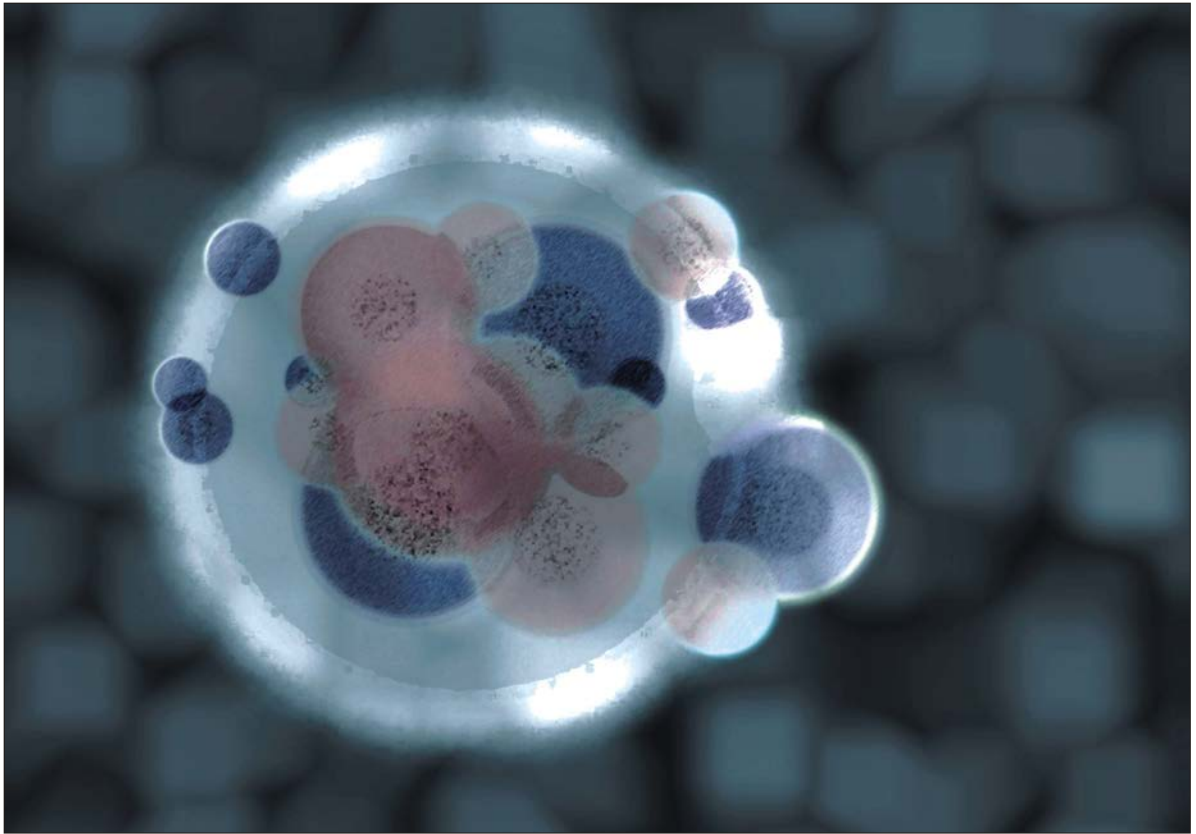

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In vitro Antioxidant Potential and Antiprotozoal Activity of Methanolic Extract of *Mentha longifolia* and *Origanum syriacum*

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Abstract: Labiatae family is represented in Saudi Arabia. It is one of the most distinctive families of flowering plants with a great diversity. This study aimed to investigate *in vitro* antioxidant and antiprotozoal activities of methanolic extract of *Mentha longifolia* (ML) and *Origanum syriacum* (OS). OS exhibited high phenolic contents (mg gallic acid equivalent g^{-1} weight) 87±3.5 mg GAE g^{-1}. ML produced 23±3.2 mg GAE g^{-1}. Using potassium ferricyanide method, OS exhibited high absorbance after 120 h (0.53±0.037) that was directly proportional to the reducing power, however, ML produced lower absorbance (0.38±0.031). Percentage scavenging activity against diphenyl-2-picryl-hydrazyl radical was 875 (OS) and 128% (ML). Absorbencies of ferric thiocyanate assay was 0.86 and 0.25 for ML and OS, respectively. Using phosphomolybdenum method, OS exhibited antioxidant activity (80%) followed by ML (55%). Percentage superoxide scavenging activities was 47 (OS) and 15% (ML). OS exhibited antimalarial activity against *P. falciparum*-K1 strain in contrast to ML. OS extract exhibited activity against *T. cruzi*. ML exhibited activity against *T. cruzi*. OS exhibited activity against *T. b. brucei*. However, ML showed no marked activity against *T. b. brucei*. ML and OS exhibited no marked activity against *L. infantum*. Both ML and OS exhibited no marked cytotoxic activity against MRC-5 cells. It was concluded that, ML and OS have the potency to act as powerful antioxidants and to some extent as antiprotozoal.

