

Chemical Investigation and Protective Effects of Bioactive Phytochemicals from *Artemisia ciniformis*

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ABSTRACT: The present study evaluates the phytochemical constituents, antimicrobial, antioxidant capacity, total phenolic content, ferrous ion chelating, tyrosinase inhibition, superoxide anion and nitric oxide radical scavenging activity of the leaf essential oil of *Artemisia ciniformis* Krasch. & Popov ex Poljakov., from Iran. Oxygenated monoterpenes (92.4%), especially camphor (32.2%), 1,8-cineole (22.4%) and trans-pinocarveol (16.8%) were the major components identified in this essential oil. Bactericidal kinetic of the essential oil of *A. ciniformis* indicated that *Acinetobacter baumannii* is the most vulnerable (MIC = 0.02 and MBC = 0.04 mg/ml, D value = 3.57 min). The total phenol content of the essential oil of *A. ciniformis* was estimated to be 206.20 ± 4.58 μ g GAE/mg of the essential oil. The ferric-reducing power of *A. ciniformis* essential oil was determined 0.315 ± 0.08 gallic acid equivalent (mg/g). The essential oil of *A. ciniformis* exhibited a dose-dependent scavenging of DPPH, nitric oxide and superoxide anion radicals with IC₅₀ values of 10.75 mg/mL, 10.63 μ g and 16.81 μ g, respectively. In the β -carotene-linoleic acid test system, oxidation of linoleic acid was effectively inhibited by *A. ciniformis* essential oil ($86.39 \pm 2.53\%$, 0.625 mg/mL essential oil). There was no correlation between ferrous ion chelating activity (IC₅₀ = 220.90 μ g) and total phenolics implying that the essential oil contains no chelating ligands. Anti-tyrosinase activity of *A. ciniformis* essential oil at 50% concentration (IC₅₀) was 6.53 mg. The leaf essential oil of *A. ciniformis* may be exploited as a natural source of bioactive phytochemicals bearing antimicrobial and antioxidant potentials.

KEYWORDS: Antimicrobial; Antioxidant; Radical scavenging activity; Anti-Tyrosinase inhibition; Ferrous ion chelating.

INTRODUCTION

Generation of free radicals is important both in life and in biological systems. Free radicals may attack life important molecules such as DNA and membrane lipids and play a key role in the pathology of numerous chronic diseases including cancer (Haddadi *et al.*, 2011 [1]); therefore, many research groups are currently screening

the different biological activities of the phytochemicals. A great number of simple phenolic compounds as well as plant flavonoids can act as antioxidants. In general, antioxidant and radical scavenging properties of plant essential oils and extracts are associated with the presence of phenolic compounds possessing the ability

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to donate hydrogen to the radical. Numerous reports indicated a good correlation between the RSA and the concentration of phenolic compounds measured by Folin-Ciocalteu method. Also, Secondary antioxidants are responsible for suppressing the formation of radicals and protecting against oxidative damage (Lim *et al.*, 2006 [2]). There are many medicinal plants with high total phenolic content and antioxidant properties. One of the most important of them is *Artemisia*. The genus *Artemisia* belongs to the important family Compositae (Asteraceae) (Rechinger, 1986 [3]). Within this family, *Artemisia* is included in the tribe Anthemideae and comprises over 500 species (Mozaffarian, 1996 [4]). The genus *Artemisia* has always been of great botanical and pharmaceutical interest and is useful in traditional medicines for the treatment of a variety of diseases and complaints (Rustaiyan & Masoudi, 2011 [5]). Among them *Artemisia ciniformis* Krasch. & Popov ex Poljakov., grows naturally in wide regions of Iran. To the best of our knowledge, very little information is available on pharmacological and biological properties of *A. ciniformis* essential oil. Therefore, the aims of this study were to study the antimicrobial property, antioxidant capacity, enzyme inhibition, chelating ability, radical scavenging properties of *A. ciniformis* essential oil from Iran.

EXPERIMENTAL SECTION

Reagents and equipments

Microbial and cell culture media and laboratory reagents were from Merck, Germany. Other chemicals were of analytical grade. Solvents, DMSO, butylated hydroxytoluene (BHT), butylated hydroxy anisole (BHA), Trolox, β -carotene, linoleic acid, gallic acid, DPPH and all other chemicals were from Sigma-Aldrich. The major equipment used were a cleverger apparatus, Shimadzu UV-2501PC spectrophotometer (Shimadzu, Japan) and DNM-9602G ELISA reader (Perlong Group, Beijing, China).

