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Full Length Research Paper

Determination of antioxidant and anti-inflammatory activities, as well as *in vitro* cytotoxic activities of extracts of *Anastatica hierochuntica* (Kaff Maryam) against HeLa cell lines

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Anastatica hierochuntica L. is distributed throughout the Arabian Peninsula, and North Africa. It is locally called "Kaff Maryam". All parts of the plant are used in folk medicine. This study aimed to investigate possible antioxidant activity of various extracts of Kaff Maryam, anti-inflammatory activities, as well as *in vitro* cytotoxic activities. Five extract types were used, namely, the whole ethanolic extracts, ethyl acetate, petroleum ether, water, and butanol extracts. The explored items included determination of total phenolics using the Folin-Ciocalteu assay and total flavonoids using Muller's method, anti-oxidant activity using three assays (2, 2'-Azinobis [3-ethylbenzothiazoline-6-sulfonic acid]-diammonium salt (ABTS), reducing power, and 2, 2-diphenyl-1-picrylhydrazyl (DPPH)), membrane stabilization test for anti-inflammatory studies, as well as *in vitro* cytotoxic activities against HeLa cell lines using the 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT) assay. The results obtained indicated high flavonoid and phenolic contents in all the five extracts types. All extracts registered high antioxidant activity using three assays, but generally, the ABTS assay recorded the highest antioxidant activity as compared to the reducing power and DPPH methods. The membrane stabilization test showed that extracts of Kaff Maryam had good anti-inflammatory activity. Extracts of Kaff Maryam also registered very good cytotoxic activities against HeLa cell lines. Kaff Maryam extracts have good antioxidant, anti-inflammatory, and cytotoxic activities and the results of this study provide the basis for further investigation of Kaff Maryam for potential identification of novel bioactive compounds with therapeutic properties.

Key words: *Anastatica hierochuntica*, Kaff Maryam, antioxidant, anti-inflammatory, HeLa cell lines, cytotoxicity.

INTRODUCTION

There is a global increase in the use of medicinal plants for health reasons. In developing countries, herbal drugs and traditional remedies are relatively more popular, because of cultural acceptability and belief that being

natural, they are safe and non-toxic. Although, there are some new approaches to drug discovery, such as combinatorial chemistry and computer based molecular modeling design, none of them can replace the

importance of natural products in drug discovery and development (Gavamukulya et al., 2015; Heinrich and Bremner, 2006; Thirumal et al., 2012). Many synthetic drugs cause severe side effects that are not acceptable except as treatments of last resort for terminal diseases, such as cancer and the metabolites discovered in medicinal plants may avoid the side effect of synthetic drugs (Valko et al., 2007), because ideally, toxicity of such drugs is yet not well understood (Barnes et al., 2007; Salah et al., 2011). The medicinal values of these plants lie in their phytochemical components which produce definite physiological actions on the human body. The most important of these components are alkaloids, tannins, flavonoid and phenolic compounds (Nakashima et al., 2010).

Anastatica is a monotypic genus with the type species *Anastatica hierochuntica*. The genus is a member of the family Brassicaceae (formerly called Cruciferae), tumbleweed and a resurrection plant (Ihsanullah, 2012; Noura et al., 2011). *Anastatica* is found in arid areas in the Middle East and Sahara Desert, including parts of North Africa and regions of Iran, Egypt, Palestine, Israel, Iraq, Jordan, and Pakistan (Ahmad et al., 2006; Masayuki et al., 2003). *A. hierochuntica* L. locally called 'Kaff Maryam', is a well-known desert zone medicinal plant. Novel melanogenesis inhibitor flavonoids with antioxidant potential were isolated from it (Law et al., 2009). The plant is widely consumed as a tea beverage. It is powdered, mixed with honey and taken for the treatment of many conditions, in particular as a remedy for difficult childbirth and uterine hemorrhage (El-Ghazali et al., 2010; Khalifa and Ahmad, 1980). In addition, it is used to treat asthma, gastrointestinal disorders, depression, high blood pressure, indigestion, headache, cold, fever, malaria, epilepsy, fatigue, diabetes, heart disease, and infertility (Batanouny, 1999; Eman et al., 2011).

Antioxidants are a group of substances that are useful for fighting cancer and other processes that potentially lead to diseases, such as atherosclerosis, Alzheimer, Parkinson, diabetes, and heart disease (Aboul-Enein et al., 2012; Valko et al., 2007). Synthetic antioxidants, such as butylated hydroxytoluene (BHT) and butylated hydroxyanisole (BHA), which are effective in their role as antioxidants, are commercially available and currently used in industrial processes. However, since suspected actions as promoters of carcinogenesis and other side effects have been reported, their use in food, cosmetic, and pharmaceutical products has been decreasing (Politeo et al., 2007). Thus, there has been an upsurge of interest in naturally-occurring antioxidants from vegetables, fruits, leaves, oilseeds, cereal crops, tree barks, roots, spices, and herbs (Rababah et al., 2004).

Inflammation is a very important aspect in membrane

injury. The mechanism of inflammation injury is attributed in part to release reactive oxygen species (ROS) from activated neutrophil and macrophages. This over release leads to tissue injury by damaging the macromolecule and lipid peroxidation of membranes. ROS propagate inflammation by stimulating the release of the cytokines, such as tumor necrosis factor, inter-leukine 1, which stimulate recruitment of additional neutrophil and macrophages. Thus, free radicals are important mediators that provoke or sustain inflammatory processes and consequently their neutralization by antioxidants and radical scavengers can attenuate inflammation (Lavanya et al., 2010).

The present study aimed to provide a comprehensive analysis of the total phenolic and total flavonoids contents, antioxidant, anti-inflammatory, and cytotoxic activities of different fractions of *A. hierochuntica* L.

MATERIALS AND METHODS

Preparation of *A. hierochuntica* extracts

The samples of *A. hierochuntica* were collected in the month of September 2014. They were identified by Dr. Narmein Shanan of the Ornamental Department at the Faculty of Agriculture in Cairo University. A voucher specimen was deposited in the Faculty Herbarium under the voucher number 14562 for future reference. The dried *A. hierochuntica* (115 g) was ground into powder and extracted with 500 ml of 70% ethanol by plant tissue homogenization method with modifications as previously described (Gavamukulya et al., 2014). The extraction was repeated twice and the total extracts were filtered, and the obtained extracts were concentrated using rotary evaporator to dryness. The crude extract was partitioned with 3×100 ml petroleum ether, and then successively partitioned with 3×100 ml of ethyl acetate, 3×100 ml of n-butanol, and 3×100 ml of water, respectively. The resulting four extracts were evaporated to dryness in vacuum.