Key words: *Organum*, *Mentha*, antioxidant, antiprotozoal, cytotoxicity, *in vitro*

INTRODUCTION

Plants have important roles in amelioration of several diseases especially in many countries. The WHO reported that 80% of the world population depend mainly on traditional medicine (WHO, 2002). Saudi Arabia medicinal plants constitute around 12% of the total number of its floral species. The total number of endogenous plant species being 2300, among them 300 species, belonging to 72 families, are considered as medicinal plants. The Labiatae, or the mint family (recently named Lamiaceae) consist of about 200 genuses and between two or 5000 species of fragrant plant and short shrubs having a large diversity and around 22 species are considered to be medicinal plants (Ronald, 1974; Heyood, 1988; Hay and Waterman, 1993; Jun *et al*., 2001). Both *Mentha longifolia* L. (ML) and *Origanum syriacum* L. (OS) belong to family Lamiaceae (Gazzer and Watson, 1970).

The ML is a rich source of polyphenolic compounds that have strong antioxidant properties used in alimentation industry that prevent the oxidation (Ansari and Goodarznia, 2012). The antimicrobial property of ML herbal essence has been proven and has a vast industrial, pharmaceutical and cosmetic use and its consumption is increasing in the world (Edris *et al*., 2003; Bimakr *et al*., 2008; Camila and Meireles, 2010; Kizil *et al*., 2010). ML has antispasmodic, antiseptic and emmenagogue effects. Besides, it is used for the different kinds of chewing gums, beverages, cosmetic products, perfumes, etc. (Leung and Faster, 2003; Dai *et al*., 2010).

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The OS which in arabic is called "doash" or "bardakush". It is a herbaceous and perennial plant characterized by its minty flavour and health promotion features. It is native to southern Europe and the Mediterranean and it is cultivated in and around the western region of Saudi Arabia particularly around the Madinah province. Traditionally, it is used in folk medicine against asthma, indigestion, headache and rheumatism. However, little is known about the biologically active compounds of marjoram as a medicinal plant, except for its essential oil (Novak *et al*., 2000). It has been claimed that, doash possesses antispasmodic, carminative, diaphoretic and diuretic properties (Jun *et al*., 2001).

Infections caused by several protozoa such as *Trypanosoma*, *Plasmodium* and *Leishmania* are a major worldwide health problem causing significant morbidity and mortality in Africa, Asia and South America. The difficulty of creating efficient vaccines underlines the continuous need for effective drugs with low toxicity and low cost. *Leishmania* parasite affects around 12 million people living mostly in tropical and subtropical areas in more than 80 countries including Saudi Arabia (WHO, 2002; Desjeux, 2004). *Leishmania* has a wide range of clinical symptoms, varying from self-healing ulcers in cutaneous leishmaniasis to progressive nasopharyngal involvement (mucocutaneous leishmaniasis) and disseminating visceral leishmaniasis. Drugs that are currently used to treat leishmaniasis (most commonly pentavalent antimonials) have many limitations including the requirement for intravenous or intramuscular injection, long courses of treatment, toxic side effects, high costs and a growing resistance of the parasite to the drugs (Vannier-Santos *et al*., 2002; Monzote, 2009). *Plasmodium falciparum* causes malaria that kills between one and two million people annually. The discovery of quinine, artemisinin and atovaquone in the last few decades for the treatment of malaria raised the hope for the discovery of even better antimalarial agents from natural sources (Snow *et al*., 2005). *Trypanosoma brucei brucei* causes sleeping sickness while *T. cruzi* causes Chagas disease in South America. It is a major cause of morbidity and mortality in Africa (Gross *et al*., 1999; Remme *et al*., 2002). It is estimated that, 11-18 million people in South America are infected by Chagas disease and 100,000 people are at risk each year (Barrett *et al*., 2003). The current antitrypanosomal drugs may cause severe side effects, require lengthy parenteral administration, lack efficacy and are unaffordable for most of the patients (Barrett *et al*., 2003).

The aim of the present study was to go insight the antioxidative potentials and antiprotozoal activity against malaria, *Leishmania* and *Trypanosoma* of methanolic extracts of ML and OS grown in Madinah province which is located in the western region, Saudi Arabia.

MATERIALS AND METHODS

Chemicals: All solvents and chemicals used in this study were of highest grade and purchased from Sigma-Aldrich (USA) and Acros (Belgium). The 1,1' Diphenyl-2-picryl-hydrazyl (DPPH) radical, α-tocopherol (vitamin E), ammonium molybdate were purchased from Acros (Belgium). Folin-Ciocalteu reagent (2N), dimethyl sulfoxide (DMSO), phenazine methosulphate (PMS), nicotinamide adenine dinucleotide (NADH) and nitroblue tetrazolium (NBT) were purchased from Sigma-Aldrich (USA). All reference drugs were either obtained from the fine chemical supplier Sigma-Aldrich (Bornem, Belgium) or from WHO-TDR.

Plant material: ML and OS leaves were collected from Madinah, Saudi Arabia. A herbarium specimen of each collected plant was prepared and kept at the herbarium of the Department of Natural Products and Alternative Medicine, Faculty of Pharmacy, King Abdulaziz University. The collected plants were identified by the staff of the Department of Taxonomy, Faculty of Science, King Abdulaziz University, Jeddah, Saudi Arabia.

