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Full Length Research Paper

Determination of antioxidant, radical scavenging activity and total phenolic compounds of *Artocarpus heterophyllus* (Jackfuit) seeds extracts

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Plants and their parts are a part of life in many brazilian communities, as observed in the jackfruit. The jackfruit seeds are consumed usually as roasted, boiled, steamed, and are eaten as a snack. The present study was carried out to identify the antioxidant, radical scavenging activity and total phenolic compounds of *Artocarpus heterophyllus* seeds using different screening tests. Total antioxidant activity was determined by ferric thiocyanate method (FTC), which was shortly followed by complexing of fosfomolybdenium method (FSB) determined in sequence with total reductive capability (TRC), hydrogen peroxide scavenging activity (HPS), DPPH free radical scavenging activity, and Total phenolic content (TPC) by Folin-Ciocalteu reagent assay method. The percentage of inhibition linoleic acid peroxidation by CEE (ethanolic extract), HF (hexanic extract), CHLF (chloroform extract) and EAF (ethyl acetate extract) (90.53, 70.23, 68.72 and 92.03%, respectively), in method using prussian blue antioxidant potential for EAF (89,05%) presented antioxidant activity greater than the standard rutin (82.3%). Highest total antioxidant activity was exerted by the ethanolic extract of *A. heterophyllus* seeds, and lowest by its EAF (188.83%). In evaluating the antioxidant activity by the hidrogen peroxide scavenging activity, it was observed that the CHLF and HF extracts showed scavenging activity above 50% (83.3 and 83.5%, respectively). The EC_{50} (free radical scavenger) values of CEE, HF, CHLF and EAF were 76.71, 399.64, 534.83 and 65.51 µg/ml, respectively, and the highest total phenolic content appeared to be present in 125 µg/ml of EAF. The results of all the sources were found to be highly significant. Further investigation for isolation and identification of the phytoconstituents responsible for antioxidant activity is desirable. *A. heterophyllus* seeds in the regular diets could improve the antioxidant status of human beings.

Key words: Jackfruit, antioxidant activity, DPPH radical, radical scavenging, seeds.

INTRODUCTION

Jackfruit (*Artocarpus heterophyllus* L.) is a shrub belonging to Moraceae family and is widely distributed in tropical countries such as Brazil, Thailand, Indonesia, India, Philippines and Malaysia (Chowdhury et al., 1997). Jackfruit is composed of several berries of yellow pulp and brown seeds encased in a hard shell and are rich in carbohydrates, complex B vitamins and minerals (Silva et al., 2007). Jackfruit is 2 to 4 cm long, and a fruit can

contain between 100 to 500 seeds. The seeds are consumed usually as roasted, boiled, steamed, and eaten as a snack. However, fresh seeds have short shelf-life.

To explore the health-promoting functionalities of locally available, culturally acceptable, and economically viable Brazilian foods, it is important to focus on their bioactive compounds. Among the various bioactive substances, phenolic compounds are the most abundant antioxidants in commonly consumed foods of plant origin.Cellular oxidants, called reactive oxygen species (ROS), are constantly produced in animal and human cells and play dual role of being both deleterious and beneficial to biological systems (Rudolf, 2001). Apart from their role in the diseased conditions in the body, ROS are also known to have a role in the spoilage of food by the autoxidation of lipids, the enzymatic oxidation, during storage and processing in fats, oils and fat-containing foods (Matthaus, 2002). Antioxidants are defined as substances present at low concentration, relative to the oxidizable substrate, which significantly delay or prevent oxidation of substrate (Godfraind, 2005). In the body, antioxidants act as free radical scavengers and thus protect cells from being exposed to free radicals and further cellular damage. This is the mechanism by which they protect the human body from several diseases attributed to the reactions of radicals.

The oxidative stress leads to serious damage to macromolecules, such as proteins and DNA. However, free radical production can be eliminated by the action of endogenous enzymes, as well as by the synthetic antioxidant activity (Halliwell 1990, 1997). Antioxidants act by several mechanisms, including the prevention of transition metal ions- chelation catalysts, peroxidase decomposition and free radicals elimination (Valko et al., 2006).

The addition of jackfruit seed flour in the preparation of biscuits, sweets and breads has been investigated as an alternative use of this by-product (Aldana et al., 2011; Bobbio et al., 1978; Mukprasit and Sajjaanantakul, 2004). Therefore, in the present study, the seeds of *A. heterophyllus* were evaluated for their antioxidant activity.

MATERIALS AND METHODS

Chemicals

Linoleic acid, α-tocopherol, butylated hydroxyanisole (BHA), butylated hydroxytoluene (BHT), nitroblue tetrazolium (NBT), phenazine methosulphate (PMS), the stable free radical 1,1-diphenyl-2-picryl-hydrazyl (DPPH·), 3-(2-pyridyl)-5,6-bis (4-phenyl-sulfonic acid)-1,2,4-triazine (Ferrozine), trichloroacetic acid (TCA) and polyoxyethylenes orbitan monolaurate (Tween-20) were obtained from Sigma (Sigma-Aldrich, Brazil). Ammonium thiocyanate was purchased from Merck. All other chemicals used were of analytical grade and obtained from either Sigma-Aldrich or Merck.

Plant materials and extraction procedures

Seeds of *A. heterophyllus* were collected in Arapongas - Brazil, in September, 2014. The material was identified by the botanist José Tadeu Weidlich Motta of the Municipal Botanical Museum of Curitiba and the exsiccate deposited under the number 397798. After drying in the shade, 80 g of seeds were submitted to ethanol extraction, following by liquid-liquid partition with hexane, cloroform and ethyl acetate, using Sohxlet, as per methodology described in previous studies (Andrade et al., 2005, 2007; Carvalho et al., 2009). After the evaporation of the solvents under reduced pressure and temperature of 40°C, a 0.53 g of hexane fraction, 1.89 g of ethyl acetate fraction, 0.3 g of cloroform, and 0.11 g of ethanol fraction were obtained.

Total antioxidant activity determination by ferric thiocyanate method (FTC)

The antioxidant activity of ethanolic extract (CEE), hexanic extract (HF), chloroform extract (CHLF) and ethyl acetate extract (EAF) and standards was determined according to the ferric thiocyanate method, with modifications (Mitsuda et al., 1996). For preparation of stock solutions, 2 mg of each CEE, HF, CHLF and EAF was dissolved in 10 ml water. HF and CHLF was previously solubilized in methanol. Then, 250 µl of the solution, which contained concentrations of stock CEE, HF, CHLF and EAF solution (200 µg/ml) or standard samples (200 µg/ml) in 2 ml of potassium phosphate buffer (0.04 mol/L, pH 7.0), was added to 550 µl of linoleic acid emulsion in potassium phosphate buffer (0.04 mol/L, pH 7.0), ethanol (PA) 250 µl, and 900 µl of water was added. The mixed solution (3.95 ml) was incubated at 50°C in a polyethylene flask. The peroxide level was determined by reading the absorbance at 500 nm in a spectrophotometer (Bio-Crom GmbH, Zurich, Switzerland) after reaction with $FeCl_2$ and thiocyanate at intervals during incubation. During the linoleic acid oxidation, peroxides are formed, which oxidize Fe^{+2} to Fe^{+3}. The latter ions form a complex with thiocyanate and this complex has a maximum absorbance at 500 nm. Five (5 ml) linoleic acid emulsion contained 17.5 µg Tween-20, 15.5 µl linoleic acid and 0.04 M potassium phosphate buffer (pH 7.0). On the other hand, the 5 ml control was composed of 2.5 ml linoleic acid emulsion and 2.5 ml 0.04 M potassium phosphate buffer (pH 7.0). This step was repeated every 5 h until the control reached its maximum absorbance value. Therefore, high absorbance indicates a high linoleic acid emulsion oxidation. Solutions without added extracts were used as blank samples. All data on total antioxidant activity is the average of triplicate analyses. The percentage inhibition of lipid peroxidation in linoleic acid emulsion was calculated by following equation.

Inhibition of lipid peroxidation Sample Control (%) $=100 \times (A_{control} - A_{sample}) / A_{control}$

Where $A_{control}$ is the absorbance of the control reaction and A_{sample} is the absorbance in the presence of the sample of CEE, HF, CHLF and EAF or standard compounds.

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Total reductive capability (TRC)

The reducing power of CEE, HF, CHLF and EAF was determined by the method of Yen and Chen (1995). HF and CHLF was previously solubilized in methanol. Concentrations of CEE, HF, CHLF and EAF (200 µg/ml) in 1 ml of distilled water were mixed with phosphate buffer (2.5 ml, 0.2 M, pH 6.6) and potassium ferricyanide [$K_3Fe(CN)_6$] (2.5 ml, 1%). The mixture was incubated at 50°C for 20 min. Aliquots (2.5 ml) of trichloroacetic acid (10%) were added to the mixture, which was then centrifuged for 10 min at 1000 × g (MSE Mistral 2000, UK). The upper layer of the solution (2.5 ml) was mixed with distilled water (2.5 ml) and $FeCl_3$ (0.5 ml, 0.1%) and the absorbance was measured at 700 nm in a spectrophotometer. Increased absorbance of the reaction mixture indicates increased reducing power.