Measurement of total phenolic content

The total phenolics content of the five different ethanolic extracts was determined with the Folin-Ciocalteu reagent (Singleton et al., 1999). Ferruleic standards were prepared (0 to 500 µg). To 50 µl of each sample (three replicates), 2.5 ml diluted Folin-Ciocalteu's reagent (1/100) and 2 ml of Na₂CO₃ (7.5%, w/v) were added and incubated at 45°C for 15 min. The absorbance of all samples was measured at 765 nm using a UV-VIS spectrophotometer (GAT UV-9100). A standard curve was plotted using different concentrations of Ferruleic and the results were expressed as Ferruleic equivalent.

Measurement of total flavonoid content

The total flavonoids content of the four different ethanolic extracts was evaluated (Christ and Muller, 1960). Rutin standards were prepared (10 to 100 µM). A standard curve was plotted using different concentrations of rutin and the results were expressed as

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Figure 1. Kaff Maryam plant (BintBatman, 2013).

rutin.

Anti-oxidant activity using the reducing power assay

The reducing power of all samples was determined as described by Dorman et al. (2003). 100 μ l were added to 1 ml of distilled water and mixed with phosphate buffer (2.5 ml, 0.2 Mol/L, pH 6.6) and potassium ferricyanide [$K_3 Fe (CN)_6$] (2.5 ml, 1%). The mixture was incubated at 50°C for 20 min. 2.5 ml of trichloroacetic acid (TCA, 10%) was added to the mixture, which was then centrifuged at 3000 rpm (MSE Mistral 2000, UK) for 10 min. The reaction was initiated by the addition of 200 μ l $FeCl_3$ (0.1%) and the mixture was shaken vigorously and left standing at room temperature for 10 min. Absorbance of the solution was then measured spectrophotometrically at 562 nm. The value is expressed as gallic acid equivalent (GAE)(Figure 1).

Measurement of 2, 2-diphenyl-1-picrylhydrazyl (DPPH) free-radical scavenging activity

DPPH radical scavenging activity of tested extracts was evaluated as described by Blois (1958). The reaction mixture contained 50 μ l of test samples (or 80% MeOH as a blank) and 5 ml of a 0.04% (w/v) solution of DPPH in methanol. Different standard antioxidants BHT and BHA were used as a positive control. Discoloration was measured at 517 nm after incubation for 30 min. Measurements were performed in triplicate.

2, 2'-Azinobis [3-ethylbenzothiazoline-6-sulfonic acid]-diammonium salt (ABTS) assay

The antioxidant activity of the samples was also measured by ABTS assay according to the method of Re et al. (1999). $ABTS^{+}$ was produced by reacting 7 mmol/L ABTS aqueous solution with 2.45 mmol/L potassium persulfate in darkness for 12 to 16 h at room temperature. Prior to assay, this solution was diluted in aqueous and equilibrated at 30°C. Sample of 0.2 ml was mixed with 3.0 ml of diluted ABTS cation radical solution. The absorbance at 734 nm was measured using spectrophotometer. The percentage inhibition was calculated of the control absorbance at 734 nm.

Anti-inflammatory activity using human red blood cell (HRBC) membrane stabilization assay

HRBC membrane stabilization assay was performed (Gandhidasan et al., 1991). The assay system consisted of 100 μ g/ml of the test methanol extract, 1 ml phosphate buffer (0.15 M, pH 7.4), 2 ml of hyposaline (0.36%), and 0.5 ml of HRBC suspension. Standard was prepared by using Diclofenac sodium (50 mg/ml). Control consisted of distilled water instead of hyposaline. The assay systems were incubated at 37°C for 30 min and centrifuged at 3000 rpm for 20 min. The hemoglobin (Hb) content of the supernatant solution was estimated spectrophotometrically at 560 nm. The percentage stabilization of HRBC membrane was calculated by using the formula: (Absorbance control-absorbance sample) / (absorbance control) \times 100.

Cytotoxic activity using the 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT) assay

The culture medium was prepared using RPMI 1640 media with 1.2 g/L sodium carbonate and L-glutamine (Gibco, Grand Island, USA), 10% inactivated fetal bovine serum (Gibco), and 100 units/ml penicillin and 100 mg/ml streptomycin were added. The cytotoxic activity of the different 4 fractions on leukemia cell line (HeLa cell line) was determined by the MTT assay (Denizot and Lang, 1986). Cells at exponentially growing phase were used. Five thousand cells per well (100 μ l) were plated in 96-well plates in the presence of various concentrations of the extracts (10, 20, and 30 μ g/ml) for 24 h at 37°C in 5% CO_2 incubator. Fytoside was used as the positive control. The activity of mitochondrial succinic dehydrogenase was measured by incubation for 4 h in the presence of 0.5 mg/ml of MTT. Absorbance reflects the viable cell number and was measured at 570 nm. % Cell death was calculated using the following formulas (Thirumal et al., 2012). % Cell death = (Control OD – Sample OD) / Control OD \times 100.

Statistical analysis

Quantitative and graphical data was analyzed using Microsoft Excel Package. The results of each series of experiments (performed in