Extraction: ML and OS were dried in open clean air environment for 3 days, followed by grinding to fine powder. Each of the ML (200 g dry weight) and OS (400 g dry weight) powder was extracted twice with 1000 mL absolute methanol using ultrataurix. The solvent was distilled off under reduced pressure using a Buchi rotary evaporator and the dried ML and OS methanolic extracts (28 and 66 g, respectively) were kept at 4°C till biological tests. The selection of methanol as the organic solvent was based on the fact that, methanol when mixed with water facilitates the solubility of the bioactive compounds compared to pure water.

Determination of total soluble phenolic contents: The total phenolic contents of ML and OS extract was determined using Folin-Ciocalteu reagent (Erkan *et al*., 2008). One milliliter of each plant extract solution, was mixed with 7.5 mL of Folin-Ciocalteu reagent which was diluted 10×with dist. H_2O. After standing at room temperature for 5 min, 7.5 mL of 60 mg mL^{-1} of aqueous Na_2CO_3 solution were added. The mixture was kept at room temperature for 2 h and then the absorbance was measured at 725 nm spectrophotometerically. The results were expressed in Gallic Acid Equivalents (GAE), per g of dry weight from the calibration curve of gallic acid. The experiment was also conducted using vitamin E (0.1 mg mL^{-1}) as a standard antioxidant. The samples were analyzed in triplicates.

Determination of the reducing power using potassium ferricyanide: One mililiter of ML or OS plant extract solution (0.1 mg mL^{-1}), was mixed with 2.5 mL of 0.2 M sodium phosphate buffer (pH = 6.6) and 2.5 mL of 1% potassium ferricyanide. The mixture was incubated in water bath at 50°C for 20 min. Then, 2.5 mL of 10% trichloroacetic acid were added to the mixture followed by centrifugation at 4000 rpm for 10 min. The supernatant (2.5 mL) was then mixed with 2.5 mL dist. H_2O and 0.5 mL of 0.1% $FeCl_3$ solution (Oyaizu, 1986). The intensity of the blue green color was measured at 700 nm spectrophotometerically. The increase in absorbance of the reaction mixture indicated higher reducing power of the sample. The experiment was also carried out using vitamin E (0.1 mg mL^{-1}) as standard antioxidant. The samples were analyzed in triplicates.

DPPH free radical-scavenging assay: A 0.06 mM solution of DPPH in ethanol was prepared. The initial absorbance of the DPPH in ethanol was measured at 517 nm and did not change throughout the period of assay. A 0.5 mL solution of the ML or OS extract at concentration of 0.1 mg mL^{-1} was added to 3.5 mL of ethanolic DPPH solution. The change in absorbance at 517 nm was measured at 30 min and free radical scavenging activity was calculated as inhibition using following equation:

$$\text{DPPH radical scavenging activity (\%)} = 1-\left[\frac{A_s}{A_c}\right]\times 100$$

where, A_s is absorbance of the DPPH solution containing samples. A_c: absorbance of the control solution without sample but with DPPH (Leong and Shui, 2002). The percentages of DPPH reduced were plotted against the samples. The experiment was also carried out using vitamin E (0.1 mg mL^{-1}) as a standard antioxidant. The samples were analyzed in triplicates.

Antioxidant activity using linoleic acid peroxidation (ferric thiocyanate test) assay: The linoleic acid emulsion was prepared by emulsification of 0.28 g linoleic acid with 0.28 g Tween 40 and 50 mL 0.2 M phosphate buffer (pH 7.0). Each of the ML or OS extract (0.1 mg mL^{-1}), was mixed with 2.5 mL linoleic acid emulsion and equal volume of phosphate buffer. The mixture was incubated at 37°C for 5 days. A negative control was conducted. Aliquots of a volume of 0.1 mL were taken from the incubation mixture every day and mixed with 75% ethanol, 0.1 mL 30% ammonium thiocyanate and 0.1 mL of 20 mM $FeCl_2$ in 3.5% HCl and allowed to stand at room temperature for 3 min followed by measuring the intensity of the developed color (resulted from oxidation of Fe^{+2} to Fe^{+3} which form a complex with thiocyanate) at 500 nm. The degree of linoleic acid peroxidation was calculated using following equation:

$$\text{Inhibition of lipid peroxidation (\%)} = 100-\left[\frac{A_s}{A_c}\right]\times 100$$

where, A_s is absorbance of sample, A_c is absorbance of control which contains only linoleic acid emulsion and sodium phosphate buffer (Yen and Hsieh, 1998). The experiment was also carried out using vitamin E (0.1 mg mL^{-1}) as a standard antioxidant. The samples were analyzed in triplicates.

Determination of antioxidant activity using phosphomolybdenum assay: An aliquot of 0.2 mL of ML or OS solution (0.1 mg mL^{-1}) was placed in a 4 mL vial with 2 mL of reagent solution (0.6 M sulfuric acid, 28 mM sodium phosphate and 4 mM ammonium molybdate). The vials were capped and incubated in water bath at 95°C for 90 min followed by cooling to room temperature and the absorbance of the mixture was measured at 695 nm against a blank. The antioxidant activity was expressed as mg of α-tocopherol equivalents g^{-1} dry weight (Prieto *et al*., 1999). The experiment was also carried out using vitamin E (0.1 mg mL^{-1}) as a standard antioxidant. The samples were analyzed in triplicates.

