against inhibition percentages of free radical formation in the different assays.

The DPPH free-radical scavenging method incorporates a free radical (DPPH•) that is capable of accepting hydrogen radicals from antioxidants in solution (Zhang et al., 2014). In its radical form, DPPH absorbs at 517 nm, in a presence of antioxidant this absorbance value decreases due to the reaction between antioxidant molecules and radical. This reaction could be observed by change of color from purple to yellow (Güder, 2016). As shown in Table 1, only methanolic extract shows a moderate activity with an IC_{50} less than 100 μg/ml (97.830 ± 2.620 μg/ml).

One of the most applied spectrophotometric methods to measure the total antioxidant activity of solution especially aliments and pure compounds is the ABTS. This technique is based on the generation of the ABTS radical cation forms (Re et al., 1999). When the chamomile extracts and essential oil were assayed with ABTS, although strong and significantly higher free radical scavenging activity was observed (IC_{50} = 22.647 ± 0.849 μg/ml) as the DPPH results only methanolic extract was active.

The FRAP assay was developed to measure the ferric reducing ability of biological fluids and aqueous solutions of pure compounds (Pulido et al., 2000). At low pH, an antioxidant reduce the ferric tripyridyltriazine (Fem-TPTZ) complex to the blue ferrous form, this reaction is measuring by spectrophotometer at 593 nm. As both previous methods, only methanolic extract reduces the Fem-TPTZ with an IC_{50} of 5.684 ± 0.328 (μmol Fe^{2+}/l). Our results

indicate that only methanolic extract showed an antioxidant capacity, this activity could be related to the highest concentration of phenolic compounds (data no shown).

The major phenolic fraction present in chamomile are flavonoids (quercetin, myricetin and rutin), coumarins (Herniarin) and phenolic acids (syringic acid, caffeic acid and ferulic acid) (Viapiana et al., 2016). Usually, most of those phenolics and flavonoids exhibit different degree of antioxidant activity. Accordingly, the extracts with strong antioxidant power would generally contain highest amount of phenolics or flavonoids. Several reports suggested correlation between all these parameters (Akkari et al., 2016; Liu et al., 2008; Sun and Ho, 2006; Pyo et al., 2004). Viapiana et al. (2016) reported a strong positive correlations between the antioxidant activity of methanolic chamomile extract, measured with DPPH and FRAP and the amount of caffeic, ferulic and syringic acids, myricetin and quercetin.

Evaluating the antiradical activity with different test systems using DPPH and ABTS give us information on the radical scavenging or antiradical activity. The antioxidant activity of the studied plant was evaluated much better with the ABTS system; in fact our results are in agreement with new findings on the one of the major compound of chamomile, chamazulene. Capuzzo et al. (2014) reported that the scavenging activity of the chamazulene measuring with ABTS was highest than DPPH.

During the bipartition step, the antioxidant activities depend especially from the type of solvent used. As shown in Table 2, the ABTS scavenging activity of different fractions was found to occur in the following order: Ethyl Acetate Fraction > Hexanic Fraction > Chloroformic Fraction > Aqueous Fraction. The ethyl acetate fraction showed the highest activities for both anti-amoeba and antioxidant and was further submitted to column chromatography. As shown in the Fig. 1, six fractions were collected. The antioxidant activities of the obtained fractions and sub-fractions were monitored during the fractionation steps. Among the obtained sub-fractions, number 5 was the most active extract and was further fractionated by different type of chromatography and analyzed by NMR. The result fraction was a mix of coumarin molecules. A decrease in IC_{50} value was observed during the fractionation process for all the test systems ABTS, DPPH and FRAP. Several reports, demonstrate the diverse pharmaceutical and biological activities namely antitumor, anti-HIV therapy, as CNS-stimulants, antibacterial, anticoagulants, antifungal, antioxidant (Salem et al., 2016).

3.3. Synergetic effect and correlation

The relative amoebicidal activity of *Matricaria recutita* L. extracts, fractions and sub-fractions against *Acanthamoeba castellanii.* Neff, was correlated to the antioxidant activity measured with ABTS and DPPH, with a regression coefficient of 0.6259 and 0.6537 ($P < 0.001$) respectively.

During the purification process a decrease in IC_{50} value was observed during the fractionation process for all both antioxidant and amoebicidal activities. The purification assay used in this study significantly ($p < 0.05$) efficient resulting in an increase of the antioxidant and amoebicidal activities compared with the crude extract (IC_{50} for ABTS = 22.647 ± 0.849 μg/ml and IC_{50} for anti-amoeba = 37.020 ± 0.636 μg/ml) (Table 2). During this study we note the absence of the synergetic effect; in fact those activities could be related to specific molecules. Same observation was found by other authors, Oldoni et al. (2016) reported the efficiency of bioassay guided used to isolate antioxidants molecules from peanut skin. However, other studies have demonstrate that the fractionation of biologically-active crude extracts can lead to the loss of their original activity, due to the synergistically and additively effects among the present components. In our previous work with olive leaf extract we observed that the original crude extract (Ethyl acetate extract) exhibit a stronger amoebicidal activity than the pure compound isolated (Sifaoui et al., 2014).

4. Conclusion

In conclusion, the obtained results suggest that the total coumarin fraction possess a strong amoebicidal and antioxidant activity. To the best of our knowledge, this is the first report on the amoebicidal activity of *Matricaria recutita* L. These results suggest a potential source of new compounds to develop novel active principles against *Acanthamoeba*. However, further studies would be necessary to identify the individual bioactive coumarin species observed.

Table 2
Antioxidant activity of chamomile extracts and fractions.

Extracts/Fractions	ABTS IC_{50} (μg/ml)	DPPH IC_{50} (μg/ml)	FRAP (μmol Fe^{2+}/l)
Essential oil	>100	>100	–
Hexanic extract	>100	>100	–
Chloroformic extract	>100	>100	–
Methanolic extract	22.647 ± 0.849	97.830 ± 2.620	5.684 ± 0.328
Aqueous extract	>100	>100	–
Fraction Hexane	16.316 ± 1.666	>100	–
Fraction Chloroforme	22.029 ± 0.958	>100	–
Fraction Ethyl Acetate	2.623 ± 0.072	12.928 ± 0.643	64.725 ± 0.610
Fraction Aqueous	29.767 ± 0.795	58.565 ± 1.152	1.342 ± 0.335
Fraction 1	31.640 ± 1.417	>100	–
Fraction 2	68.467 ± 1.374	>100	–
Fraction 3	>100	>100	–
Fraction 4	52.270 ± 0.991	>100	–
Fraction 5	3.363 ± 1.157	6.417 ± 1.006	91.084 ± 0.147
Fraction 6	6.975 ± 0.766	14.539 ± 0.623	23.202 ± 2.811
Fraction 5-F1	3.592 ± 0.668	15.926 ± 2.181	28.526 ± 0.571
Fraction 5-F2	4.288 ± 0.654	17.402 ± 0.370	11.902 ± 0.689
Fraction 5-F3	8.662 ± 0.643	7.204 ± 0.194	13.857 ± 0.559
Fraction 5-F4	14.540 ± 1.676	11.551 ± 0.790	14.801 ± 0.492
Fraction A	6.538 ± 0.859	16.871 ± 0.450	27.098 ± 0.305
Fraction B	7.318 ± 0.930	14.651 ± 0.631	11.677 ± 2.331
Fraction C	9.799 ± 0.792	18.133 ± 0.890	12.527 ± 0.492
Fraction D	2.575 ± 0.490	7.575 ± 0.490	48.462 ± 0.828

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