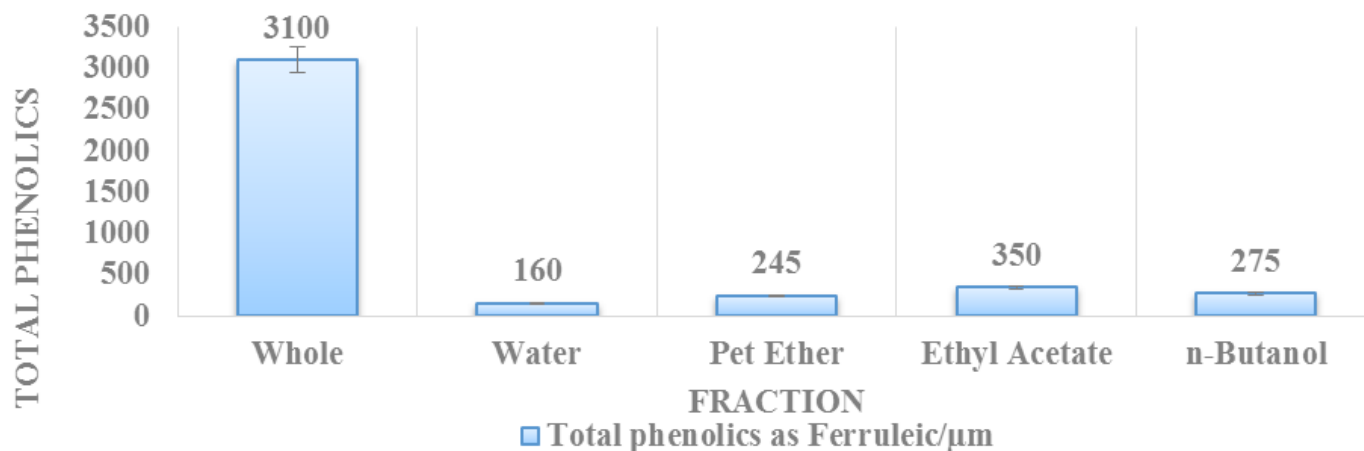


Figure 2. The total phenolic content of the different extracts of Kaff Mariam (Standard curve equation: $y=1\text{E}-04x$; $R^2=0.9357$).

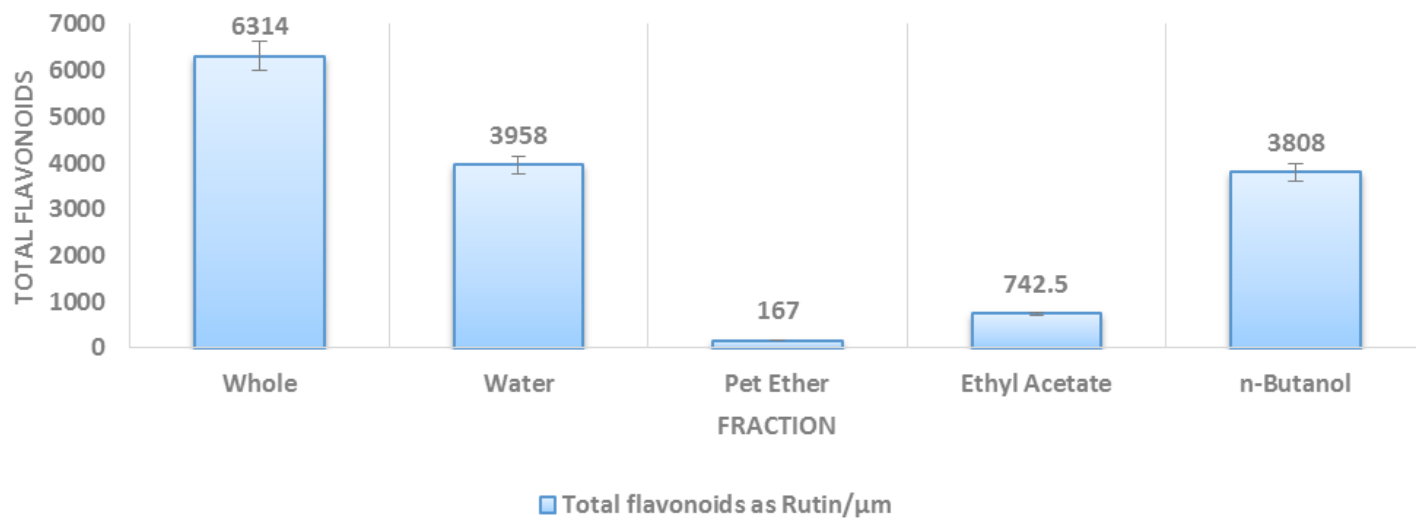


Figure 3. Total flavonoids of different fractions of Kaff Mariam (Standard curve equation: $y=0.0016x$; $R^2=0.9762$).

triplicates) were expressed as the mean \pm standard deviation. Graphs of percentage activity against logarithm of concentration were generated for the different parameters (Reducing power, DPPH, ABTS, anti-inflammatory, and cytotoxic activity), and the IC_{50} values estimated therein. P-values less than 0.05 were considered statistically significant.

RESULTS

Determination of total phenolics

It was noted that the highest content was registered in the whole extract at 3100 ferruleic acid equivalents (FAE), while the lowest was in the water extract at 160 FAE. Phenols play a crucial role in the antioxidant activity of plant extracts and an increase in the content is

a direct indication of the expected activity (Figure 2).

Determination of total flavonoids

A standard curve for rutin was generated for the determination of total flavonoid content. Figure 3 shows the total flavonoid content of the different extracts of Kaff Mariam. The highest content was registered in the whole extract at 6314 μm as rutin, while the lowest was in the pet ether extract at 167 μm as rutin. As shown in Figure 2, the whole, water and n-butanol extracts had relatively higher flavonoid content of more than 3000 μm as rutin, while that of ethyl acetate and pet ether had slightly lower content. The role of flavonoids in health has been well covered in various literatures, and thus this recorded high

Table 1. Reducing power of different fractions of Kaff Mariam.

| Sample Concentration ($\mu\text{g/ml}$) | % Activity | | | IC ₅₀ |
|--|------------------|------------------|-------------------|------------------|
| | 100 | 200 | 400 | |
| Whole extract | 73.32 \pm 0.06 | 100 \pm 0.12 | 100 \pm 0.31 | 23.63 |
| Pet ether | 0 | 0 | 0 | 0 |
| Ethyl acetate | 16.77 \pm 0.02 | 29.09 \pm 0.07 | 47.33 \pm 0.12 | 472.1 |
| Butanol | 6.29 \pm 0.02 | 8.12 \pm 0.003 | 19.55 \pm 0.009 | 11401.9 |
| Water | 0 | 5.08 \pm 0.004 | 23.53 \pm 0.05 | 2168.99 |
| BHA | 100 \pm 0.14 | 100 \pm 0.05 | 100 \pm 0.08 | N/A |
| BHT | 100 \pm 0.20 | 100 \pm 0.09 | 100 \pm 0.06 | N/A |

Standard curve equation: $y=0.0039x$; $R^2=0.7283$

Table 2. DPPH activity of different fractions of Kaff Mariam.

| Sample Concentration ($\mu\text{g/ml}$) | % Activity | | | IC ₅₀ |
|--|-------------------|------------------|-------------------|----------------------|
| | 100 | 200 | 400 | |
| Control | 0 | 0 | 0 | 0 |
| Whole extract | 35.34 \pm 0.003 | 64.88 \pm 0.06 | 72.41 \pm 0.06 | 150.85 |
| Pet ether | 0 | 0 | 14.84 \pm 0.004 | 13448.6 |
| Ethyl acetate | 0 | 32.11 \pm 0.02 | 68.73 \pm 0.04 | 278.3 |
| Butanol | 0 | 12.89 \pm 0.02 | 52.70 \pm 0.03 | 419.18 |
| Water | 0 | 12.17 \pm 0.02 | 49.20 \pm 0.008 | 459.72 |
| BHA | 59.61 \pm 0.14 | 58.55 \pm 0.18 | 65.01 \pm 0.18 | 11.71 |
| BHT | 75.29 \pm 0.04 | 75.3 \pm 0.09 | 75.37 \pm 0.07 | 6x10 ⁻¹⁸⁹ |

content projects higher antioxidant, scavenging, and cytotoxic activity in the corresponding fractions.