Determination of anion superoxide-scavenging activity: The superoxide anion was generated in a PMS-NADH system by oxidation of NADH and assayed by the reduction of NBT. The reaction mixture, containing 0.2 mL of each of ML or OS (0.1 mg mL^{-1}), 0.2 mL of 60 μM PMS, 0.2 mL of 677 μM NADH and 0.2 mL of 144 μM NBT, was incubated at room temperature for 5 min. Then the absorbance was read at 560 nm (Ozsoy *et al*., 2008). All solutions were prepared in a 0.1 M phosphate buffer pH 7.4. The experiment was also carried out using vitamin E (0.1 mg mL^{-1}) as a standard antioxidant. The samples were analyzed in triplicates. The scavenging activity was calculated as follows:

$$\text{Inhibition of superoxide ion (\%)} = \left[\frac{(A_c - A_s)}{A_c}\right]\times 100$$

where, A_c is absorbance of control reaction and A_s is absorbance in presence of sample.

Biological assays: Whole-cell *in vitro* assays were performed according to procedures reported by Vik *et al.* (2009) at the WHO-TDR supported screening center at the Laboratory of Microbiology, Parasitology and Hygiene (LMPH), Faculty of Pharmaceutical Sciences, Biomedical and Veterinary Sciences- University of Antwerp, Belgium using the standard protocols used in WHO-TDR Drug Discovery Network.

Reference drugs: For the different tests, appropriate reference drugs were used as positive control. Tamoxifen for MRC-5, chloroquine for *P. falciparum*-K1, miltefosin for *L. infantum*, benznidazole for *T. cruzi* and suramin for *T. b. brucei*.

Test plate production: The experiments were performed in 96-well plates (Greiner, Bio-One, Wemmel, Belgium), each plate containing 16 samples at 4-fold dilutions in a dose-titration range of 64-0.25 μg mL^{-1}. Dilutions were carried out by a programmable precision robotic station (Biomek 2000, Beckman, Brea, CA 92822, USA). Each plate also contained medium-controls (blanks: 0% growth), infected untreated controls (negative control: 100% growth) and reference controls (positive control). All tests were run in duplicate.

Antiplasmodial activity: The chloroquine-sensitive *P. falciparum*-K1strain was used to test plant extracts according to procedures reported by Vik *et al.* (2009) and Makler *et al.* (1993). Percentage growth inhibition was calculated compared to the negative blanks.

Antitrypanosomal activity of *T.b. brucei*: Trypomastigotes of *T.b. brucei* Squib-427 strain (suramin-sensitive) were cultured at 37°C and 5% CO_2 in Hirumi-9 medium (Hirumi and Hirumi, 1989), supplemented with 10% Fetal Calf Serum (FCS). Assay was performed according to procedures reported by Vik *et al.* (2009) and Raz *et al.* (1997). *T. cruzi*: Tulahuen CL2 strain (nifurtimox-sensitive) is maintained on MRC-5 cells in Minimal Essential Medium (MEM). The *in vitro* antitrypanosomal activity was determined according to procedures reported by Vik *et al.* (2009) and Buckner *et al.* (1996). The color reaction was read at 540 nm and absorbance values were expressed as a percentage of the blank controls.

Antileishmanial activity: *L. infantum* amastigotes (MHOM/MA(BE)/67) were collected from an infected donor hamster and used to infect primary peritoneal mouse macrophages. The *in vitro* antileishmanial activity was determined according to procedures reported by Vik *et al.* (2009). Parasite burdens were determined microscopically after Giemsa staining and expressed as a percentage of the blank controls without sample.

Cytotoxicity assay: MRC-5 cells were cultivated in MEM medium, supplemented with L-glutamine (20 mM), 16.5 mM $NaHCO_3$ and 5% FCS at 37°C and 5% CO_2. For the assay, 10^4 MRC-5 cells/well were seeded onto the test plates containing the pre-diluted samples and incubated at 37°C and 5% CO_2 for 72 h. Cells viability were determined fluorimetrically after addition of resazurin.

Data analysis: Data are reported as the Mean±SD. of three measurements. The standard error was calculated and the figure were presented using the GraphPad Prism software version 4.0 (San Diego, USA).