Evaluation of antioxidant activity by complexing fosfomolybdenum (FSB) method

This method, described by Prieto et al. (1999) is based on the reduction of molybdenum (VI) to molybdenum (V) which occurred in the presence of certain substances with antioxidant capacity, with formation of a complex between phosphate green/molybdenum (V), acid pH, which is determined spectrophotometrically at 695 nm. The phosphomolibdenium complex was formed by the reaction of Na_3PO_4 solution (28 ml, 0.1 mol/L) with solution of $(NH_4)6Mo_7O_{24}.4H_2O$ (12 ml, 0,03 mol/L) H_2SO_4 solution (20 ml, 3 mol/L) in an aqueous medium. The final volume was adjusted with distilled water to 100 ml, and the yellow color turned green as it reduced. For the preparation of the phosphomolibdenium complex, all reagents were transferred into a volumetric flask and the volume was completed to 100 ml with distilled water. CEE, HF, CHLF and EAF were prepared in aqueous solutions with final concentration of 200 µg/ml, using a minimum amount of methanol to dissolve the sample when necessary. Of these, 0.3 ml were taken and added to 1 ml of reagent solution fosfomolibdenio complex.

The tubes were closed and kept in a water bath at 95°C for 90 min. After cooling, the reading was carried out at 695 nm in a Shimadzu UV-1601 spectrophotometer, to obtain the absorbance, using distilled water as the blank. The antioxidant capacity of the samples was expressed relative to ascorbic acid (200 µg/ml) which was used as standard and rutin (200 µg/ml) whose antioxidant activity was considered a reference. Ascorbic acid is one of the most effective and best known biological antioxidant (Darr et al., 1996). For this test we used the following samples in triplicate: ethanol crude extract, and fractions hexane, chloroform and ethyl acetate.

The results of the evaluation of the antioxidant activity were expressed as relative antioxidant activity AAR% (ascorbic acid) and AAR% (rutin). The calculations were established by means of the equations:

$$AAR\%\ (\text{Ascorbic acid}) = 100 \times (A_{sample} - A_{blank}) / (A_{ascorbic\ acid} - A_{blank})$$
$$AAR\%\ (\text{rutin}) = 100 \times (A_{sample} - A_{blank}) / (A_{rutin} - A_{blank})$$

Hydrogen peroxide scavenging activity (HPS)

The hydrogen peroxide scavenging ability of CEE, HF, CHLF and EAF was determined according to the method of Ruch et al (1989). A solution of H_2O_2 (40 mM) was prepared in phosphate buffer (pH 7.4). Extracts (500 µg/ml) in distilled water, using as amount minimum methanol to dissolve the sample were added to a H_2O_2 solution (0.6 ml, 40 mM). The absorbance value of the reaction mixture was recorded at 230 nm. Blank solution contained the phosphate buffer without H_2O_2. The percentage of H_2O_2 scavenging of CEE, HF, CHLF and EAF and standard compounds was calculated as:

$$\%\ \text{Scavenging effect} = 100 \times (A_{control} - A_{sample}) / A_{control}$$

Where $A_{Control}$ is the absorbance of the control, and A_{Sample} is the absorbance in the presence of the sample of CEE, HF, CHLF and EAF or standards (Gülçin et al., 2003).

1,1-Diphenyl-2-picryl-hydrazyl (DPPH) free radical scavenging activity

The free-radical scavenging capacity of CEE, HF, CHLF and EAF was evaluated with the DPPH· stable radical following the methodology described by Blois (1958). This method is described extensively elsewhere (Elmastas et al., 2006), wherein the bleaching rate of a stable free radical, DPPH· is monitored at a characteristic wavelength in the presence of the sample. In its radical form, DPPH was absorbed at 517 nm, but upon reduction by an antioxidant or a radical species, its absorption decreased (Elmastas et al., 2006). Briefly, 0.1 mmol/L solution of DPPH· in ethanol was prepared and 1 ml of this solution was added to 3 ml of CEE, HF, CHLF and EAF solution in water at different concentrations (25 to 500 µg/ml). Thirty minutes later, the absorbance was measured at 517 nm. A lower absorbance of the reaction mixture indicates higher free radical scavenging activity. The DPPH· concentration (mM) in the reaction medium was calculated from the following calibration curve, determined by linear regression (R2 = 0.990):

$$\text{Absorbance} = 9.2872 \times [\text{DPPH}\cdot] + 0.097$$

The capability to scavenge the DPPH· radical was calculated using the following equation:

$$\text{DPPH scavenging} = 100 \times (A_{control} - A_{sample}) / A_{control}$$

Where $A_{Control}$ is the absorbance of the control reaction and A_{Sample} is the absorbance in the presence of CEE, HF, CHLF and EAF (Gülçin et al., 2005).

Total phenolic compounds (TPC)

The content of phenolic compounds in CEE, HF, CHLF and EAF was performed based on colorimetric Folin-Ciocalteu method (Kujala et al., 2000; Wu et al., 2005; Meda et al., 2005), with some modifications. Rates of CEE, HF, CHLF and EAF were diluted with distilled water to obtain concentrations of 25 to 100 mg/ml. To the 0.5 ml of each sample was added 0.5 ml of 2N Folin-Ciocalteau reagent and 1.0 ml of water. After a period of 2 to 5 min, to the tubes was added 0.5 ml of sodium carbonate (Na_2CO_3) at 10%. After 1 h incubation at room temperature, absorbance was measured in spectrophotometer at 760 nm using distilled water as the blank. Gallic acid (2.5 to 12.5 mg/ml) dissolved in distilled water was used for standardize the phenolic concentration curve preparation, and values were expressed as gallic acid equivalents (mg gallic acid/g sample).

Statistical analysis

All data on total antioxidant activity are the average of triplicate analyses. The data were recorded as mean ± standard deviation and analysed by Graph Pad Prism (version 6.0). Oneway analysis of variance was performed by ANOVA procedures. Significant differences between means were determined by Duncan's multiple range tests. Values of $p < 0.05$ were regarded as significant and $p < 0.01$ very significant.

Table 1. Antioxidant activity by complexing method fosfomolibdenio in the crude ethanolic extract (CCE), and hexanic (HF), chloroformic (CHLF) and ethyl acetate (EAF) extracts in *A. heterophyllus* seeds.

Parameter	AAR% rutin (x±dp)	AAR % ascorbic acid (x±dp)
CEE	701.00±4.52	219.62±1.41
HF	635.24±4.24	199.01±1.33
CHLF	607.29±4.69	190.26±1.47
EAF	602.74±2.34	188.83±0.73

RESULTS AND DISCUSSION

Results of different assays performed for all the extracts are presented in tables and graphs.

Total antioxidant activity determination by ferric thiocyanate method (FTC).

The antioxidant effects of *A. heterophyllus* seeds prevented the peroxidation of linoleic acid, as measured by thiocyanate method (Figure 1). We have found the anti-oxidative property of these four extracts (CEE, HF, CHLF and EAF). The percentage of inhibition linoleic acid peroxidation by CEE, HF, CHLF and EAF (90.53, 70.23, 68.72 and 92.03%, respectively) were simmilar with the pattern BHT (di-terc-butil metil fenol) (78.32%) as shown in Figure 1. These results demonstrate the importance of the extracts in preventing oxidation as substances such as cell membranes.

Methods to evaluate the lipid peroxidation are important since they measure the action of a free radical on cell membrane lipids, leading to destruction of its structure, the failure mechanism of exchange metabolites, and in an extreme condition, to cell death (Benzie, 1996). In the ferric thiocyanate method, the present peroxides oxidize Fe^{2+} to Fe^{3+}, which is determined by colorimetry (500 nm) as ferric chloride or thiocyanate (Silva et al., 1999). In this assay, hydroperoxides generated during the oxidation of linoleic acid react with ferrous sulfate to give ferric sulfate and then ferric thiocyanate and blood red color. The decrease in absorbance discloses interrupting the oxidation due to the unavailability of linoleic acid in the reaction medium and the appearance of secondary products derived from the degradation of hydroperoxides (Chen et al., 1996).

Total reductive capability (TRC).

The reduction of a capacity fraction serves as an indicator of its antioxidant potential. In evaluating the antioxidant activity by reducing power method (Prussian Blue), an antioxidant potential for EAF (89.05%) presenting antioxidant activity upper to the standard rutin (82.3%) was observed. The other extracts did not show 50% activity compared to Rutin, as showed in Figure 2.

Methods based on the reduction of $Fe^{3+,}$ determining the reducing power are also used to evaluate antioxidant activity. These methods assess the ability of phenolic compounds to reduce Fe^{3+}, with consequent formation of a colored complex with Fe^{2+} (Roginsky et al., 2005). The reducing power was observed by direct donation of electrons in the reduction of potassium ferricyanide $[Fe(CN)_6]^{3-}$ potassium ferrocyanide $[Fe(CN)_6]^{4-}$. The product was visualized by the addition of Fe^{3+} ions which form complex Prussian Blue, $Fe_4[Fe(CN)_6]^3$. This method is considered very effective when the substances present phenolic compounds. Thus this test is to quantify either total phenols, and to determine the antioxidant activity (Monteiro et al., 2005). *In vitro* studies indicate that phenolic compounds found in plants can effectively participate in processes that may have anti-carcinogenic implications. Among these processes, the most obvious is the antioxidant capacity of these compounds reducing power allocated to the aromatic hydroxyl group, which reduces reactive free radical, and radical fenoxila produces stabilized resonance. Antioxidant capacity of phenolic compounds is influenced by the number and position of the OH groups, as well as by glycosylation positions (Cerqueira, 2007).