Determination of antioxidant activity

Determination of reducing power

Gallic acid was used as a standard for the determination of reducing power of the extracts. As shown in Table 1, the reducing power of the different extracts was determined across the ranges of concentrations (100, 200, and 400 $\mu\text{g/ml}$). Generally, with exception of pet ether, which did not register any reducing power activity by this method, all the remaining extracts registered some good activity. There was a general increase in reducing power activity with increase in concentration of the extracts. From the different extracts activity, the whole extract had the highest IC₅₀ at 23.63, since the lower the IC₅₀, the higher the reducing power and eventually the antioxidant activity. Ethyl acetate followed in activity, while the remaining fractions had graded lower activity, water and butanol, respectively. Standard antioxidants BHA and BHT were used to compare the effectiveness of the method and as shown in the Table 1, registered the highest activity.

DPPH radical scavenging activity

Table 2 shows the antioxidant activity of the different extracts using the DPPH radical scavenging assay. The control registered no activity at all, as expected, and the standard antioxidants BHA and BHT showed very good antioxidant activity across the range of concentration as shown, resulting in very low IC₅₀ values and resultantly very high antioxidant activity. All the extracts showed good activity which increased with increase in concentration of the extracts, giving on average IC₅₀ values of 150 to 460, with exception of pet ether extract that only showed activity at 400 $\mu\text{g/ml}$, and a staggering IC₅₀ of 13448.6. The highest antioxidant activity was registered by the whole extract with an IC₅₀ of 150.85.

ABTS anti-oxidant activity

The antioxidant activity of the extracts was also determined using the ABTS assay. Generally, the best activity was registered using this method as indicated in Table 3. With exception of the pet ether extract which showed the lowest activity (IC₅₀ 1085), the rest of the extracts had very good activity which increased with increasing concentration. The whole extract had the

Table 3. ABTS activity of different fractions of Kaff Mariam.

| Sample | % Activity | | | IC ₅₀ | |
|---------------|-----------------------|-------------|-------------|------------------|---------------------|
| | Concentration (µg/ml) | 100 | 200 | | 400 |
| Control | | 0 | 0 | 0 | |
| Whole extract | | 91.47±0.006 | 91.68±0.001 | 93.18±0.02 | 3x10 ⁻¹³ |
| Pet ether | | 36.25±0.006 | 34.75±0.002 | 45.42±0.13 | 1085 |
| Ethyl acetate | | 65.78±0.01 | 70.36±0.02 | 92.53±0.02 | 51.388 |
| Butanol | | 78.14±0.03 | 80.28±0.03 | 98.51±0.004 | 17.69 |
| Water | | 74.31±0.05 | 85.29±0.008 | 96.16±0.009 | 21.37 |
| BHA | | 99.25±0.002 | 99.57±0.001 | 100±0.18 | 3x10 ⁻³⁸ |
| BHT | | 68.23±0.01 | 79.85±0.008 | 99.36±0.002 | 47.09 |

Table 4. *In vitro* anti-inflammatory activity of tested ethanolic extracts of Kaff Maryam by membrane stabilization test.

| Plant extracts (100 µg/ml) | % Anti-inflammatory activity |
|----------------------------|------------------------------|
| Whole | 57±14.73 |
| Water | 48±10 |
| Pet ether | 16.67±0.58 |
| Ethyl acetate | 7±3.46 |
| Butanol | 28±0 |
| Sodium diclofenac | 71.33±2.31 |

highest activity registering an IC₅₀ value of 3x10⁻¹³, far higher than that of one of the positive controls, BHT which was recorded at 47.09. Similarly, the water and butanol extracts had IC₅₀ values higher than that of BHT.

Figure 4 is a general comparison of the antioxidant activity of the five extracts using the different assays. In all the methods used, the whole extract recorded the highest activity with the lowest IC₅₀ values, while the pet ether extract recorded the lowest activity in all the assays. A general summary indicates that the best method of assay was the ABTS method which generated the highest activities, followed by the DPPH assay, and least on the list was the reducing power assay. All methods show high antioxidant activity of the plant, Kaff Mariam.

Anti-inflammatory activity by HRBC membrane stabilization test

Table 4 summarized the HRBC membrane stabilization effect of the whole ethanolic extract and the different extraction fractions. Accordingly, this is one of the primary reports on the *in vitro* membrane stabilization potential of Kaff Maryam extracts. In this study, the maximum anti-inflammatory activity was found in the whole ethanolic extracts of Kaff Maryam at a concentration of 100 µg/ml (57±14.73), whereas the standard drug diclofenac showed 71.33±2.31%

protection. The other four ethanolic extracts have weak anti-inflammatory activity, with water extracts having a slightly higher one at 48±10%, while the ethyl acetate fraction registered the lowest activity at 7±3.46%.

Cytotoxic activity against HeLa cell line

The cytotoxic activity of the extracts of Kaff Mariam against HeLa cell lines was determined using the MTT assay. With exception of the negative control which showed no cytotoxic effect, all the extracts had an increase activity with increasing concentration. Fytoside, the positive control had the highest activity with an IC₅₀ of 0.00002, and it is anticipated as this is usually in a purified form. The level of increasing activity was registered in a graded level with ethyl acetate fraction having the highest activity, followed by water and pet ether, then the whole extract and finally the butanol fraction registered the lowest activity. The results, as shown in Table 5 indicate a generally high cytotoxic activity in the Kaff Mariam extracts.