RESULTS

Total soluble phenolic contents and reducing power: The determination of total phenolics based on the absorbance values of ML or OS extracts that react with Folin-Ciocalteu reagent and followed by comparing with the standard solution of gallic acid equivalents. The standard curve of gallic acid (Fig. 1a) was done by using gallic acid concentration ranging from 0.01-0.30 mg mL^{-1}. The following equation expressed the absorbance of gallic acid standard solution as a function of concentration:

$$A = 10.071\ C+0.314$$

whereas, A is absorbance at 765 nm and C is gallic acid concentration (mg mL^{-1}). OS exhibited the greater phenolic contents as mg gallic acid equivalent/g weight (mg GAE g^{-1}) for a value of 87±3.5 mg GAE g^{-1} (Fig. 1b). On the other hand, ML produced 24±3.2 mg GAE g^{-1}. The standard antioxidant vitamin E used in this experiment elicited a value of total phenolic contents equal 22±2.2 mg GAE g^{-1}. Figure 1c showed the reducing power of OS and ML at concentration of 0.1 mg mL^{-1} expressed as absorbance at 700 nm, using potassium ferricyanide method. OS exhibited the highest absorbance (0.53±0.037) that was directly proportional to the reducing power. However, ML produced lower absorbance (0.38±0.031).

DPPH free radical-scavenging assay: Free radical scavenging activity of OS and ML extracts were tested using the DPPH method and the results were shown in Fig. 2. In this study, the free radical scavenging ability of each sample was evaluated through recording the change of absorbance produced by the reduction of DPPH. The percentage scavenging activity of against DPPH were 875 and 128% for OS and ML extracts, respectively.

Antioxidant activity using linoleic acid peroxidation (ferric thiocyanate test) assay: The absorbencies of the ferric thiocyanate assay system with ML and OS (0.1 mg mL^{-1}) as well as blank reaction and vitamin E at 500 nm were plotted against time intervals (Fig. 3). It was noticed that the absorbance directly proportional with the time, the highest increase was pronounced after 120 h for each system. The absorbance at 500 nm was 1.1, 0.86, 0.29 and 0.25 for blank, ML, vitamin E and OS, respectively. As expected, the highest absorbance was noticed for the

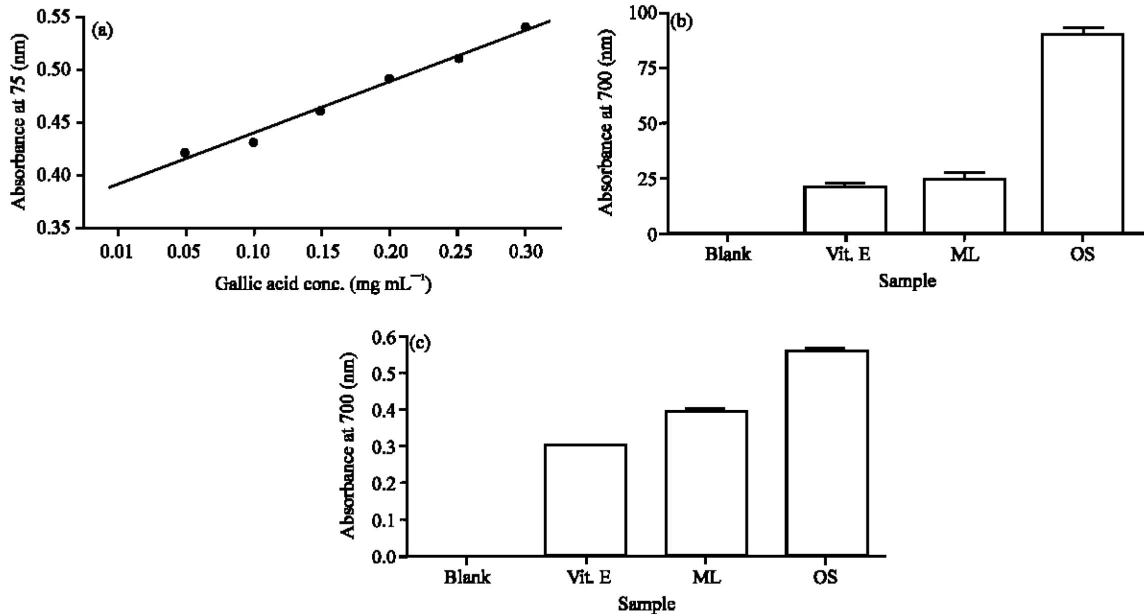

Fig. 1(a-c): (a) Gallic acid standard curve using gallic acid concentrations ranging from 0.01-0.30 mg mL^{-1}, (b) Total soluble phenolic contents of ML and OS extracts determined by Folin-Ciocalteu reagent expressed as gallic acid equivalents (GAE) utilizing absorbance versus concentration curve for gallic acid and (c) Reducing power of ML and OS extracts determined using potassium ferricyamide method and expressed as absorbance at 700 nm. Vitamin E was used as reference antioxidant. The samples were analyzed in triplicates. Values were expressed as Mean±SD

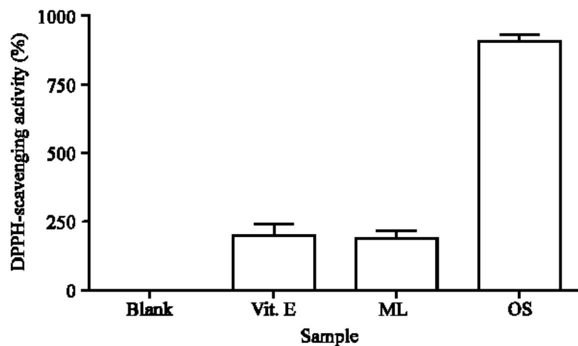

Fig. 2: DPPH free radical scavenging activity of ML and OS extracts determined using diphenyl picrylhydrazyl radical and expressed as % inhibition of DPPH radical. Vitamin E (0.1 mg mL^{-1}) was used as reference antioxidant. The samples were analyzed in triplicates. Values were expressed as Mean±SD