The assay of reducing power becomes important in the analysis of substances for purposes of comparison with the activity of DPPH. The advantage of this test lies in the fact that trials in less soluble fractions of activity may have their particular activity. Huang et al. (2005) found that extracts obtained from edible fuits have excellent antioxidant power reduction, and considering that the reducing power of the ethyl acetate extract was superior to commercial standard rutin, it can be considered that this has a high antioxidant activity.

Evaluation of antioxidant activity by complexing method fosfomolibdenio (FSB)

Different extracts showed the total antioxidant activity in a wide range as shown in Table 1. Highest total antioxidant

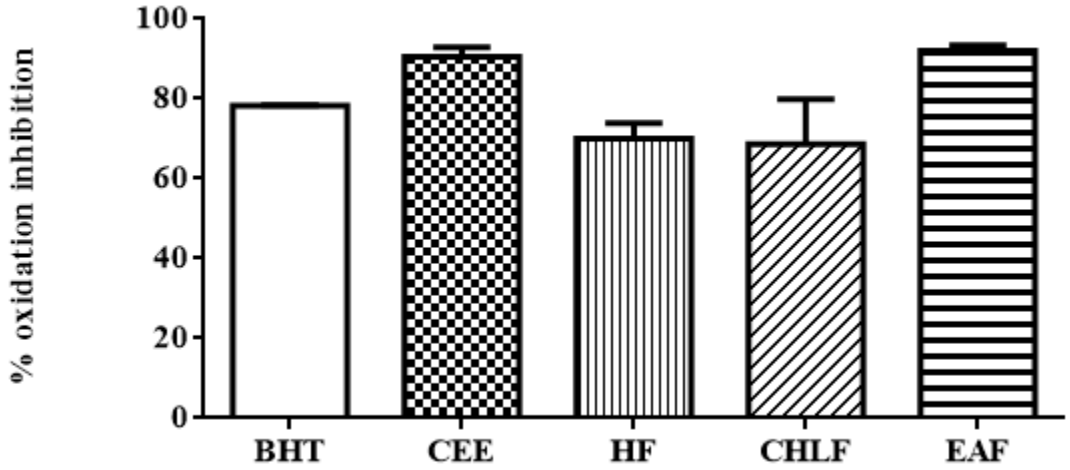

Figure 1. Antioxidant activity of standard BHT, crude ethanolic extract (CCE), and hexanic (HF), chloroformic (CHLF) and ethyl acetate (EAF) extracts in *A. heterophyllus* seeds by thiocyanate method. None of statements presented statistical difference when compared to standard bht. Dunnet test ($p < 0.05$).

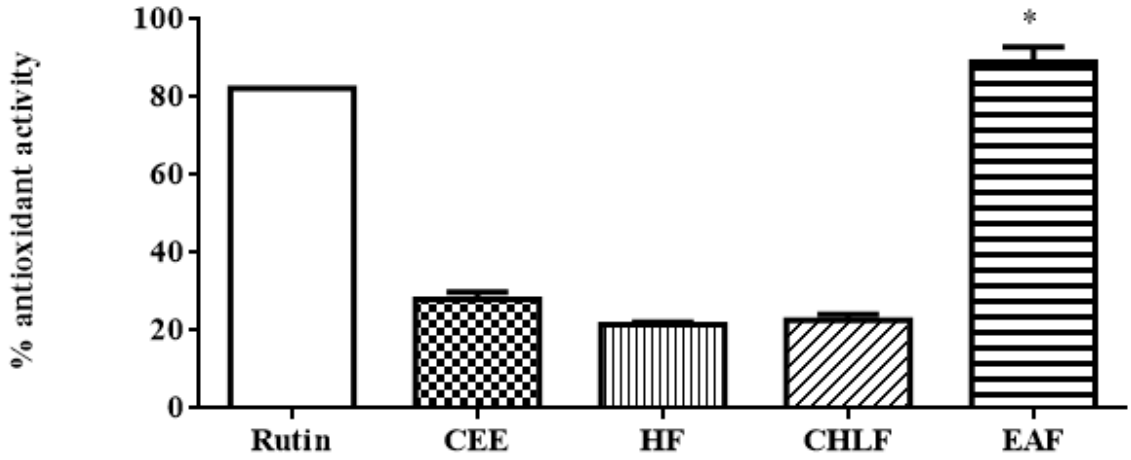

Figure 2. Antioxidant activity of pattern Rutin, crude ethanolic extract (CCE), and hexanic (HF), chloroformic (CHLF) and ethyl acetate (EAF) extracts in *A. heterophyllus* seeds by reducing power (Prussian blue). *significantly different when compared to Rutin. Dunnet test ($p < 0.05$).

activity was exerted by the ethanolic extract of *A. heterophyllus* seeds, and lowest by its EAF (188.83%). All extracts showed that close activities were all included possible antioxidants. Ascorbic acid (200 µg/ml), used as a positive control, registered an activity 100%. In the test of the antioxidant fosfomolibdenio, the results indicate that all extracts from *A. heterophyllus* seeds, to reduce the molybdenum VI to molybdenum V, can inhibit the action of xanthine oxidase. Xanthine oxidase (XO) is the enzyme responsible for processing both hypoxanthine to xanthine to uric acid, as this, in the physiological environment, is in the form of urate. The hypoxanthine and xanthine are much more soluble than uric acid and the latter may deposit as sodium urate, a major contributor to the development of the condition known as gout (Tsutomu et al., 1991). Thus, extracts and fractions which had antioxidant activity by this method may have their activities investigated in the treatment of gout disease.

Hydrogen peroxide scavenging activity (HPS)

In evaluating the antioxidant activity by the hydrogen peroxide scavenging activity, it was observed that the CEE and EAF extracts did not show activity similar to

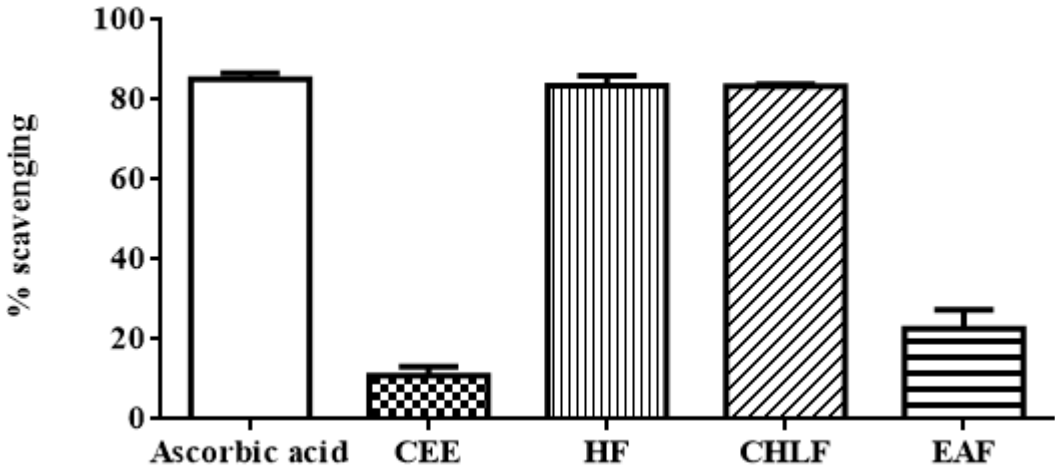

Figure 3. Antioxidant activity by , crude ethanolic extract (CCE), and hexanic (HF), chloroformic (CHLF) and ethyl acetate (EAF) extracts in *A. heterophyllus* seeds compared with Ascorbic acid by H_2O_2 scavenging method. Dunnet test ($p < 0.05$).

standard ascorbic acid. However, the CHLF and HF extracts showed scavenging activity above 50% (83.3 and 83.5%, respectively) (Figure 3). Although hydrogen peroxide is not a free radical, it is involved directly or indirectly in different pathologies. In chemical terms, H_2O_2 is poorly reactive, however it plays an important role in oxidative stress by being able to cross cellular membranes easily and generate the hydroxyl radical (•OH) (Aruoma et al., 1989). Hidrogen peroxide can readily cross cell membranes and once inside the cell, the hydrogen peroxide can react with Fe^{2+} ions to form hydroxyl radicals which may cause toxic effects. Therefore, it is advantageous to biologically cells which control the amount of hydrogen peroxide (Nagulendran et al., 2007). Thus, the fractions of CHLF and HF with higher antioxidant activity have shown to be promising in the study of antioxidant activity in the scavenging of H_2O_2.

1,1-Diphenyl-2-picryl-hydrazyl (DPPH) free radical scavenging activity

DPPH, a stable free radical with characteristics of absorption at 515 nm, was widely utilized to appraise the radical scavenging activity of extracts. Highest and lowest activity was exerted, respectively, in EAF and HF extract of *A. heterophyllus* seeds (Figure 4). Extracts prepared in polar solvents are better free radical scavengers than those prepared in less polar solvents, as evident from the fact that hexane extracts exhibited least DPPH scavenging ability. The potency of the extracts in scavenging the radicals is due to the number of hydrogens available for donation by the hydroxyl groups (Chen and Ho, 1995). Higher activity of the EAF extract may be due to the presence of high hydroxyl groups, which is proportionate to their phenolic content. Free radicals act as a trigger to a number of degenerative diseases. Therefore, samples having free radical scavenging activity can be of potent medicinal importance. The significant increase in DPPH free radical scavenging power of CEE, HF, CHLF and EAF were observed in a concentration-dependent fashion (Figure 4), confirming the free radical scavenging activity of *A. heterophyllus* seeds. The EC_{50} (free radical scavenger) values of CEE, HF, CHLF and EAF were 76.71, 399.64, 534.83 and 65.51 µg/ml, respectively. Ascorbic acid, a potent antioxidant, had lower EC_{50} value (4.92 µg/ml) than EAF. Ascorbic acid and rutin was used as a positive control.