DISCUSSION

It is well-known that plant phenolic compounds are highly effective free radical scavengers and antioxidants. Polyphenolic compounds have inhibitory effects on

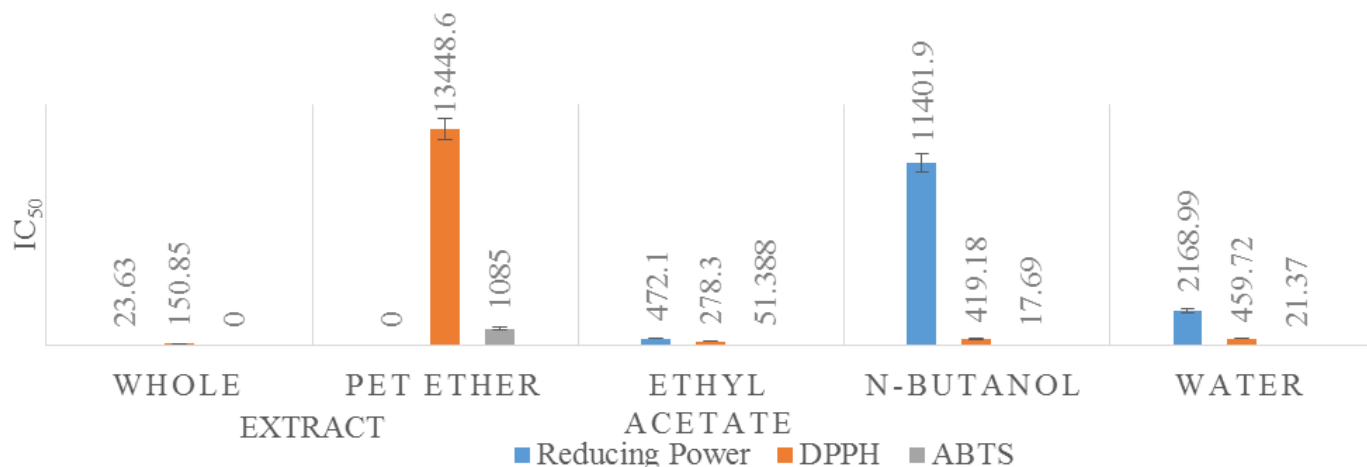


Figure 4. Comparison of IC₅₀ values of the different anti-oxidant assays.

Table 5. Cytotoxic activity of the different ethanolic extracts of Kaff Mariam against HeLa cell lines.

| Sample | % Activity (Cell death) | | | | |
|------------------|-------------------------|------------|------------|------------|------------------|
| | Concentration (µg/ml) | 10 µg/ml | 20 µg/ml | 30 µg/ml | IC ₅₀ |
| Negative control | | 0 | 0 | 0 | 0 |
| Whole extract | | 60.67±1.16 | 64±1.17 | 68±1 | 2.02 |
| Pet ether | | 77.67±3.06 | 81.67±1.53 | 84.67±1.53 | 0.12 |
| Ethyl acetate | | 81.67±2.31 | 82.67±3.51 | 86.33±1.16 | 0.0036 |
| Butanol | | 55.67±12.2 | 83.67±0.58 | 85±1.73 | 7.54 |
| Water | | 78.67±1.16 | 82.33±1.53 | 85.67±0.58 | 0.12 |
| Fytoside | | 77.75±0.5 | 78±3.65 | 80.25±1.26 | 0.00002 |

mutagenesis and carcinogenesis in humans, when up to 1.0 g daily is ingested from a diet rich in fruits and vegetables (Tanaka et al., 1998). The total phenolic content of Kaff Maryam was high and the extracts exhibited potent antioxidant activity. It has also been recognized that flavonoids show antioxidant activity and their effects on human nutrition and health are considerable. The action mechanisms of flavonoids are through the scavenging or chelating process (Kessler et al., 2003). Compounds, such as flavonoids, which contain hydroxyl functional groups, are responsible for the antioxidant effect in the plants (Das and Pereira, 1990). Carotenoids may act as a singlet oxygen quencher and can transfer one electron to the radicals, giving rise to a stable carotenoid radical cation regenerating the original molecule (Mortensen and Skkibsted, 1997). All these factors taken together may influence the performance of the plant with respect to phytochemical content. The total phenolics and total flavonoids of the Kaff Maryam extracts indicate high content within the range of solvents used. The highest content registered by the whole extract could be as a result of the ability of the ethanolic extract

to extract more of the bioactive components, as compared to the rest of the solvents.

Ferric-Ferrous transition to measure the antioxidant activity of Kaff Maryam extracts using the reducing power assay was investigated. The reducing capacity of a compound may serve as a significant indicator of its potential antioxidant activity (Meir et al., 1995). The antioxidant activities of putative antioxidants have been attributed to various mechanisms, among which are prevention of chain initiation, binding of transition metal ion catalysts, decomposition of peroxides, prevention of continued hydrogen abstraction, and radical scavenging (Diplock, 1997). The reducing power of Kaff Maryam showed significant activity across the different ranges of extraction solvent fractions used. However, the activity was low compared to that of the remaining two assays, DPPH and ABTS as subsequently discussed in details. Generally, high reducing power registered indicates a very high antioxidant potential of the plant under study. It exhibited a dose dependent reducing activity within the applied concentrations.

The proton radical scavenging action is known as an

important mechanism of antioxidants. The model of scavenging the stable DPPH radical is a widely used method to evaluate antioxidant activity in a relatively short time compared with other methods. The effect of antioxidants on DPPH radical scavenging was thought to result from their hydrogen donating ability (Shimada et al., 1992). DPPH is a stable free radical and accepts an electron or hydrogen radical to become a stable diamagnetic molecule (Soares et al., 1997). The decrease in absorbance of the DPPH radical caused by antioxidants, because of the reaction between antioxidant molecules and the radical, progresses, which results in the scavenging of the radical by hydrogen donation. It is visually noticeable as a discoloration from purple to yellow. Hence, DPPH is usually used as a substrate to evaluate the anti-oxidative activity of natural antioxidants. Phenolic compounds of Kaff Maryam extracts are probably involved in their antiradical activity (Hsu et al., 2006). Although, the activity is relatively lower than that of BHT and BHA, the extracts may be viable source of bioactive compounds with better activities after fractionation. The results obtained using this method indicate a relatively high antioxidant activity of the plant. The differences in activity arise from the ranges of extraction solvents used. However, in general, all the extracts showed high activity as measured by the DPPH method, an indication of their high antioxidant activity.