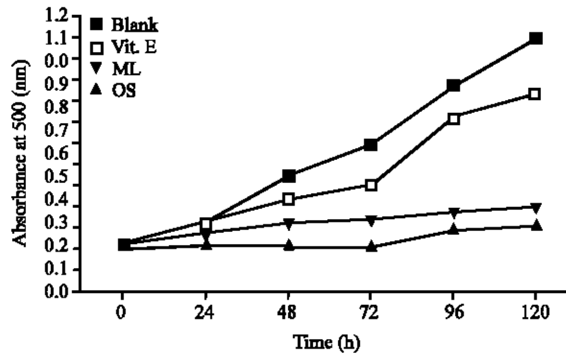

Fig. 3: Absorbance versus time plots for the antioxidant activities of ML and OS extracts at 500 nm using the ferric thiocyanate/linoleic acid peroxidation *in vitro* assay. Vitamin E (0.1 mg mL^{-1}) was used as reference antioxidant. The samples were analyzed in triplicates. Values were expressed as Mean±SD

blank reaction at all time intervals. The ratios of absorbance of OS, ML and vitamin E to the absorbance of control system at 24, 48, 72, 96 and 120 h were presented in Table 1. The increasing order of the ratio was vit. E>OS>ML.

Antioxidant activity using phosphomolybdenum assay: The antioxidant activity of OS and ML (0.1 mg mL^{-1}) was measured using the phosphomolybdenum method. It seemed that, OS exhibited the higher antioxidant activity (80%) followed by ML (55%) and vitamin E (77.1%) (Fig. 4a).

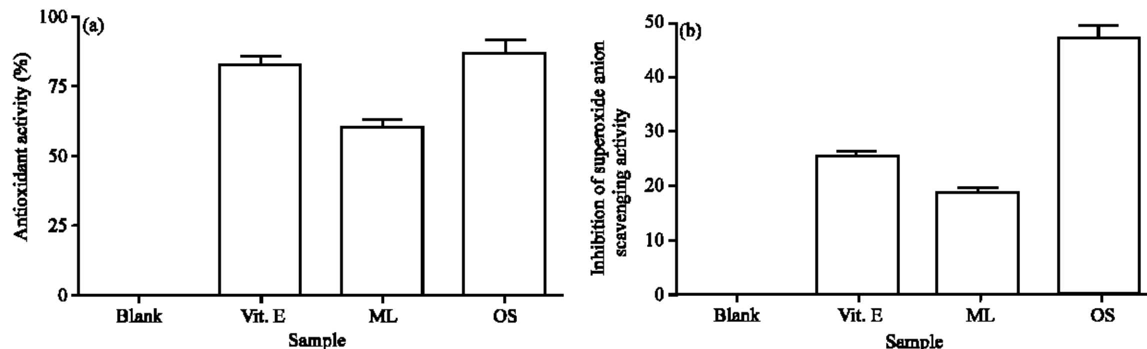

Fig. 4(a-b): (a) Percentage of antioxidant activity of ML and OS extracts using the phosphomolybedenum method and (b) Percentage inhibition of superoxide scavenging activity of ML and OS (0.1 mg mL^{-1} each) using the PMS-NADH system. Expressed as % inhibition of superoxide radical. Vitamin E (0.1 mg mL^{-1}) was used as reference antioxidant. The samples were analyzed in triplicates. Values were expressed as Mean±SD

Table 1: The ratios of absorbance of OS, ML and vitamin E (0.1 mg mL^{-1} for each) at 24, 48, 72, 96 and 120 h to the absorbance of control system at 500 nm determined by linoleic acid/ferric thiocyanate peroxidation *in vitro* assay

	$A_{sample}/A_{control}$				
Sample	24 h	48 h	72 h	96 h	120 h
ML	0.96±0.18	0.80±0.13	0.72±0.16	0.84±0.16	0.78±0.13
OS	0.86±0.20	0.70±0.14	0.65±0.11	0.57±0.09	0.60±0.90
Vit. E	0.80±0.16	0.58±0.07	0.48±0.09	0.38±0.86	0.33±0.06

The absorbance directly proportional with the time, the highest increase was pronounced after 120 h. The increasing order of the ratio was vit. E>OS>ML. Vitamin E (0.1 mg mL^{-1}) was used as a standard antioxidant as a positive control. The samples were analyzed in triplicates. Values were expressed as Mean±SD

Table 2(a-b): (a)Antiprotozoal activity of the methanolic extracts of ML and OS and their cytotoxicity against MRC-5 cell line and (b) Reference validation of certain drugs against MRC5 and antiprotozoal activities