Plant material

The aerial parts of *A. ciniformis* were collected in October 2011 from Baam village, after Gahreman abad in Esfarayen, Province of Khorasan, northeastern Iran. Voucher specimens have been deposited at the herbarium of the Research Institute of Forests and Rangelands, Tehran, Iran. Plant specimen were identified by Dr. Vali-Aallah Mozaffarian

from the same institute. The voucher specimen (No. 12569) has been deposited in the herbarium, Department of Pharmacognosy, Faculty of Pharmacy, Mashhad University of Medical Sciences, Mashhad, Iran.

Isolation of the essential oil

The aerial parts of *A. ciniformis* were dried at room temperature for several days. Air-dried leaves of *A. ciniformis* (100 g) were separately subjected to hydrodistillation using a cleverger-type apparatus for 3 h. After decanting and drying the essential oil over anhydrous sodium sulfate, the essential oil was recovered. Results showed that essential oil yield was 1.05% (w/w).

Gas chromatography

GC analysis was performed on Shimadzu 15A gas chromatograph equipped with a split/splitless injector (25°C) and a flame ionization detector (250°C). Nitrogen was used as carrier gas (1 mL/min) and the capillary column used was DB-5 (50 m \times 0.2 mm, film thickness 0.32 μ m). The column temperature was kept at 60°C for 3 min, and then heated to 220°C with a 5°C/min rate and kept constant at 220°C for 5 min. Relative percentage amount were calculated from peak area using a Shimadzu C-R4A chromatopac without the use of correction factors.

Gas chromatography-Mass spectrometry

Analysis was performed using a Hewlett-Packard 5973 with a HP-5MS column (30 m \times 0.25 mm, film thickness 0.25 μ m). The column temperature was kept at 60°C for 3 min. and programmed to 220°C at a rate of 5°C/min and kept constant at 220°C/min for 5 min. The flow rate of helium as carrier gas with (1 mL/min). MS were taken at 70 eV. The retention indices for all the components were determined according to the Van Den Dool method, using *n*-alkanes as standards. The compounds were identified by (RRI, DB5) with those reported in the literature and by comparison of their mass spectra with the Wiley library or with the published mass spectra (Adams, 2001 [6]).

Antimicrobial activity

Essential oil dilution solvent

Bacterial strains were streaked on Mueller Hinton agar plates using sterile cotton swabs. Five microlitres of dimethylsulphoxide (DMSO), loaded on sterile blank disks,

were placed on the agar plates and were incubated at 37°C for 24 h. There was no antibacterial activity on the plates and hence DMSO was selected as a safe diluting agent for the essential oil. Each essential oil dilution (5mL), followed by sterilization, using a 0.45 µm membrane filter, were added to sterile blank discs. The solvent also served as control (Allahghadri *et al.*, 2010 [7]).

Microbial strain and growth media

Escherichia coli (ATCC25922), *Staphylococcus aureus* (ATCC25923), *Pseudomonas aeruginosa* (ATCC8830), *Candida albicans* (ATCC 5027) and *Acinetobacter baumannii* (ATCC 17978) were employed in the study. Nutrient agar was used. Bacterial suspensions were made in Brain Heart Infusion (BHI) broth to a concentration of approximately 10⁸ cfu/mL. Subsequent dilutions were made from the above mentioned suspension, which were then used in the tests.

Essential oil sterility test

In order to ensure sterility of the essential oils, geometric dilutions, ranging from 0.036 to 72.0 mg/mL of the essential oil, were prepared in a 96-well microtitre plate, including one growth control (BHI + Tween 80) and one sterility control (BHI + Tween 80 + test oil). Plates were incubated under normal atmospheric conditions, at 37°C for 24 h. The contaminating bacterial growth, if at all, was indicated by the presence of a white “pellet” on the well bottom (Allahghadri *et al.*, 2010 [7]).