Total phenolic compounds (TPC)

Plant phenolics present in fruits and vegetables have received considerable attention because of their potential antioxidant activity. Total phenolic content of different extracts was recorded in the range of 3.034 to 5.72 µg GAE of samples tested. The highest total phenolic content appeared to be present in 125 µg/ml of EAF. The concentrations of 25 and 50 µg/ml of the samples appeared to be poor in extracting phenolics. Fruits contain phytochemicals which have antioxidants known as polyphenols, which can strengthen the defense response in the body through different antioxidant mechanisms such as eliminating harmful free radicals in cells and likely tissues. Consequently, the antioxidant activity of a fruit is an important parameter to consider for the establishment of its nutritional value (Rice-Evan and Miller, 1996).

Epidemiological research has established an association between high intake of polyphenols derived

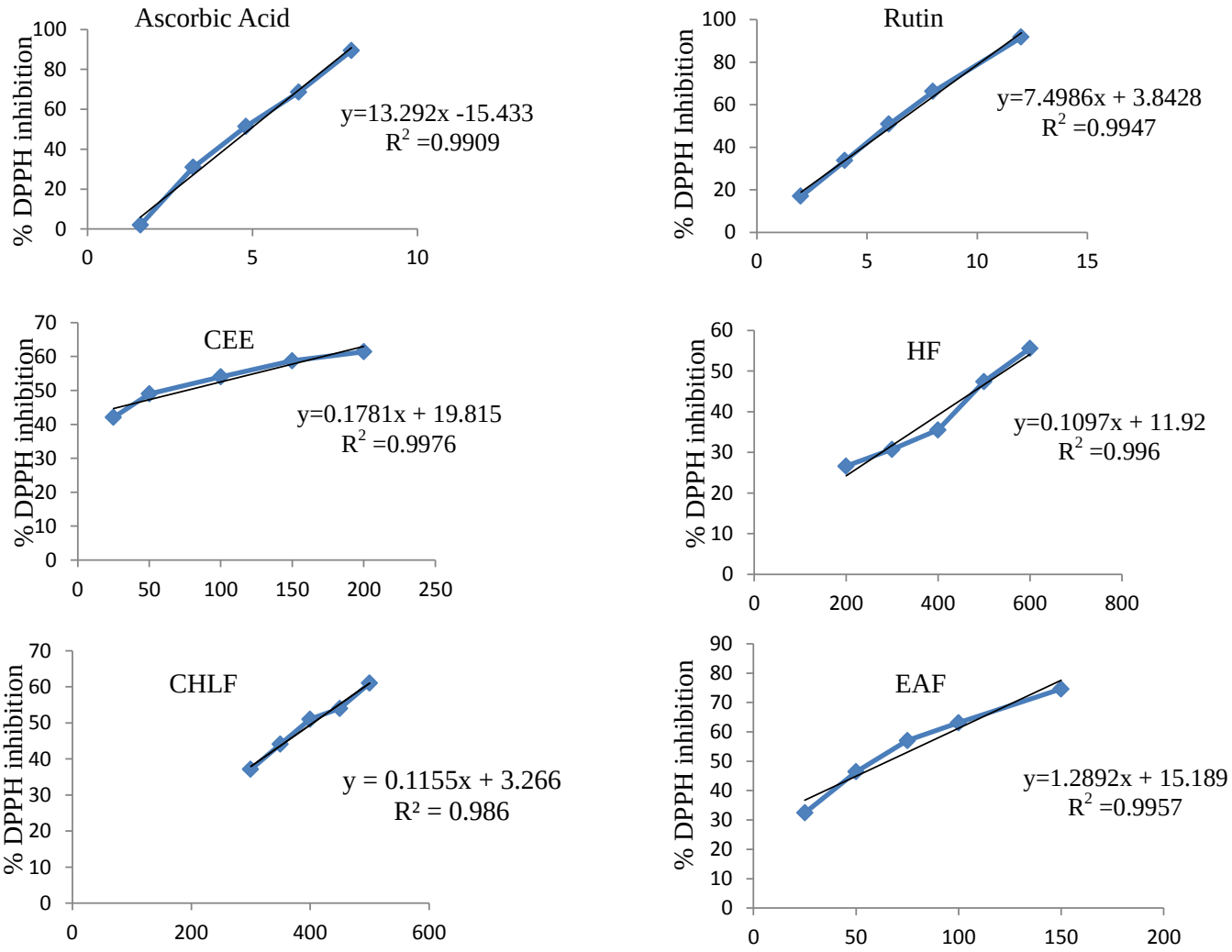

Figure 4. Curves of Ascorbic acid, Rutin, crude ethanolic extract (CCE), and hexanic (HF), chloroformic (CHLF) and ethyl acetate (EAF) extracts in *A. heterophyllus* seeds in DPPH inhibition.

from fruit and a reduced risk of certain cancers and cardiovascular disease (Fuhrman et al., 1995; Potter, 2005). These compounds exert their bioactivity of protection due to its antioxidant capacity. Polyphenols can wipe out the spread of harmful free radicals to the body, which causes cellular lipid peroxidation by transferring an equivalent electron radical. Although the role that these substances play in maintaining health are neglected in most food composition surveys. Thus, these studies show that, possibly the polyphenols present in the samples tested exert the different antioxidant activity and may act in the scavenging of H_2O_2, inhibition of peroxidation rate of lipids and inhibition of xanthine oxidase. Subsequent isolation studies and identification of chemical substances with antioxidant capacity should be investigated.

Other species of the *Artocarpus* genus have this parts studied for antioxidant activity, but the parts studied were leaves and fruit. *A. heterophylus* exhibited significant antioxidant activity studied its pulp presenting antioxidant capacity against DPPH radical, ferric reducing power and radical DMPD was studied. The jackfruit is a rich source of phytochemicals including phenolic compounds and offers opportunities for development of value-added products from jackfruit (Jagtap et al., 2010).

Conclusion

The results of the present study indicated that *A. heterophyllus* seeds showed antioxidant activity. The recorded remarkable antioxidant property in terms of reducing power, DPPH and superoxide radical scavenging activities indicate that incorporation of the seeds with food is complementary. Finally, this study has identified that CHLF and CEE extract of *A. heterophyllus*

seeds has high antioxidant activity (attributable to its high phenolic content), and EAF and CHLF extracts has high radical scavenging activity. The presence of antioxidant activity in extracts prepared in all the different solvents (which differ widely in their polarity) indicate that phytochemicals contributing to the antioxidant activity of the seeds tested belong to different groups of plant metabolites and varies widely with respect to their chemical properties. Further investigation for isolation and identification of the phytoconstituents responsible for antioxidant activity is desirable. *A. heterophyllus* seeds in the regular diets could improve the antioxidant status of human beings.

Conflict of Interests

The authors have not declared any conflict of interests.

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Essential oil of parsley and fractions to *in vitro* control of cattle ticks and dengue mosquitoes

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The essential oil of *Petroselinum crispum* was extracted by hydrodistillation and its chemical composition was determined by gas chromatography-mass spectrometry (GC/MS). The GC/MS method detected 31 constituents of the essential oil extracted from *P. crispum*, from which 26 were identified by the analysis. Phenylpropanoids were the major compounds, comprising 52.07% of the oil and consisting mainly of apiole (41.05%). The essential oil was subjected to classical chromatography and its four fractions (FR1, FR2, FR3 and FR4) showed acaricidal and larvicidal activity. The FR1 consists of myrcene (10.15%); limonene (9.72%); p-mentha-1,4,(8)-diene (20.99%) and p-mentha-1,5,8-triene (59.14%). The FR2 consists of ρ-cymen-8-ol (72.43%); α-terpineol (16.65%); trans-carveol (7.44%); citronellol (1.37%) and carveol (2.11%). The FR3 consists of myristicin (99.90%) and the FR4 of elemicin (1.87%); carotol (0.43%) and apiole (96.80%). Lethal concentrations (LC50 and LC99.9) of parsley's essential oil on engorged female of Rhipicephalus (Boophilus) microplus were of 73, 400 µg/ml and of 109, 760 µg/ml, respectively. The values of LC50 and LC99.9 were 0.82 and 9.62 µg/ml, respectively for the fraction FR4. The larvicidal activity against *Aedes aegypti* showed the best results in the fraction FR4, with values of LC50 and LC99.9 of 0.01 and 0.07 µg/ml, respectively. Results demonstrated that the parsley's essential oil and its major compound apiole are potentially useful in the bio-control of R. (B.) microplus and A. aegypti. This work should support complementary studies to explore the use of the parsley's essential oil in the bio-control of R. (B.) microplus and A. aegypti.

Key words: Apiole, phenylpropanoids, monoterpenes, essential oil, parsley, *Aedes aegypti*, Rhipicephalus (Boophilus) microplus, anticholinesterase.