(a)	MRC-5			*T. cruzi*			*L. infantum*			*T.b. brucei*			*P. falciparum*-K1		
	CC_{50}	SI	Sc	IC_{50}	SI	Sc	IC_{50}	SI	Sc	IC_{50}	SI	Sc	IC_{50}	SI	Sc
ML	>64	11.35	<1	38.4	3.41	1	>64	7.13	<1	>64	0.03	<1	>64	0.36	<1
OS	>64	11.35	<1	56.4	3.41	1	>64	7.13	<1	32.81	0.03	1	50.80	0.36	1

(b)	Reference validation	
Screening panel	Reference drug	IC_{50} (µM)
MRC-5	Tamoxifen	11.35
T. cruz	Benznidazol	3.41
L.infantum	Miltefosine	7.13
T.b. brucei	Suramine	0.03
P.falciparum-K1	Chloroquine	0.36

Scores adopted by LMPH (µg mL^{-1}) for evaluation of antiprotozoal and cytotoxic activities of the crude methanolic extracts: *T. cruzi,* score 1: >15, *T.b. brucei,* score 1: >10, *L. infantum,* score 1: >15, *P. falciparum*-K1, score 1: >10, cytotoxicity, score 1: > 30. MRC-5: diploid human embryonic lung fibroblasts, CC_{50}: concentration causing 50% cytotoxicity, IC_{50} (µM): concentration causing 50% inhibition, SI: selectivity index, Sc: Score

Superoxide anion-scavenging activity: The percentage of superoxide scavenging activities of the OS and ML (0.1 mg mL^{-1}) on superoxide radicals are showed in Fig. 4b. The percentage superoxide scavenging activities was 47% in case of OS and 15% in case of ML. Vitamin E produced a percentage = 24%.

Cytotoxic and antiparasitic activity: The extracts were tested *in vitro* for their potential antiprotozoal activity against *T. cruzi, T. b. brucei, L. infantum* and *P. falciparum*-K1, as well as against MRC-5 cell lines for cytotoxicity and evaluation of selectivity. To facilitate interpretation of the IC_{50} values in the different models, semi-quantitative activity scores were introduced (Table 2).

Antimalarial activity: The data in Table 2 revealed that, OS extract exhibited antimalarial activity (score 1) against *P. falciparum*-K1 strain (IC_{50} 50.8 µg mL^{-1}, SI 0.36). However, ML showed no marked antimalarial activity (IC_{50}>64 µg mL^{-1}, SI 0.36).

Antitrypanosomal activity: The data in Table 2 revealed that, OS extract exhibited activity (score 1) against *T. cruzi* (IC_{50}<56.4 μg mL^{-1}, SI 3.41). The ML extract exhibited activity (score 1) against *T. cruzi* (IC_{50}<38.4 μg mL^{-1}, SI 3.41).The OS extract exhibited activity against *T. b. brucei* (IC_{50}<32.81 μg mL^{-1}, SI 0.03). However, ML showed no marked activity against *T.b. brucei* (IC_{50}<64 μg mL^{-1}, SI 0.03).

Antileishmanial activity: The data in Table 2 revealed that, ML and OS extract exhibited no marked activity against *L. infantum* (IC_{50}>64 μg mL^{-1}, SI 7.13).

Cytotoxicity: The results in Table 2 revealed that, both ML and OS extract exhibited no marked cytotoxic activity against MRC-5 cells (IC_{50}>64 μg mL^{-1} l, SI 11.35).

DISCUSSION

Since, ancient times, mankind has used plants to treat common diseases and some of these traditional medicines are still included as part of the habitual treatments of various maladies (Rios and Recios, 2005). The analysis of the plant extracts activities may make possible the design of less expensive therapies to be used in economically unprivileged regions (De Sousa *et al*., 2004). The number and efficacy of drugs available for the treatment of human and animal trypanosomiasis, leishmaniasis, amoebiasis and malaria are limited. In this study we aimed to investigate *in vitro* antioxidant and antiparasitic activity of the crude methanolic extract of ML and OS.

In general, the antiradical and antioxidant activities of plant extract are associated to their phenolic contents (Baydar *et al*., 2006). The phosphomolybdate complex can be reduced to blue products when reacted with phenolic compounds (Vasco *et al*., 2008). In our study, the OS and ML extracts elicited high total phenolic contents may be due to the fact that, it contain phenolic compounds such as flavonoids. It was reported from other study that, ML contains the flavonoid, quercetin-3-O-glycoside which possesses antioxidant activity (Akroum *et al*., 2009). It is clear that, the number of phenolic groups present in the structure of an antioxidant molecule is not always the only factor to determine its antioxidant activity. Positions of the phenolic groups, presence of other functional groups in the molecules such as double bonds and conjugation to phenolic and ketone groups, also play important roles in antioxidant activities and have been reported by another study that studied the activity relationship of antioxidant activity of flavonoids and phenolic acid (Rice-Evans *et al*., 1996). In general, the mentioned factors can be presented as the tension at the phenolic groups. The two phenolic groups of quercetin-3-O-glycoside are attached to different unadjacent benzene rings that give the two OHs the mobility to work freely without hindrance. Additionally, polarity and hydrophobicity of antioxidants plays an important factor in the antioxidant activity especially in the biomembrane systems (Wu *et al*., 2007). The antioxidant activity has been reported to be associated with the reducing power (Tanaka *et al*., 1998). The OS extract exhibited the higher reducing power than ML extract that was higher than vit. E. The antioxidant action of reductones (a class of compounds that has been associated with the reducing property) is based on the breaking of the free radical chain by donating a hydrogen atom. Reductones also react with certain precursors of peroxides, thus preventing peroxide formation (Gordon, 1990). The data presented in this study indicated that, the marked antioxidant activity of OS and ML extracts seemed to be as a result of their reducing power due to the presence of flavonoids.