Disc diffusion method

The agar disc diffusion method was employed for the determination of antimicrobial activities of the essential oils in question. Briefly, 0.1 mL from 10⁸ cfu/mL bacterial suspension was spread on the Mueller Hinton Agar (MHA) plates. Filter paper discs (6 mm in diameter) were impregnated with 5 µL of the undiluted essential oil and placed on the inoculated plates. These plates, after remaining at 4°C for 2 h, were incubated at 37°C for 24 h. The diameters of the inhibition zones were measured in millimeters. All tests were performed in triplicate (Allahghadri *et al.*, 2010 [7]).

Determination of minimum inhibitory (MIC) and bactericidal (MBC) concentrations

All tests were performed in Brain Heart Infusion (BHI) broth supplemented with Tween 80 detergent

(final concentration of 0.5% (v/v)). Test strains were suspended in BHI broth to give a final density of 10⁷ cfu/mL and these were confirmed by viable counts. The Minimal Inhibitory Concentration (MIC) and Minimal Bactericidal Concentration (MBC) were assessed according to our modified procedure (Rasooli & Mirmostafa, 2003 [8]). MIC was determined by a broth dilution method in test tubes as follows: 40 µL from each of various dilutions of the essential oils were added to 5 mL of Brain Heart Infusion (BHI) both in tubes containing 10⁷ cfu/mL of live bacterial cells. The tubes were then incubated on an incubator shaker to evenly disperse the essential oil throughout the broth in tubes. The highest dilution (lowest concentration), showing no visible growth, was regarded as the MIC. Cell suspensions (0.1 mL) from the tubes showing no growth were subcultured on BHI agar plates in triplicate to determine if the inhibition was reversible or permanent. MBC was determined as the highest dilution (lowest concentration) at which no growth occurred on the plates.

Bactericidal kinetics of the essential oil

Forty microlitres of each essential oil at the dilution determined by MBC, was added to each 5 mL of Brain Heart Infusion (BHI) broth in tubes containing bacterial suspension of 10⁷ cfu/mL and were then incubated at 37°C in an incubator shaker. Samples (0.1 mL) were taken after 5, 10, 15, 20, 25, 30, 45, 90, 120, 150, 180, 210 and 240 min. The samples were immediately washed with sterile phosphate buffer, pH 7.0, centrifuged at 10000 rpm, resuspended in the buffer and were then spread-cultured on BHI agar for 24 h at 37°C. Phosphate buffer was used as diluent when needed. Bactericidal experiments were performed three times. Microbial colonies were counted from triplicates after the incubation period and the mean total number of viable cells per mL was calculated. The mean total number of viable bacteria from bactericidal kinetics experiments at each time interval was converted to log₁₀ viable cells using routine mathematical formulae. The trend of bacterial death was plotted graphically. Decimal reduction value (D-value) is calculated as the time (min) required to reduce the viable microbial population by 90%, or a logarithmic value of 1 (Rasooli & Mirmostafa, 2003 [8]).

Total phenolic content assay

Total Phenolic Content (TPC) of essential oil was determined using the Folin-Ciocalteu assay (Kahkonen *et al.*, 1999 [9]). Samples (300 μ L) were introduced into test tubes followed by 1.5 mL of a Folin-Ciocalteu's reagent (10 x dilutions) and 1.2 mL of sodium carbonate (7.5% w/v). The tubes were allowed to stand for 30 min before measuring absorbance at 765 nm. TPC was expressed as gallic acid equivalent (GAE) in mg per 100 g material ($y = 0.001x + 0.0708$; $R^2 = 0.996$).

Antioxidant activity

Ferric-Reducing Antioxidant Power (FRAP) assay of the essential oil

The FRAP assay was carried out according to the procedure employed by Lim and co workers (Lim *et al.*, 2009 [10]). One millilitre of the extract dilution was added to 2.5 mL of 0.2 M potassium phosphate buffer (PH 6.6) and 2.5 mL 1% potassium ferricyanide. The mixture was incubated for 20 min at 50°C, after which 2.5 mL of 10% trichloroacetic acid was added. The mixture was then separated into aliquots of 2.5 mL and mixed with 2.5 ml of deionized water. Then, 0.5 mL of 0.1% (w/v) FeCl_3 was added to each tube and allowed to stand for 30 min. Absorbance for each tube was measured at 700 nm. The FRAP was expressed as gallic acid equivalents (GAE) in mg/g of samples used ($y = 16.263x - 0.0699$; $R^2 = 0.9944$).