The ABTS antioxidant assay can be used to determine the total antioxidant capacity of biological fluids, cells, and tissue. It can also be used to assay the antioxidant activity of naturally occurring or synthetic compounds for use as dietary supplements, topical protection, and therapeutics. The assay measures ABTS⁺ radical cation formation induced by metmyoglobin and hydrogen peroxide. This method was used to assay for the antioxidant activity of the different fractions of Kaff Maryam. A strong dose dependent activity was registered by all the extracts, with the highest activity being shown by the whole extract of ethanol. In general, the ABTS assay generated the highest antioxidant activity as compared to the remaining the assays of reducing power and DPPH assay. The ABTS method could have generated the highest activity due to its various advantages as compared to the former methods of reducing power and DPPH assays (Shalaby and Shanab, 2013).

Results of anti-inflammatory activity by HRBC membrane stabilization demonstrated that ethanolic extracts of Kaff Maryam inhibit heat-induced hemolysis of erythrocytes. This indicates that this extract possess biological membrane stabilization properties preventing stress-induced destruction of the plasma membrane. Stabilization of lysosomal membrane is important in limiting the anti-inflammatory response by preventing the release of lysosomal constituents of activated neutrophils such as bactericidal enzymes and proteases, which cause further tissue inflammation and damage upon

extracellular release (Murugasan et al., 1981). The erythrocyte membrane is analogous to lysosomal membrane and its stabilization implies that the extract may as well stabilize lysosomal membranes.

The *in vitro* cytotoxic activity of extracts of Kaff Maryam against HeLa cell lines was determined using the MTT assay. With exception of the negative control, which showed no effect on the HeLa cell lines, the rest of the extracts showed very high cytotoxic activity, a good step towards the authentication of the use of this plant in therapeutics include cancer management. All extracts showed high cytotoxic activity as compared to their corresponding low antioxidant activity, such as that of the pet ether fraction. This trend of where extracts which show high cytotoxic activity have antioxidant activity, but not vice versa is not a new phenomenon as it has been earlier on demonstrated (Aboul-Enein et al., 2012; Gavamukulya et al., 2014).

An integrated part of cancer cell development is the resistance to programmed cell death (apoptosis) and therefore re-establishment of apoptosis in cancer cells is a target mechanism for anticancer agents (Joshi et al., 1999). Some plant derived products are known to selectively induce apoptosis in cancer cells, which represent the ideal property for successful anticancer agents (Joshi et al., 1999; Wamidh, 2011). The current study showed the highly effective action of the ethanolic extracts of Kaff Maryam and can be used in the management and treatment of cancer and other common diseases. This is in line with a study which showed that any extract had anticancer and cytotoxic activity if it had an IC₅₀ value less than 1000 µg/ml after 24 h contact time, and that the smaller the IC₅₀ value of a test compound, the more toxic the compound (Hirano et al., 1995). The results obtained in this study all have IC₅₀ values far lower than 100, indicating the strong cytotoxic activity of Kaff Maryam.

The present findings exhibited a relation between antioxidant, cytotoxic and anti-inflammatory activities of ethanolic extracts of Kaff Maryam. In this regard, the antioxidant compounds play an important role in the later stages of cancer development. There is increasing evidence that oxidative processes promote carcinogenesis, although the mechanisms are not well understood. The antioxidants may be able to cause the regression of premalignant lesions and inhibit their development into cancer. Several herbs and spices in addition to plenty of other medicinal plants are reportedly exhibiting antioxidant activity (Abou-Elella and Ahmed, 2015; Rajapakse and Kim, 2011; Souza et al., 2011). Majority of the active antioxidant compounds are flavonoids, isoflavones, flavones, anthocyanins, coumarins, lignans, catechins, and isocatechins. In addition, vitamins C and E, β-carotene, and - tocopherol present in natural foods, are known to possess anticancer potential (Abou-Elella and Ahmed, 2015; Genovese et al., 2012). Thus, potential antioxidant, anti-

inflammatory, and anticancer properties of plant extracts (or isolated products of plant origin) can possibly be explored for developing the anticancer drugs.

Conclusion

The objective of this study was to compare the antioxidant activity of extracts of Kaff Maryam using various ethanolic extract fractions, determining anti-inflammatory activities, as well as investigating the *in vitro* cytotoxic activities of the same extracts on HeLa cell lines. Very good antioxidant activity was recorded across the three assays used, as well as relatively high total phenolics and total flavonoids. The extracts were also found to have good anti-inflammatory activity, and high cytotoxic activity against HeLa cancer cell lines. The identification of novel bioactive compounds with cytotoxic properties, and the elucidation of the mechanisms by which the cytotoxic properties derived from the natural products work are of immense importance. The results of this study provide the basis for further investigation of Kaff Maryam for potential identification of novel bioactive compounds with therapeutic and anti-cancer properties.

Conflict of interests

The authors have not declared any conflict of interest exists.

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Full Length Research Paper

Effect of frost on yield and composition of *Aloysia triphylla* essential oil

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Aloysia triphylla, also known as lemon verbena is a medicinal plant with aromatic leaves which produce essential oil rich in citral and has great importance for pharmaceutical and cosmetic industries. The objective of this study was to evaluate the effect of frost on the chemical composition and yield of *A. triphylla* essential oil. The experiment was conducted at the Campus of UFSM in Frederico Westphalen, Rio Grande do Sul, in July 2014. A complete randomized design with three repetitions was used where three random collections and extractions occurred before and after the occurrence of frost. The oil was obtained via hydrodistillation and analyzed by a process of gas chromatography and mass spectrometry. The total yield obtained from leaves before frost was 0.162% and 0.187% after frost. Essential oil composition was largely not affected by the presence of frost; however, of the macro components, limonene was observed to be most sensitive to frost, accounting for 14.36% of essential oil content before and 10.15% after frost.

Key words: Chemical composition, secondary metabolites, low temperatures.

INTRODUCTION

Aloysia triphylla is synonymous with *Aloysia citriodora* Palau, *Lippia citriodora* (Lam.) Kunth, and *L. triphylla* (L. Her.) Kuntze and also known as Lemon verbena. Native to South America, it is an herb rich in essential oil with known astringent properties (Lira et al., 2013). Its leaves have great medicinal importance and have been shown to have antipyretic and antispasmodic properties, and are known to aid in digestion (El-Hawary et al., 2012). One of

the chemicals of greatest interest of this essential oil is citral, due to its importance in the development of pharmaceuticals (Rojas et al., 2012).