Plants play a significant role in health promotion through free radical scavenging activity (Sanchez-Moreno, 2002). In DPPH assay, the antioxidant were able to reduce the stable DPPH radical to the yellow-colored DPPH (Frankle and Meyer, 2000). The results obtained from this study indicated that, OS extract yielded high percentage of DPPH scavenging activity than ML that may be attributed to the high reducing power and higher total phenolic contents present in OS extract. Besides, the ferric thiocyanate test determine the antioxidant activity through measurement of the amount of peroxides formed in linoleic acid emulsion of antioxidant during incubation (Singh *et al*., 2007). The present study indicated that, ML extract formed high amount of peroxides than in case of OS extract. The explanation of these findings can be clarified on the basis that, hydrophobicity of antioxidants plays an important role in their solubility in the oil. The hydrophobic antioxidant mixed well with the oil better than the hydrophilic antioxidant(Frankle and Meyer, 2000). The OS extract contains high amount of essential oils comparing with ML extract. The hydrophilic-OH group of polyphenols may not free, so that the hydrophilicity of OS and ML molecule seemed to be more pronounced. The antioxidant activity of ML and OS extracts were measured using the phosphomolybdenum method. The phosphomolybdenum method based on the reduction of Mo^{+4} to Mo^{+5} by the antioxidant compounds and the formation of green Mo^{+5} complex that can be absorbed at 695 nm (Parejo *et al*., 2002). It seemed that, OS extract exhibited higher antioxidant activity followed by vitamin E and ML extract. The antioxidants breaks the free radical

chain by donating a hydrogen atom (Gordon, 1990). This may depend on the presence of polyphenols that were freely present. Superoxide has been observed to directly initiate lipid peroxidation. It has also been reported that antioxidant properties of some flavonoids are effective mainly through scavenging of superoxide anion radical (Yen and Duh, 1994). In this study, the increasing order of the percentage superoxide scavenging activities was OS>vitamin E>ML. These effects may be attributed to the antioxidant activity of the OS and ML extracts.

The OS and ML extracts were tested *in vitro* for their potential antiprotozoal activity against *T. cruzi, T. b. brucei, L. infantum* and *P. falciparum*-K1 strains, as well as against MRC-5 cell lines for cytotoxicity evaluation. To facilitate interpretation of the IC_{50} values in the different models, semi-quantitative activity scores were introduced (Table 2). The ML and OS extracts exhibited score 1 against *T. cruzi*, however, OS extract exhibited activity against *T. cruzi*, *T.b. brucei* and *P. falciparum*-K1. Several investigations attributed the medicinal activity of plant extract to the presence of phenolic compounds (Khan *et al*., 2011) like flavonids and saponins with antifungal activities (Mohanta *et al*., 2007). It was reported that, ML extract possessed antibacterial and antifungal activity (Camila and Meireles, 2010). In our study, both the MO and OS extracts exhibited no pronounced cytotoxic activity against MRC-5 cells. In contrary, another study showed that ML extract reduced the viability of McCoy cells (Razavi *et al*., 2012). The leishmanicidal and trypanosomicidal activity of essential oil derived from OS species (especially eugenol and oregano), were observed (Ueda-Nakamura *et al*., 2006). The flavonoids of ML extract allowed the inhibition of some microorgamisms indicating that they could be used to cure diseases. Among the identified flavonoids, the quercetin-3-O-glycoside that showed the best antibacterial activity and acute toxicity effect which encouraged its use (Akroum *et al*., 2009). We observed that, the ML or OS crude extracts were less active than standard drugs used. These extracts are composed of a large number of different compounds and the active principles might show higher activity in their pure form. Further detailed studies of the active plants for the isolation of major active constituents are in progress. The OS and ML crude extracts will be subjected for further fractionation and these fractions were re-evaluated biologically.

CONCLUSION

OS extract was found to be more effective antioxidant more than ML extract, using *in vitro* assays such as the determination of the total soluble phenolic contents, reducing power, DPPH scavenging activity, superoxide scavenging activity and antioxidant activity estimation using the phosphomolybdenum method. However, determination of antioxidant activity using ferric thiocyanate/linoleic system revealed higher antioxidant power in case of ML due to hydrophobicity of ML that allow proper dissolution in the linoleic acid. The OS extract exhibited activity against *T. cruzi, T. b. brucei* and *P. falciparum*-K1 strains. On the other hand, the ML extract exhibited activity against *T. cruzi*. Based on results achieved, we can concluded that, ML and OS extracts have the potency to act as powerful antioxidants and to some extent as antiprotozoal.

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