Bleaching of 2,2-diphenylpicrylhydrazyl (DPPH)

The hydrogen atom or electron donation abilities of the corresponding extracts and some pure compounds were measured from the bleaching of the purple-colored methanol solution of 2,2-diphenylpicrylhydrazyl (DPPH). This spectrophotometric assay uses the stable radical DPPH as a reagent. Fifty microlitres of 1:5 concentrations of the essential oils in methanol were added to 5 mL of a 0.004% methanol solution of DPPH. Trolox (1 mM) (Sigma-Aldrich), a stable antioxidant, was used as a synthetic reference. The essential oil from *Thymus x-porlock* was used as a natural reference. After a 30 min incubation period at room temperature, the absorbance was read against a blank at 517 nm. Inhibition of free radical by DPPH in percent (I%) was calculated in the following way (Yadegarinia *et al.*, 2006 [11]):

$$I\% = [A_{\text{blank}} - (A_{\text{sample}} / A_{\text{blank}})] \times 100;$$

Where A_{blank} is the absorbance of the control reaction (containing all reagents except the test compound), and A_{sample} is the absorbance of the test compound. The tests were carried out in triplicate ($y = 3.4266x + 13.145$; $R^2 = 0.9838$).

β -Carotene-linoleic acid assay

Antioxidant activity of essential oils was determined using the β -carotene bleaching test (Taga *et al.*, 1984 [12]). Approximately 10 mg of β -carotene (type I synthetic, Sigma-Aldrich) was dissolved in 10 mL of chloroform. The carotene-chloroform solution, 0.2 mL, was pipetted into a boiling flask containing 20 mg linoleic acid (Sigma-Aldrich) and 200 mg Tween 40 (Sigma-Aldrich). Chloroform was removed using a rotary evaporator at 40°C for 5 min and to the residue, 50 ml of distilled water was added, slowly with vigorous agitation, to form an emulsion. The emulsion (5 mL) was added to a tube containing 0.2 mL of essential oil solution, prepared and the absorbance was immediately measured at 470 nm against a blank, consisting of an emulsion without β -carotene. The tubes were placed in a water bath at 50°C and the oxidation of the emulsion was monitored spectrophotometrically by measuring absorbance at 470 nm over a 60 min period. Control samples contained 10 μ L of water instead of essential oils. Butylated hydroxy anisole (BHA; Sigma-Aldrich), a stable antioxidant, was used as a synthetic reference. The antioxidant activity was expressed as inhibition percentage with reference to the control after 60 min of incubation, using the following equation ($y = 78.719x + 38.643$; $R^2 = 0.9795$):

$$AA = 100 (DR_C - DR_S) / DR_C ; \text{ where}$$

AA = antioxidant activity,

DR_C = degradation rate of the control = $[\ln (a/b) / 60]$,

DR_S = degradation rate in presence of the sample = $[\ln (a/b) / 60]$,

a = absorbance at time 0, b = absorbance at 60 min.

Ferrous-Ion Chelating (FIC) assay

FeSO_4 (2 mM) and ferrozine (5 mM) were prepared and diluted 20 times oil (250, 500 and 1000 μ L, diluted to 1 mL) was mixed with 1 mL diluted FeSO_4 , followed by 1 mL of diluted ferrozine. The tubes were mixed well and allowed to stand for 10 min at room temperature. Absorbance of each oil was measured against blank at 562 nm (Lim *et al.*, 2009 [10]). The ability of the sample

to chelate ferrous-ions was calculated and expressed as (oil curve; $y = 0.1638x + 13.815$; $R^2 = 0.99$), (Standard curve for EDTA; $y = 16.226x + 34.893$; $R^2 = 0.9871$):

$$\text{Chelating effect (\%)} = (1 - (A_{\text{sample}} / A_{\text{control}})) \times 100\%$$