INTRODUCTION

The veterinary industry is continuously challenged to develop new chemical solutions because of pests' resistance to anti-parasitic products. Acaricide resistance is a big issue to the Brazilian cattle livestock since ticks

are responsible for significant economic losses and transmission of hemoparasites (Olivo et al., 2008). The continuous use of chemical acaricide, such as pyrethroid, organophosphate, amitraz, ivermectin and fipronil (Kafle et al., 2012), has caused tick resistance. The resistance to chemical larvicides is also a serious problem in the combat of *Aedes aegypti* L. (Diptera, Culicidae). For the culicidae larva control, chemical insecticides such as temephos®, malathion® and fenitrothion® are the main products applied currently. These chemicals cause mosquito resistance repeatedly described in Brazil and worldwide (Magalhães et al., 2010). Furthermore, environmental impacts caused by these substances have demanded urgent alternatives for manufacturing commercial acaricides and larvicides with environmental appeal (Chagas et al., 2003).

The *Petroselinum crispum* (Mill.) Nym. ex A. W. Hill belongs to the Apiaceae family. It is a well-known spice plant widely cultivated in Brazil. The parsley is used in both the cosmetic industry and alternative medicine. The parsley's essential oil (EO) obtained from seeds and leaves has medicinal properties as anti-hepatotoxic, anti-hypertensive, anti-coagulant (Petropoulos et al., 2009), anti-microbial (Petropoulos et al., 2010) and anti-oxidant (Zhang et al., 2006). In the parsley's EO there are two main phenylpropanoids: apiole and myristicin (Petropoulos et al., 2009). Song et al. (2011) showed that apiole obtained from *Petroselinum sativum* seeds has high acaricidal activity against the house-dust and stored-food mites (*Dermatophagoides farinae*, *Dermatophagoides pteronyssinus* and *Tyrophagus putrescentiae)*. A number of phenylpropanoid derivatives (isoeugenol, eugenol and safrole) also had acaricidal activity against *D. farinae* and *D. pteronyssinus* (Chu et al., 2011). Ferraz et al. (2010) suggested that phenylpropanoids of the EO obtained from *Piper amalago*, *Piper mikanianum* and *Piper xylosteoides* were the main reason for acaricidal activity against *R. (B.) microplus*. However, there was no conclusive study on the acaricidal and larvicidal activities of apiole and phenylpropanoids against cattle ticks (*R.* (*B.*) *microplus*) and dengue mosquitos (*A. aegypti*).

Therefore, the aim of this study was to isolate the main chemical compounds of the EO of *P. crispum*, which is a source of phenylpropanoides and apiole, and evaluate its acaricidal and larvicidal activities against *R.* (*B.*) *microplus* and *A. aegypti*. Alternative solutions like the insect and tick control from the parsley's EO reported in this study are highly demanded for the production of pro-environmental products to control pests as well as to avoid pest resistance.

MATERIALS AND METHODS

Plant material and essential oil

P. crispum was identified and the exsiccate (numbered 192) was maintained at the Educational Herbarium of the Paranaense University (*Herbário Educacional da Universidade Paranaense* - HEUP). The crop was cultivated in the Medicinal Garden of the Paranaense University (*Horto Medicinal da Universidade Paranaense*), Umuarama city, Paraná, Brazil, at coordinates S 23° 46, 225' and WO 53° 16 730', at 391 m high. The planting occurred in January, 2013. Non-tillage in lines was the crop system used, which is recommended for short-cycle and medium-to-small species (up to 1 m height) like *P. crispum*. Three planting lines were used and the spacing between them was 0.40 m. Spacing between plants was 0.30 m. Three seeds were planted per hill at a depth of 0.02 m (Kurowska and Galaska, 2006). Plants were harvested at 30 cm height and at the peak of flowering, when the inflorescence was visible (Kurowska and Galaska, 2006). Aerial parts were cut at 2 to 3 cm above soil, yellow and crushed leaves were removed. Plant material was harvested in the morning, the dew and rainy days were avoided. Plant material was subjected to hydrodistillation in Clevenger apparatus by 2 h (Stankovic et al., 2004) and then the EO was removed from equipment by a Pasteur pipette, filtered with anhydrous sodium sulfate (NA_2SO_4) and stored in amber vials at -20°C.

Separation of essential oil compounds

The technique used to obtain the EO compounds was classical chromatography (CC) with silica gel 60 (0.063 to 0.200 mm) at 1:25 and pre-heated at 90°C for 45 min. The essential oil of *P. crispum* (2.0 g) was subjected to column chromatography (Diogolab, 30 × 3.5 cm) and sequentially eluted with hexane (100%), hexane: dichloromethane (7:3, 1:1 and 3:7), dichloromethane (100%), dichloromethane: ethyl acetate (7:3, 1:1 and 3:7), ethyl acetate (100%), ethyl acetate: methanol (7:3 1:1 and 3:7), and methanol (100%). Fractions (FRs) were concentrated in rotary evaporator (Tecnal TE-210) to reduce the volume to approximately 2 ml.

Chemical composition of essential oil

Chemical composition of EO and their FRs was obtained by GC-MS with 5973 N Mass Selective Detector and Agilent 6890N GC System equipped with a 5% phenylmethylsiloxane capillary column (DB-5) (30 m × 0.25 mm × 0.25 µm film thickness), using helium carrier gas at 4.8 ml/min and 2.1:1 split mode. The temperatures of transference line, ion source and quadrupole were maintained at 320, 230 and 150°C, respectively. The column was programmed to heat at 6°C/min, initially from 40°C to reach 300°C (maintained for 1 min). The total time of analysis was of 44.33 min. The mass spectra

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were obtained in a range of 50 to 550 (*m/z*) provided through scanmode with solvent delay time of 3.5 min. In addition to GC-MS results, eo chemical compounds were also identified by comparing retention indexes (RI) using a homologous series of n-alkanes (C7-C26). Mass spectra were then compared with the Wiley 275 L mass spectral library and literature (Adams, 2007).

Acaricidal effect on the *R. (B.) microplus*

The acaricidal effect of EO and FRs (FR1, FR2, FR3 and FR4) of *P. crispum* on *R. (B.) microplus* was determined by the adult immersion test (AIT) originally developed by Drummond et al. (1973); and also by the larval pocket test (LPT) described by Stone and Haydock (1962). The EO of *P. crispum* and its FRs were diluted in water solution added with 2% of polysorbate 80 (v/v). The 2% polysorbate 80 (v/v) solution was used for negative control. The positive control was made with a commercial acaricide containing 150,000 µg/ml of cypermethrin, 250,000 µg/ml of chlorpyrifos and 100,000 µg/ml of citronellal. The commercial acaricide was prepared in 2% polysorbate 80 at 1,250 µg/ml. In the AIT, approximately 100 fully engorged females of *R. (B.) microplus* were collected from infested cattle in a farm at Umuarama, Parana, Brazil. Then, ticks were cleaned with water purified and selected according to their aspects of normal appearance, regular motility, integrity of body, and full engorgement. Groups of 30 females each were immersed in different concentrations of EO for 5 min. Each female was individually weighed and maintained in petri dishes at 28°C and 80% relative humidity (RH) for 14 days to monitor oviposition. Egg masses were obtained from each female, placed in sample tubes, and kept at 28°C and 80% (RH) for 21 days to hatching. Larvae were killed with sulfuric ether and counted with magnifying glass in order to obtain the rate of hatching. From the tick mass, egg mass and hatching rate data, the reproductive efficiency was computed as RE = [(egg mass (g) × % hatching × 20,000)/(tick mass)(g)]; and product efficiency as PE = [(RE (negative control) × RE (treatment group) × 100)/RE(negative control)] (Drumond et al., 1973). For the larval packet test (LPT), 100 larvae were placed between filter papers (2 × 2 cm) freshly impregnated with EO dilutions to form envelopes, which were sealed and kept in petri dishes. The envelopes were maintained at 28°C and 80% (RH). After 24 h, living larvae were isolated from dead with the help of a magnifying glass. All tests were performed in triplicate and larvae mortality was computed as the following: LM = [(dead larvae × 100)/(total larvae)]; and mean mortality as MM = (LM 1st repetition + LM 2nd repetition + LM 3rd repetition)/3.

Larvicidal effect on *A. aegypti*

The third stage larvae of *A. aegypti* were used for bio-tests. They were originated from the Control Center of Endemic Diseases Transmitted by Vectors - Umuarama City, Paraná. The EO and FRs from *P. crispum* were diluted on 2% polysorbate 80 (v/v) solutions. The negative control was a 2% polysorbate 80 solution and the positive control was made with an organophosphate-based temephos®. Ten third stage larvae of *A. aegypti* were taken on Pasteur pipette and placed into 250 ml vials with 10 ml solution containing different concentrations of EO or the isolated. The number of dead larvae was counted after 24 h (Cavalcanti et al., 2004).

Anticholinesterase activity

The method described by Yang et al. (2009) was used to determine the anticholinesterase activity through an autobiographic assay. The EO and FRs obtained by CC were solubilized in methanol indifferent concentrations from 100,000 to 0.012 µg/ml. The samples were applied to aluminum chromatoplates (10 × 10 cm, silica Gel 60 F_{254} with 0.2 mm thickness) where a decreasing series of spots with different amounts of EO and FRs was obtained. For chromatographic development, a mixture of 9:1 dichloride methane/methanol was utilized. After sample migration and drying to eliminate solvents, an enzyme solution (Acetylcholinesterase in Tris buffer solution) was sprayed on the chromatoplate, followed by a solution of α-naphthyl acetate. Partial drying was done in the plate that was kept in water bath (37°C) for 20 min for enzyme incubation. Then, the chromatoplate was sprayed with colorimetric Fast Blue B salt reagent resulting in a purple color surface on the chromatoplate for 5 min. By analyzing the absence of purple color, the anticholinesterase activity was determined.