In recent years, several studies have been conducted around the world with regards to the production of this species (Ali et al., 2011; Rojas et al., 2012; Felgines et al., 2014). Great differences in the yield and composition of essential oil distilled from the leaves of *A. triphylla* have

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been noted in plants collected from different seasons of the year and in different regions of the world (Lira et al., 2013; Díaz et al., 2007).

The biosynthesis of essential oils in plants is influenced by seasonal variation, such as temperature, photoperiod, relative humidity, precipitation, and solar intensity, and can be determinants of optimal harvest times used to analyze periods of greater essential oil yield or the yield of a specific component. In the state of Rio Grande do Sul, the temperature most favorable to the growth and development of *A. triphylla* occurs between September and April where the average temperature ranges from 12 to 25°C. During winter there is a general reduction in the plant's growth rate, likely related to a decrease in average daily temperatures.

According to Paulus et al. (2013), *A. triphylla* cultivated in the slightly more northern state of Paraná began losing leaves between the months of May and June, at a gradual rate of 40%; the authors observed a total loss of leaves in July due to the occurrence of frost which made it impossible to harvest leaves for analysis.

Leaves damaged by frost can display lower rates of respiration, the inhibition of protein synthesis, and a degradation of proteins which disrupts the plasma membrane during cooling (Taiz and Zeiger, 2013). Some studies claim that when subjected to stress conditions, the plants produce a greater amount of essential oil (Abreu and Mazzafera, 2005). The frequency and duration of these stress periods often results in more serious consequences which can threaten the overall health of a plant. The chemical composition of essential oil is genetically determined; however several abiotic factors such as light, temperature, and water availability can significantly modify the production of secondary metabolites. For species of *A. triphylla*, production and composition of essential oil appears to be due to climatic conditions, especially temperature and radiation (Paulus et al., 2013). The effect of frost on essential oil composition and production is relatively unknown.

The identification of limiting climatic conditions for essential oil production is an important aspect for systems of commercial production. This study aimed to evaluate the effect of frost on the composition and yield of essential oil production, as well as identify major compounds in the essential oil of *A. triphylla*.

MATERIALS AND METHODS

The experiment was conducted in fields belonging to the Federal University of Santa Maria, Campus of Frederico Westphalen, in Rio Grande do Sul, Brazil, latitude 27° 23'26" S; longitude 53° 25'43", altitude 4613 m in the city of Frederico Westphalen. The climate, according to Koppen climate classification is Cfa, with distinct seasons throughout the year and mean temperatures for the hottest month exceeding 22°C and for the coldest month near 3°C, with the possible formation of frost during the winter (mainly in July and August). Rainfall is well distributed over the months of the year, and the local soil is a typical dystrophic Red Latosol.

The seedlings were originally grown from cuttings 15 to 20 cm

long, which were taken from healthy branches of mature plants; a 1000 ppm dose of indole butyric acid was applied to these cuttings. The cuttings were then placed to root in trays with 96 seedling tubes of medium size, which were filled with a mixture of commercial substrate and vermiculite in a 1:1 ratio and placed in a protected environment with sprinkler irrigation until the full development of the root system within the core of the plant.

After the preparation of seedlings, transplanting occurred in previously prepared plant beds. Each experimental unit was composed of an *A. triphylla* plant, with 0.8 m of space between plants and 1.0 m between rows. The transplant was performed on November 23, 2011. The plants were hand weeded and provided water through drip irrigation.

The experiment used a complete randomized design which resulted in three collections from different plants before frost, and three collections from additional plants after frost; each collection resulted in an independent process of hydrodistillation. The collection of samples was performed in July 2014, which is considered the coldest month of the year in the region. Samples were collected in different weather conditions: the first in a typical winter day with low temperatures (the average temperature was 4.6°C) but without the occurrence of frost, and the second collection after the occurrence of frost (average temperature for the day was 2.6°C).

In order to assess the effect of frost on the yield (%) and composition of the essential oil, the date of collection for the two groups (before and after the presence of frost) was based on a forecast from the previous day and the probability of occurrence of frost (on July 23rd). After the frost, additional samples of plant material were unable to be taken as a result of the total leaf senescence due to the formation of frost.

The air temperature values were obtained from the Climatological Station INMET (National Institute of Meteorology) located 50 m from the experiment, the station is linked to the Agroclimatology Laboratory of the Federal University of Santa Maria. For collection the day before frost, mean maximum temperature was 5.2°C and the mean minimum temperature was 4.2°C with a minimum of 2.2°C; for collection after the presence of frost, the mean maximum was 3.5°C and the mean minimum was 1.9°C with a minimum recorded temperature of -1.8°C.

The essential oil was obtained from fresh material (leaves) by hydrodistillation, as accepted in the literature (Argyropoulou, et al., 2007; Gomes et al., 2006). Three samples consisting of 200 g of fresh leaves were used in the calculation of the total yield; the leaves were removed from the apical, middle and basal branches and chopped into fractions of approximately 1 cm. The collection of plant material took place at 14:00 h in order to let the leaves dry after the morning frost and dew. The extraction time was 3 h (Schwerz et al., 2015). After obtaining the essential oil, the yield (%) was measured and calculated by the formula: % T = oil mass (g) / 200 g x 100. Three extractions were performed for the calculation of each total yield (before and after frost).

The chemical composition of the essential oil was determined with a gas chromatograph-mass spectrometry system: Agilent Technologies 6890N GC-FID equipped with a capillary column DB-5 (30 x 0.25 mm; 0.25 mm film thickness) and connected to a FID detector. The temperatures of the detector and the injector were adjusted to 280°C. Helium gas was used at a flow rate of 1.3 ml min⁻¹. The temperature program was from 50 to 300°C at a rate of 5°C min⁻¹. One microliter of essential oil was added to the chromatograph.