Tyrosinase inhibition

Tyrosinase inhibitory activity was determined by a spectrophotometric method, as described by Chan and co workers (Chan *et al.*, 2008 [13]) using a modified dopachrome method with L-DOPA as the substrate. A 5 mg aliquot of the oil was weighed and dissolved in 2 mL of 50% DMSO. Then, 40 μL of sample was added to 80 μL of 0.1 M phosphate buffer (pH 6.8), 40 μL of 0.02 mg/mL tyrosinase and 40 μL of L-DOPA (2.5 mM) in a well of a 96-well microtiter plate. The samples were incubated for 30 min at 37°C. Each sample was accompanied by a blank that contained all components except L-DOPA. Absorbance was measured at 475 nm. Results were compared with the control containing 50% DMSO instead of the sample solution. Quercetin was used as the positive control. The percentage of tyrosinase inhibition was calculated as:

$$\text{Tyrosinase Inhibition(\%)} = [(A_{\text{control}} - A_{\text{sample}}) / A_{\text{control}}] \times 100$$

Anti-tyrosinase activity of the essential oil was also expressed as Quercetin Equivalent (QE) in mg/g of essential oil ($y = 8.6355x - 6.4394$; $R^2 = 0.986$), which were obtained from the following standard (Quercetin) curve: ($y = 11.872x + 16.506$; $R^2 = 0.993$), where, y represents % inhibition and x represents concentration in mg.

Radical scavenging activity

Superoxide anion radical scavenging

The ability of the essential oil to scavenge superoxide anion radicals was determined by the method described by Lee and co workers (Lee *et al.*, 2002 [14]). In brief, to a 100 μL aliquot of dissolved oil the following was added: 100 μL (30 mmol/L) Na_2EDTA , 100 μL (3 mmol/L) hypoxanthine in 50 mmol/L NaOH and 200 μL (1.42 mmol/L) nitroblue tetrazolium (NBT) in NaH_2PO_4 -NaOH (50 mmol/L, pH 7.4). After a 3-min incubation period at room temperature, 100 μL (0.5 U/mL) xanthine oxidase in the NaH_2PO_4 -NaOH buffer was added followed by 2.4 mL NaH_2PO_4 -NaOH buffer. The resulting solution was incubated at room temperature for 20 min and the absorbance at 560 nm was measured. The absorbance

was also measured at 293 nm to detect if the oil inhibited uric acid generation. Once it was confirmed that uric acid formation is not inhibited, then the percentage of inhibition at 560 nm was calculated using the following equation and IC_{50} values were estimated using a non-linear regression ($y = 2.0852x + 14.944$; $R^2 = 0.9865$).

$$\text{Inhibition (\%)} = [(A_{\text{control}} - A_{\text{sample}}) / A_{\text{control}}] \times 100$$

Nitric oxide radical scavenging

The ability of the extract to scavenge nitric oxide free radicals was determined using a modification of the method described by Marcocci and co workers (Marcocci *et al.*, 1994 [15]). In brief, a 0.5 mL aliquot of extract (1 mg/mL) or positive control (1 mg/mL) dissolved in KH_2PO_4 -KOH (50 mmol/L, pH:7.4) was mixed with 0.5 mL of (10 mmol/L) sodium nitro prusside solution. The mixture was incubated at 37°C for 2.5 h under normal light condition. After incubation the sample was placed in dark for 20 min. Thereafter, 1 mL of Griess reagent (1g/IN-(1-naphtyl) ethylenediamine and 10 g/L sulphanilamide dissolved in 20 mL/L aqueous H_3PO_4) was added and the absorbance was taken after 40 min at 546 nm. The percentage of inhibition was calculated using the following equation ($y = 2.1582x + 27.049$; $R^2 = 0.9824$):

$$\text{Inhibition (\%)} = [(A_{\text{control}} - A_{\text{sample}}) / A_{\text{control}}] \times 100$$

Statistical analysis

All analyses and tests were run in triplicate and mean values recorded. All the experimental data are presented as mean \pm SEM of three individual samples. Data are presented as percentage of inhibition or radical scavenging on different concentration of *A. ciniformis* essential oil. IC_{50} (the concentration required to scavenge/inhibit 50% of free radicals/tyrosinase or lipid peroxidation) value was calculated from the dose-response curves. All of the statistical analyses were performed by means of Microsoft Office Excel 2007 software.