Statistical analysis

Tests were performed in triplicate. Acaricidal and larvicidal activities were analyzed by frequency distribution with Bioestat® (version 5.3) (Kamirauá Institute for Sustainable Development, Manaus, Tefé). Values for LC_{50} and $LC_{99.9}$ were obtained in Microsoft Excel® (versionExcel® 2010) (Microsoft, Redmond, Washington, United States) by linearization of the response curve. The confidence intervals were calculated using Assistat (version 7.7) INPI 0004051-2 developed by prof. Dr. Francisco de A. S. e Silva from Federal University of Campina Grande, Brazil distributed for free.

RESULTS

Chemical identification

In the essential oil - extracted from *P. crispum*, 31 constituents were detected and 26 were identified from GC-MS analysis and listed by elution time (Table 1). Phenylpropanoides were the dominant compounds, comprising 52.07% of the oil and consisting mainly of apiole (41.05%) and myristicin (5.08%). The percentage of monoterpene hydrocarbons was 20.30% that were represented by *p*-mentha-2,4,(8)-diene (6.06%) and *p*-mentha-1,5,8-triene (5.95%). Oxygenated monoterpenes comprised 18.76% of the total EO; for this group the main constituents were neral (4.56%) and *cis*-carveol (4.04%). Sesquiterpene hydrocarbons comprised just 8.87% of the total EO. The classical chromatography technique clustered 7 FRs from EO. Among these, four FRs showed acaricidal and larvicidal activities and were submitted to GC/MS for identification. Fraction 1 (FR1) was obtained with eluent hexane (100%) and presented: myrcene (10.15%); limonene (9.72%); *p*-mentha-1,4,(8)-diene (20.99%) and *p*-mentha-1,5,8-triene (59.14%). Fraction 2 (FR2) was obtained with dichloromethane: ethyl acetate (7:3) and presented the following compounds: ρ-cimen-8-ol (72.43%); α-terpineol (16.65%);

Table 1. Chemical composition of the essential oil obtained from leaves and stems of *P. crispum.*

Peak	[a]Constituent	RI[b]	Area (%)	Methods of identification
1	α-pinene	929	0.73	b,c
2	β-Pinene	952	0.57	b,c
3	Myrcene	974	1.29	b,c
4	Limonene	1012	2.75	b,c
5	*p*-mentha-2,4,(8)-diene	1048	6.06	b,c
6	*p*-cymenene	1065	2.95	b,c
7	*p*-mentha-1,5,8-triene	1097	5.95	b,c
8	*m*-Cymen-8-ol	1143	1.37	b,c
9	n.i	1144	0.82	b,c
10	ρ-cimen-8-ol	1150	1.15	b,c
11	α-terpineol	1170	1.03	b,c
12	*trans*-carveol	1174	1.44	b,c
13	α- methy-Cinnamaldehyde	1193	3.27	b,c
14	n.i	1199	0.53	b,c
15	Citronellol	1202	2.86	b,c
16	*Cis*-carveol	1231	4.04	b,c
17	n.i	1237	0.96	b,c
18	Neral	1245	4.56	b,c
19	α-copaene	1370	0.72	b,c
20	β-elemene	1397	0.91	b,c
21	α-guaiene	1427	2.32	b,c
22	α-humulene	1440	0.99	b,c
23	cis-β-farnesene	1460	0.83	b,c
24	β-ionone	1481	0.55	b,c
25	β-bisabonele	1500	1.68	b,c
26	Germacrene A	1516	0.87	b,c
27	Myristicin	1521	5.08	b,c
28	Elemicin	1553	0.79	b,c
29	Apiole	1641	41.05	b,c
30	n.i	1695	0.82	b,c
31	n.i	1721	1.06	b,c
Total identified			95.81%	-
Compoundgroups (%)				
Monoterpene hydrocarbons		-	20.30	-
Oxygenated monoterpenes		-	18.76	-
Sesquiterpene hydrocarbons		-	8.87	-
Oxygenated sesquiterpenes		-	0.00	-
Phenylpropanoids		-	52.07	-

[b]Identification based on retention index (RI) using n-alkane C7 - C26 on DB-5 column; [c]identification based on comparison of mass spectra; [a]Compounds listed in order of elution from DB-5 column. n.i. = not identified.

trans-carveol (7.44%); citronellol (1.37%) and carveol (2.11%). Fraction 3 (FR3) was obtained with hexane:dichloromethane (3:7) and presented myristycin (99.90%) mostly. Fraction 4 (FR4) was obtained with dichloromethane (100%) and presented Myristycin (0.90%); elemicin (1.87%); carotol (0.43%) and

Table 2. Means ± (standard error) of female mortality (%), egg mass (g), percentage of hatching (%) and product efficiency (%) from engorged females of *Rhipicephalus (Boophilus) microplus* subjected to adult immersion test at different concentrations of essential oil of *P. crispum*.

Essential oil concentration (µg/ml)	Female mortality (%)	Egg mass (g)	Percentage of hatching (%)	Product efficiency (%)
PC	100 ± 0.00	0 ± 0.00	0 ± 0.00	100.00
100,000	100 ± 0.00	0 ± 0.00	0 ± 0.00	100.00
80,000	70.8 ± 0.35	0.05 ± 0.00	13.2 ± 0.53	65.53
70,000	48.4 ± 0.15	0.06 ± 0.00	25.7 ± 4.49	60.03
60,000	27.3 ± 0.10	0.07 ± 0.00	37.6 ± 1.79	59.79
50,000	16.7 ± 0.02	0.07 ± 0.00	44.89 ± 1.89	58.18
40,000	5.3 ± 0.01	0.073 ± 0.00	65.15 ± 0.61	32.45
25,000	0 ± 0.00	0.09 ± 0.00	80.45 ± 1.60	11.32
12,500	0 ± 0.00	0.08 ± 0.00	80.86 ± 5.00	7.45
NC	0 ± 0.00	0.08 ± 0.00	93.49 ± 3.24	0.00

PC = positive control [commercial solution containing 150,000 µg/ml cypermethrin, 250,000 µg/ml of chlorpyrifos and 10,000 µg/ml of citronellal prepared in 2% polysorbate 80 at 1,250 µg/ml]; NC = negative control [2% polysorbate 80 (v/v) water solution].

apiole (96.80%).

Acaricidal and larvicidal effect on *R. (B.) microplus*

Results to acaricidal activity (Table 2) indicate that the EO of *P. crispum* at 100,000 µg/ml caused 100% mortality in the tick females; similar results were obtained with the positive control that is a commercial mixture of 150,000 µg/ml of cypermethrin, 250,000 µg/ml of chlorpyrifos and 10,000 µg/ml of citronellal. The hatching rates were reduced by the increasingly oil concentrations: at 100,000 µg/ml of EO no hatching larvae was found (Table 2). The product efficiency ranged from 100 to 58.18% as the concentrations varied from 100,000 to 50,000 µg/ml. Within the concentration range from 40,000 to 12,500 µg/ml, product efficiency was lower than 50%. Lethal concentrations (LC_{50} and $LC_{99.9}$) of parsley's EO on engorged female were of 73,400 µg/ml (71,240 to 78,450) and of 109,760 µg/ml (106,540 to 117,320), respectively. The EO and FRs of *P. crispum* were active in the control of *R. (B.) microplus* larvae (Tables 3 and 5). The FR4, which presented apiole as the main compound (96.80%), showed the highest larvicidal activity with LC_{50} of 0.82 µg/ml and $LC_{99.9}$ of 9.62 µg/ml. These results were similar to the positive control that presented LC_{50} and $LC_{99.9}$ of 0.45 and 4.62 µg/ml, respectively.

Larvicide effect on *A. aegypti*

EO and FRs had high mortality potential against *A. aegypti* larvae (Tables 4 and 5). The best result was for FR4 with LC_{50} of 0.01 µg/ml and $LC_{99.9}$ of 0.07 µg/ml and 96.80% of apiole. The FR4 results were better than the positive control, which showed LC_{50} and $LC_{99.9}$ of 0.05 and 0.30 µg/ml, respectively. Intermediate activity was verified for FR1 and FR3 with LC_{50} of 0.49 and 0.88 µg/ml, and $LC_{99.9}$ of 1.28 and 2.73 µg/ml, respectively (Table 5). The FR1 is composed of: myrcene: 10.15%; limonene: 9.72%; *p*-mentha-1,4,(8)-diene: 20.99% and *p*-mentha-1,5,8-triene: 59.14%; while FR3 has 99.90% of myristycin.

Anticholinesterase activity

Preliminary lab tests for EO and FRs demonstrated that these substances have inhibited the acetyl cholinesterase activity (Table 6). The quantitative analysis of acetylcholinesterase (AChE) activity revealed that the highest enzymatic inhibition occurred for EO and FR4 at 0.012 µg/ml, which agrees with the highest activities against *A. aegypti* larvae. The FR3 showed low potential for enzyme inhibition, suggesting that the larvicide action against *A. aegypti* is related to another mechanism as perhaps the opening of the sodium channels (Soderlund et al., 2002).