The percent area of the compound was calculated based on the areas of the GC peaks without the use of correction factors. In addition to GC-MS results, essential oil 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, 2009). The results were submitted to analysis of variance

Table 1. Chemical composition and yield of essential oil (%) for leaves of *Aloysia triphylla* before and after the occurrence of frost in Frederico Westphalen – RS, 2014.

| Peak | ^a Constituent | ^b IK Reference | Treatments | | | |
|-----------------------------------|--------------------------|---------------------------|---------------------------|--------------|---------------------------|--------------|
| | | | Before frost | | After frost | |
| | | | ^c IK calculate | Area (%) | ^c IK calculate | Area (%) |
| Others | | | | 4.51 | | 6.69 |
| 1 | 1-Octen-3-ol | 974 | 982 | 0.74 | 982 | 0.68 |
| 2 | 6-Metil-5-hepten-2-ona | 986 | 991 | 3.77 | 991 | 6.01 |
| Monoterpene hydrocarbons | | | | 16.36 | | 12.68 |
| 3 | Limoneno | 1028 | 1027 | 14.36 | 1027 | 10.16 |
| 4 | β -Ocimeno | 1053 | 1049 | 2.00 | 1048 | 2.52 |
| Oxygenated monoterpenes | | | | 57.33 | | 53.70 |
| 5 | Linalol | 1097 | 1101 | 0.56 | 1101 | 0.51 |
| 6 | α -Ciclocitral | 1102 | 1103 | 0.81 | 1103 | 0.79 |
| 7 | Z-p-Menten-2,8-dienol | 1122 | 1122 | 0.53 | - | - |
| 8 | E-Verbenol | 1144 | 1139 | 2.19 | 1139 | 1.96 |
| 9 | E-Crisantemal | * | 1150 | 0.88 | 1151 | 1.18 |
| 10 | Z-Isocitral | 1164 | 1167 | 2.21 | 1166 | 2.11 |
| 11 | E-Isocitral | 1180 | 1184 | 4.43 | 1184 | 3.83 |
| 12 | Z-Geraniol | 1230 | 1230 | 1.05 | 1230 | 1.19 |
| 13 | β -Citral (Neral) | 1240 | 1242 | 15.89 | 1242 | 13.26 |
| 14 | E-Geraniol | 1260 | 1256 | 0.81 | 1256 | 1.05 |
| 15 | α -Citral | 1270 | 1272 | 27.21 | 1271 | 26.77 |
| 16 | Acetato de geranila | 1381 | 1385 | 0.76 | 1385 | 1.05 |
| Sesquiterpene hydrocarbons | | | | 7.37 | | 7.52 |
| 17 | α -Cedreno | 1412 | 1413 | 0.41 | 1412 | 0.35 |
| 18 | β -Cariofileno | 1420 | 1420 | 4.08 | 1421 | 3.1 |
| 19 | Elixeno | 1492 | 1498 | 1.48 | 1498 | 1.76 |
| 20 | β -Curcumeno | 1516 | 1513 | 0.91 | 1514 | 1.18 |
| 21 | δ -Cadineno | 1524 | 1524 | 0.49 | 1525 | 1.13 |
| Oxygenated sesquiterpenes | | | | 5.57 | | 8.86 |
| 22 | E-Nerolidol | 1563 | 1565 | 0.99 | 1565 | 2.32 |
| 23 | Espatuleno | 1580 | 1579 | 2.05 | 1579 | 2.61 |
| 24 | Óxido de cariofileno | 1584 | 1585 | 1.22 | 1585 | 1.55 |
| 25 | Helifolen-12-al A | 1593 | 1594 | 0.34 | 1594 | 0.59 |
| 26 | δ -Cadinol | 1646 | 1643 | 0.97 | 1643 | 1.79 |
| Total identified (%) | | | | 91.14 | | 89.45 |
| Yield (%) | | | | 0.162 | | 0.187 |

^aConstituent listed in order of elution from DB-5 column. ^bIdentification based on Kovats Index (IK); ^cidentification based on comparison of mass spectra; * Kovats index not reported.

using the computational program 'Statistical Analysis System' (SAS, 2003).

RESULTS

In the essential oil extracted for leaves of *A. triphylla*, 26

constituents were detected identified from GC-MS analysis and listed by elution time (Table 1). Limonene, citral, and neral were the dominant chemical constituents of the essential oil making up 57.56% of the oil before frost and 50.19% of the oil after frost. Oxygenated monoterpenes made up the majority of the essential oil

with 57.33% composition before frost and 53.70% after frost.

The major compounds identified in the essential oil were citral, neral, and limonene: citral varied between 27.21% before and 26.77% after frost, neral between 15.89% before and 13.26% after frost, and Limonene 14.36% before and 10.15% after frost. The analysis of variance showed no significance between the before and after frost test samples.

DISCUSSION

The essential oil yield of 0.162 and 0.187% observed before and after the formation of frost (Table 1), respectively, is in agreement with results found by Paulus et al. (2013) who found lower essential oil content of *A. triphylla* during the winter, ranging from 0.07 to 0.27%. Taiz and Zeiger (2013) argue that a reduction in temperature and other weather elements can reduce the primary and secondary metabolism of the plant, causing cellular stress, and a lower rate of essential oil synthesis. According to Correa (1994), under stress conditions there is an increase in the production of trichomes on the leaves, with an inverse relationship in essential oil production.

The main visual observation was of tissue necrosis and subsequent senescence and death of leaves, as observed on the 23rd of July, 2014 where temperatures ranged from 0.9 to -1.8°C for a total of eight hours; this characterizes the species as sensitive to frost.

The essential oil storage of Lamiaceae family plants usually occurs in peltate glandular trichomes which are located both on the adaxial and abaxial leaf faces (Gattuso et al., 2008). As the essential oil does not freeze, it is likely that the cells of these trichomes are not as strongly affected. The cells contain concentrations of oil and low concentration of water; this helps to keep the structure of their biomembranes intact in the presence of frost. Similarly, as noted in this study, frost only had an effect on leaf necrosis and senescence; it had no influence on the yield and composition of the oil, likely because of its storage in trichomes, allowing its later extraction.

The commercial importance of essential oil of *A. triphylla* depends on the percentage neral among other constituents, as well as a low percentage of other undesirable components such as nerol and geraniol, which are oxidative forms of neral and geraniol and affect overall composition (Tabatavaie and Nazari, 2007). These oxidative components (nerol and geraniol) were found in low amounts in the essential oil described in this study.

The results of this study demonstrate the importance of crop management on a commercial scale during the winter. Under conditions of low temperatures and frost it is common for leaf necrosis and senescence to occur. As the results showed, it is possible to harvest and extract oil

after the presence of frost; however, within two days was the observed natural rate of ensuing leaf senescence. Thus it is possible to optimize the extraction of essential oil, without a decrease in strength and composition, after the occurrence of frost.

Conclusions

The yield and overall chemical composition of essential oil from *A. triphylla* are not significantly, by analysis of variance, influenced by the occurrence of frost; however, of the macro components, limonene was observed to be most sensitive to frost, accounting for 14.36% of essential oil content before and 10.15% after frost.

Conflict of Interests

The authors have not declared any conflict of interest.

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