RESULTS AND DISCUSSION

Chemical composition of the essential oil

Water-distilled essential oil from the leaves of *A. ciniformis* was analyzed by GC and GC-MS (Table 1). Thirteen components, which representing 97.6% of the total composition were identified. The leaf

Table 1: Composition of the leaf essential oil of *A. ciniformis* Krasch. & Popov ex Poljakov.

Compound	*RI	Percentage (%)
1,8-Cineole	1033	22.4
<i>cis</i> -Sabinene hydrate	1068	2.3
<i>trans</i> -Pinocarveol	1139	16.8
Camphor	1143	32.2
Pinocavone	1162	4.7
<i>endo</i> -Borneol	1165	3.6
Terpinen-4-ol	1177	5.8
α -Terpineol	1189	2.1
<i>trans</i> -Carveol	1217	1.1
Carvacrol	1298	1.4
(<i>Z</i>)-Caryophyllene	1404	1.2
Cedrane	1436	1.6
α -Guaiene	1439	2.4
Monoterpene hydrocarbons		-
Oxygenated monoterpenes		92.4
Sesquiterpene hydrocarbons		5.2
Oxygenated sesquiterpenes		-
Others		-
Total		97.6

*RI, Retention indices were as determined on a DB-5 column using the homologous series of *n*-alkanes.

essential oil of *A. ciniformis* consists of ten oxygenated monoterpenes (92.4%) and three sesquiterpene hydrocarbons (5.2%). Camphor (32.2%), 1,8-cineole (22.4%) and *trans*-pinocarveol (16.8%) were the major components identified in this essential oil. As can be seen from Table 1, oxygenated monoterpenes were reported as major constituents from leaf essential oil of *A. ciniformis*.

In contrast, the aerial parts of *A. ciniformis* Krasch. & M. Pop. ex Poljak, which were collected from the Bojnourd area of Iran in November 2005, consisted of ten monoterpene hydrocarbons (35.5%), seven oxygenated monoterpenes (23.1%), and four sesquiterpenes (33.9%). Davanone (29.6%), myrcene (14.4%), camphor (10.6%), *p*-cymene (9.6%) and linalool (8.6%) were found to be the major components among the 22 constituents characterized, comprising 92.5% of the total components detected (Firouzni *et al.*, 2008 [16]).