DISCUSSION

In this work, the main compound of parsley's EO was the apiole. Kurowska and Galazka (2006) found α-pineno (32.0%) as the major constituent of the EO of *P. crispum* cultivated in Poland, followed by β-pineno (19.0%), myristicin (18.3%) and apiole (10.1%). Stankovic et al.

Table 3. Mortality rates (%) of larvae of *Rhipicephalus (Boophilus) microplus* subjected to larval immersion test at different concentrations of EO, FR1, FR2, FR3 and FR4 (%) obtained from *P. crispum.*

Concentration (µg/ml)	EO (%)	FR1	FR2	FR3	FR4	PC (%)	NC (%)
100,000	100.00	100.00	100.00	100.00	100.00	100.00	0.00
50,000	100.00	100.00	100.00	100.00	100.00	100.00	0.00
25,000	100.00	90.62	100.00	100.00	100.00	100.00	0.00
12,500	100.00	72.21	83.33	100.00	100.00	100.00	0.00
6,250	100.00	60.71	70.00	100.00	100.00	100.00	0.00
3,125	100.00	49.99	50.00	100.00	100.00	100.00	0.00
1,562	100.00	38.51	40.00	100.00	100.00	100.00	0.00
781.00	100.00	34.91	22.70	100.00	100.00	100.00	0.00
390.00	100.00	15.62	10.00	70.10	100.00	100.00	0.00
195.00	100.00	9.37	8.33	47.40	100.00	100.00	0.00
97.65	100.00	0.00	0.00	33.70	100.00	100.00	0.00
48.83	100.00	0.00	0.00	12.00	100.00	100.00	0.00
24.41	100.00	0.00	0.00	9.75	100.00	100.00	0.00
12.21	100.00	0.00	0.00	4.33	100.00	100.00	0.00
6.10	76.20	0.00	0.00	0.00	88.20	100.00	0.00
3.05	58.33	0.00	0.00	0.00	85.70	97.20	0.00
1.52	23.50	0.00	0.00	0.00	73.20	88.70	0.00
0.76	13.50	0.00	0.00	0.00	58.40	72.90	0.00
0.38	0.00	0.00	0.00	0.00	37.80	50.00	0.00
0.19	0.00	0.00	0.00	0.00	25.40	35.50	0.00
0.09	0.00	0.00	0.00	0.00	12.90	15.70	0.00
0.047	0.00	0.00	0.00	0.00	5.23	2.37	0.00

FR1: myrcene (10.15%); limonene (9.72%); *p*-mentha-1,4,(8)-diene (20.99%) and *p*-mentha-1,5,8-triene (59.14%).FR2: ρ-cimen-8-ol (72.43%); α-terpineol (16.65%); *trans*-carveol (7.44%); citronellol (1.37%) and carveol (2.11%). FR3: myristicin (99.90%).FR4: myristicin (0.90%); elemicin(1.87%); carotol (0.43%); apiole (96.80%).PC = positive control [commercial solution containing 150,000 µg/ml cypermethrin, 250,000 µg/mL of chlorpyrifos and 10,000 µg/ml of citronellal prepared in 2% polysorbate 80 at 1,250 µg/ml]; NC = negative control [2% polysorbate 80 (v/v) water solution].

(2004) found that apiole (57.0%), α-thujone (10.7%), benzene-1,2-dicarboxylic acid (9.0%) and myristicin (9.7%) were the major constituents of essential oil of *P. crispum* cultivated in Serbia and Montenegro. Zhanget al. (2006) evaluated the oil composition of parsley cultivated in China and observed that myristicin (32.75%), apiole (17.54%), α-pinene (16.64%), β-pinene (11.54%) and 2,3,4,5-tetramethoxy-allylbenzene 1 (10.0%) were the major oil constituents. Knioetal. (2008) reported sabinene (21.14%), *p*-Mentha-1,5,8-triene (8.25%) and apiole (7.65) as the main compounds of parsley's EO from the local market in Beirut, Lebanon. This diverse chemical composition could be a result of genetic and environmental factors that can affect the secondary metabolism. Among these factors, it is worth mentioning the plant germplasm diversity, their ages and development stages, as well as their interactions with microorganisms and insects. In addition, abiotic factors like luminosity, temperature, rainfall, nutrition, time of the day for harvest and post-harvest techniques are also important to form the chemical composition of Eos (Morais and Castanha, 2012). Although many variations in the EO composition of *P. crispum* were already reported in literature, the apiole and myristicin have always appeared as the major constituents of parsley's EO, which agrees with the results of this work.

Literature has shown a series of advantages in the use of EO for the control of ticks. Among them, low toxicity to mammalian, rapid degradation and slow development of resistance are common benefits of acaricides extracted from plants. Such benefits make bio-acaricides commercially attractive to control *R. (B.) microplus* with reduced hazard to environment. Essential oils of *Eucalyptus citriodora, Eucalyptus globules* and *Eucalyptus staigeriana* (Myrtaceae) have showed *in vitro* 100% control over cattle ticks at concentrations of 175,000, 150,000 and 125,000 µg/ml, respectively (Chagas et al., 2002). Olivo et al. (2008) observed *in vitro* 89.5% control at 100,000 µg/ml of EO of *Cymbopogon nardus* (Poaceae) on the cattle tick larvae. In our work,

Table 4. Mortality rates (%) of larvae of *Aedes aegypti* subjected to different concentrations of EO, FR1, FR2, FR3 and FR4 (%) obtained from *P. crispum.*

Concentration (µg/ml)	EO (%)	FR1	FR2	FR3	FR4	PC (%)	NC (%)
97.65	100.00	100.00	100.00	100.00	100.00	100.00	0.00
48.83	100.00	100.00	100.00	100.00	100.00	100.00	0.00
24.41	100.00	100.00	100.00	100.00	100.00	100.00	0.00
12.21	90.00	100.00	75.00	100.00	100.00	100.00	0.00
6.10	70.00	100.00	50.00	100.00	100.00	100.00	0.00
3.05	40.00	100.00	32.00	100.00	100.00	100.00	0.00
1.52	20.00	100.00	20.00	70.00	100.00	100.00	0.00
0.76	12.60	90.00	13.70	58.00	100.00	100.00	0.00
0.38	5.00	47.00	8.77	21.66	100.00	100.00	0.00
0.19	0.00	32.00	0.00	12.00	100.00	92.00	0.00
0.09	0.00	18.70	0.00	5.33	100.00	73.00	0.00
0.047	0.00	9.30	0.00	0.00	90.00	50.00	0.00
0.024	0.00	3.78	0.00	0.00	75.00	43.00	0.00
0.011	0.00	0.00	0.00	0.00	55.00	37.20	0.00
0.0046	0.00	0.00	0.00	0.00	43.50	17.80	0.00
0.0023	0.00	0.00	0.00	0.00	36.30	5.00	0.00
0.0012	0.00	0.00	0.00	0.00	18.70	0.00	0.00
0.0006	0.00	0.00	0.00	0.00	9.08	0.00	0.00

FR1: myrcene (10.15%); limonene (9.72%); *p*-mentha-1,4,(8)-diene (20.99%) and *p*-mentha-1,5,8-triene (59.14%).FR2: ρ-cimen-8-ol (72.43%); α-terpineol (16.65%); *trans*-carveol (7.44%); citronellol (1.37%) and carveol (2.11%).FR3: myristicin(99.90%). FR4: myristicin (0.90%); elemicin (1.87%); carotol(0.43%); apiole (96.80%).PC = positive control [commercial solution of organophosphate-based Temephós®].NC = negative control [2% polysorbate 80 (v/v) water solution].

the efficiency of *P. crispum* EO over females of *R. (B.) microplus* (100% with 100,000 µg/ml) was higher than those reported in literature. Thus, the EO of *P. crispum* presented considerable acaricide activity on the cattle tick observed *in vitro* and such activity should be deeply investigated for field applications to control *R. (B.) microplus*.

In our work, apiole had the highest larvicidal activity with LC_{50} of 0.82 µg/ml and $LC_{99.9}$ of 9.62 µg/ml. The EO of *Pipper mikanianum* and *Pipper xylosteoides* (with 67.89 and 48.53% of phenylpropanoides, apiole and safrole, respectively) showed LC_{50} of 2.33 µg/ml and 6.15 µg/ml against *R*. (*B*.) *microplus* larvae (Ferraz et al., 2010). For EO of *Pipper aduncum* (with 94.84% of dillapiole) just 0.1 µg/ml induced 100% mortality in larvae of *R*. (*B*.) *microplus* (Silva et al., 2009). Single chemical control is a concern, since populations of *R. (B.) microplus* are acquiring resistance to chemical acaricides. The chemical resistance is often found in regions favorable to tick development, as the major countries of Latin America, Central America, Australia and South Africa. In the Brazilian States of São Paulo, Mato Grosso do Sul, Rio Grande do Sul and Paraná, where the largest livestock producers are located, the tick populations are becoming increasingly resistant to different conventional acaricides (Madalena et al., 2012). Therefore, the EO of parsley and its main constituent apiole could be useful as part of an integrated pest management. Future studies are in progress by our group to search for synergistic effects of essential oils and chemical acaricides to increase the efficacy of these compounds in the control of *R. (B.) microplus*.

Research results on larvicidal activity of essential oils and isolated compounds to control A*. aegypti* are scarce. Rafael et al. (2008) isolated the dillapiole from leaves of *Piper aduncum* and the insecticidal activity against larvae and pupae of *A. aegypti* occurred at 200 and 400 µg/ml, respectively. In our work, the FR4 (with 96.80% of apiole) presented a small $LC_{99.9}$ value of 0.07 µg/ml, which was lower than that reported by Rafael et al. (2008). So apiole seems to have a real potential to help the *A. aegypti* control; note that Rafael et al. (2008) also found that apiole performed better than the positive control. For EO of *Citrus limon* (with high limonene content) was reported low insecticidal activity against *A. aegypti* larvae with LC_{50} of 95.8 mg/ml and LC_{90} of 102.7 mg/ml (Furtado et al., 2005). Also, for limonene obtained from EO of *Clausena excavate,* Cheng et al. (2009) reported LC_{50} of19.4 µg/ml. In our work, the FR1 (in which *p*-mentha represents 80.13%) showed $LC_{99.9}$ of 1.28 µg/ml, which

Table 5. Means of lethal concentrations (LC_{50} and $LC_{99.9}$ in µg/ml) of the essential oil of *Petroselinum crispum* and its fractions applied to larvae of *R. (B.) microplus* and *Aedes aegypti* followed by confidence interval by probit analysis.

Parameter	EO of *P. crispum*		FR1		FR2		FR3		FR4		PC	
	LC_{50}	$LC_{99.9}$	LC_{50}	$LC_{99.9}$	LC_{50}	$LC_{99.9}$	LC_{50}	$LC_{99.9}$	LC_{50}	$LC_{99.9}$	LC_{50}	$LC_{99.9}$
	CI		CI		CI		CI		CI		CI	
Mortality larvae(%)	2.67	10.80	3,703.55	40,125.42	3,753.17	20,777.31	247.37	707.16	0.82	9.62	0.45	4.62
R. (B.) microplus	(2.59-2.85)	(10.49-11.55)	(3,595.00-3,958.80)	(38,949.35-42,890.87)	(3,643.16- 4,011.84)	(20,168.33-22,209.29)	(240.12-264.42)	(686.43 -755.90)	(0.80-0.88)	(9.33- 10.28)	(0.44-0.48)	(4.48- 4.94)
Mortality larvae (%)	4.19	19.92	0.49	1.28	6.61	21.79	0.88	2.73	0.01	0.07	0.05	0.30
Aedesaegypti	(4.07-4.48)	(19.34-21.30)	(0.48-0.53)	(1.24-1.37)	(6.41-7.07)	(21.15-23.29)	(0.86-0.95)	(2.65- 2.92)	(0.011-0.012)	(0.07- 0.08)	(0.04-0.05)	(0.29-0.32)

LC_{50}: Lethal concentration, 50%; $LC_{99.9}$: Lethal concentration, 99.9%.CI: Confidence interval.FR1:myrcene(10.15%); limonene (9.72%); *p*-mentha-1,4,(8)-diene (20.99%) and *p*-mentha-1,5,8-triene (59.14%).FR2: ρ-cimen-8-ol (72.43%); α-terpineol (16.65%); *trans*-carveol (7.44%); citronellol (1.37%) and carveol (2.11%). FR3: myristicin (99.90%).FR4: myristicin(0.90%); elemicin (1.87%); carotol (0.43%); apiole (96.80%).PC = Positive control on *R. (B.) microplus* [commercial solution containing 150,000 µg/ml cypermethrin, 250,000 µg/ml of chlorpyrifos and 10,000 µg/ml of citronellal prepared in 2% polysorbate 80 at 1,250 µg/ml]; Positive control on *Aedesaegypti* [commercial solution of organophosphate-based Temephós®].

was lower than the values reported in the literature. So the larvicidal activity of FR1 to *A. aegypti* seems to be related to the presence of *p*-mentha. However, there is no report on the larvicidal activity of the *p*-mentha against *A. aegypti*. The EO of Pink Chablis bluebeard (*Caryopteris* × *clandonensis* 'Durio') compounded by 4.6% of *trans*-*p*-mentha-2,8-dien-1-ol, 4.5% of *trans*-*p*-mentha-1(7),8-dien-2-ol and 4.0% of *cis*-*p*-mentha-2,8-dien-1-ol exhibited weak activity against mosquito larvae (Blythe et al., 2015). Although the larvicidal effect of FR1 (80.13% of *p*-mentha compounds) to *A. aegypti* was 18 times less effective than FR4 (96.80% of apiole). Simas et al. (2004) showed that phenylpropanoids and sesquiterpene alcohols have higher larvicidal effect against *A. aegypti*. Myristycin, the main constituent of FR3, is the phenylpropanoid responsible by the larvicidal activity of this fraction. The other phenylpropanoids showed larvicidal activity to *A. aegypti* with LC_{50} values as the following: 28 µg/ml for anethole (Morais et al., 2006), 67 µg/ml for E-methyl-cinnamate and 60 µg/ml for eugenol. Knio et al. (2008) evaluated the Parsley's seed oil in the control of the seaside mosquito (*Ochlerotatus caspius)*. The EO results showed LC_{50} values of 34.3 and 23.4 mg/ml and LC_{90} values of 62.2 and 42.2 mg/ml, for 24 and 48 h, respectively.

In this present work, an efficient *in vitro* control of larvae of *A. aegypti* was observed for the reduced concentrations of *P. crispum* EO and its FRs, mainly for FR4 (apiole). Thus, these substances are promising to substitute or complement synergistically chemical insecticides such as temephos®, malathion®, fenitrothion® on the control of *A. aegypti* mosquitoes. Importantly, the use of these commercial insecticides is the main action adopted by the Public Health Programs for mosquito control. However, chemical products cause pollution in the environment and also insect resistance to conventional insecticides was already observed in different regions of the world, including Brazil (Magalhães et al., 2010).

In this study, the high acaricide and larvicide potential of the parsley's EO and its FRs to control *R. (B.) microplus* and *A. aegypti* may be related to the inhibition of AChE (Souza et al., 2012). This enzyme hydrolyzes the acetylcholine neurotransmitter, being the major action of commercial acaricides, such as organophosphates chlorpyrifos. Differently, pyrethroids can prevent the closure of the voltage-gated sodium channels in the axonal membranes so that the nerves cannot repolarize, leaving the axonal membrane permanently depolarized and thereby paralyzing the organism (Soderlund et al., 2002). Many essential oils and primarily monoterpenes (Picollo et al., 2008), sesquiterpenes (Fujiwara et al., 2010) and phenylpropanoids (Dohi et al., 2009) have AChE inhibitory activity. This could explain the high anticholinesterase activity for FR4, composed mostly for phenylpropanoid apiole. The phenylpropanoids were also the major compounds

Table 6. Inhibitory concentration of *Petroselinum crispum* essential oil and fractions of the acetylcholinesterase after separation the autobiographic assay.

Concentration (µg/ml)	EO	FR1	FR2	FR3	FR4	PC (I)	PC (II)
100,000	+	+	+	+	+	+	+
75,000	+	+	+	+	+	+	+
50,000	+	+	+	+	+	+	+
25,000	+	+	+	+	+	+	+
12,500	+	+	+	+	+	+	+
6,250	+	+	+	+	+	+	+
3,125	+	+	+	+	+	+	+
1,562	+	+	+	+	+	+	+
781.00	+	+	+	+	+	+	+
390.00	+	+	+	+	+	+	+
195.00	+	+	+	+	+	+	+
97.65	+	+	+	-	+	+	+
48.83	+	+	+	-	+	+	+
24.41	+	+	+	-	+	+	+
12.21	+	+	+	-	+	+	+
6.10	+	+	+	-	+	+	+
3.05	+	+	+	-	+	+	+
1.52	+	+	+	-	+	+	+
0.76	+	+	+	-	+	+	+
0.38	+	+	+	-	+	+	+
0.19	+	+	+	-	+	+	+
0.095	+	+	+	-	+	+	+
0.047	+	+	+	-	+	+	+
0.024	+	-	-	-	+	+	+
0.012	-	-	-	-	-	+	+

(+) inhibitionof –acetylcholinesterase; (-)no enzyme inhibition. FR1: myrcene (10.15%); limonene (9.72%); *p*-mentha-1,4,(8)-diene (20.99%) and *p*-mentha-1,5,8-triene (59.14%). FR2: *ρ*-cimen-8-ol (72.43%); α-terpineol (16.65%); *trans*-carveol (7.44%); citronellol (1.37%) and carveol (2.11%).FR3: myristicin (99.90%). FR4: myristicin (0.90%); elemicin (1.87%); carotol (0.43%); apiole (96.80%). PC (I) = positive control [commercial solution containing 150,000 µg/mlcypermethrin, 250,000 µg/ml of chlorpyrifos and 10,000 µg/ml of citronellal prepared in 2% polysorbate 80 at 1,250 µg/ml]. PC (II) = positive control [commercial solution of organophosphate-based Temephós® 0.005%].

of EO (52.07%).

Conclusion

The EO and the FRs containing apiole and myristicin as the major compounds of *P.crispum* presented larvicidal and acaricidal activitiy on the *R*. (*B*.) *microplus* (cattle tick) and high larvicide potential to control the *A*. *aegipty* (dengue mosquito). The parsley's EO is promising to be used in the bio-control of *R*. (*B*.) *microplus* and *A*.*aegipty*. Further studies in living animals at field conditions are needed to confirm the acaricide and larvicide potential of the parsley's EO. In addition, proper concentrations and mode of application should be tested by exploring surfactant and synergistic effects from the mixture with other acaricides and larvicides.

Conflict of Interests

The authors have not declared any conflict of interest.

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