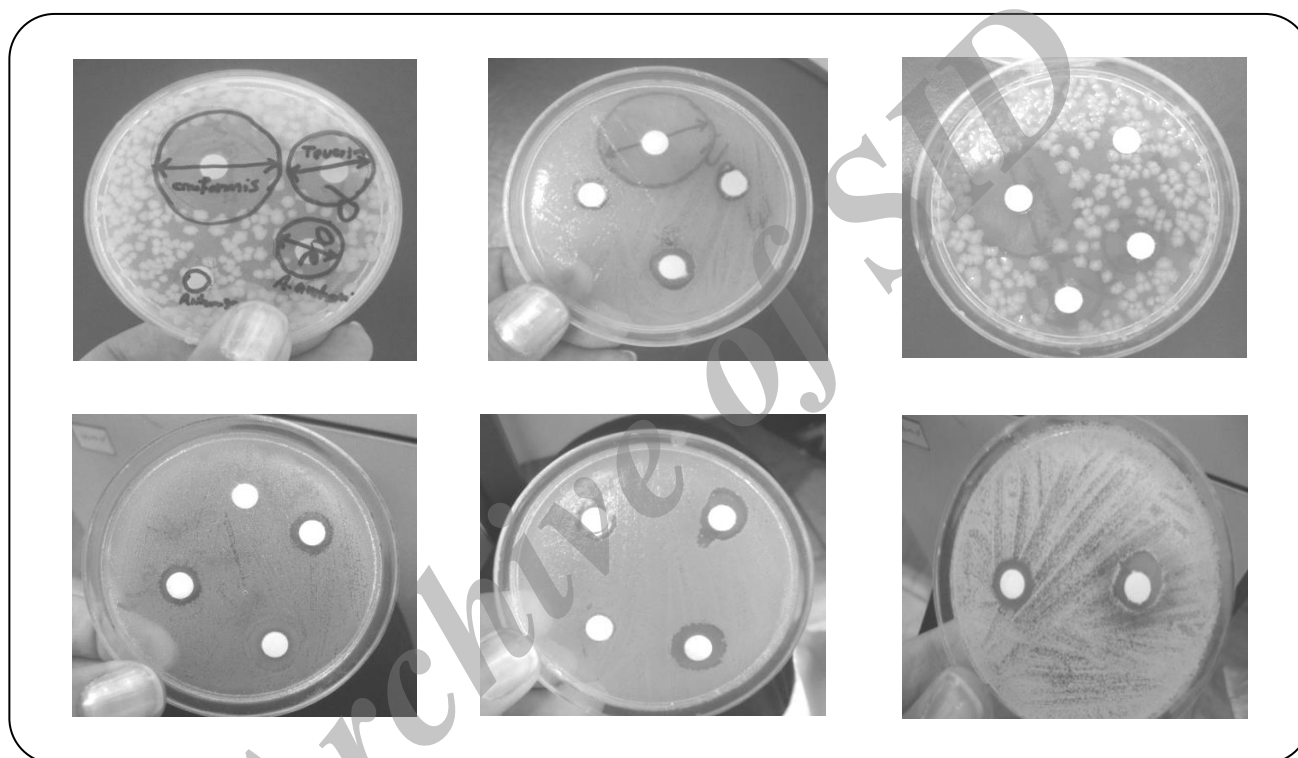
Antibacterial Activity

Antibacterial and antifungal activities of the essential oil of *A. ciniformis* were tested by three methods: 1. agar diffusion method, 2. determination of minimum inhibitory (MIC) and 3. bactericidal (MBC) concentrations and bactericidal kinetics of the essential oil, using different dilutions viz., 2.5 mg/mL, 5 mg/mL and 10 mg/mL. The results of antibacterial activities of the leaf essential oil of *A. ciniformis* are presented in Table 2. Maximum inhibition was obtained against *Acinetobacter baumannii* (51.83 mm), *Escherichia coli* (34.83 mm) and *Staphylococcus aureus* (34.50 mm) followed by *Pseudomonas aeruginosa* (30.17 mm) and *Candida albicans* (28.50 mm) at a concentration of 10 mg/mL of the essential oil (Figs. 1, 2). The essential oil of *A. ciniformis* indicated moderate inhibitory activity against all tested microorganisms. Complete death time on exposure to *A. ciniformis* essential oil was 3.57 min for *A. baumannii*.

Table 2: Antimicrobial activity of the leaf essential oil of *A. ciniformis* Krasch. & Popov ex Poljakov.

Microorganisms			<i>Escherichia coli</i> ATCC25922	<i>Staphylococcus aureus</i> ATCC25923	<i>Pseudomonas aeruginosa</i> ATCC8830	<i>Candida albicans</i> ATCC 5027	<i>Acinetobacter baumannii</i> ATCC 17978
IZ* (mm)	Oil (mg/mL)	10	34.83±1.65	34.50±1.32	30.17±0.76	28.50±1.00	51.83±1.76
		5	20.33±2.02	21.00±1.00	21.50±1.50	17.00±0.50	41.50±0.87
		2.5	12.67±1.15	11.50±1.32	13.33±0.58	10.33±1.76	29.67±1.53
MIC*-MBC* (mg/mL)			1-2.5	2.5-5	1-2.5	1-2.5	0.02-0.04
D value* (min)			6.43	17.14	8.57	8.57	3.57

IZ*: Inhibition Zone (mm); MIC*: Minimum Inhibitory Concentration (mg/mL); MBC*: Minimum Bactericidal Concentration (mg/mL); D-value*: Decimal Reduction Time (minutes)

**Fig. 1: Determination of antimicrobial activity by disk diffusion method.**

In the present study, determination of MBC and MIC from the essential oil of *A. ciniformis* indicated that all the test organisms were approximately sensitive to the essential oil, but *A. baumannii* is the most vulnerable. Determination of the inhibition zone showed the essential oil of *A. ciniformis* was bactericidal in order of *A. baumannii* > *E. coli* > *S. aureus* > *P. aeruginosa* > *C. albicans*. The results showed that *A. baumannii* has the minimum MIC and MBC values against *A. ciniformis* essential oil. Bactericidal kinetics of the essential oil of *A. ciniformis* indicated that *A. baumannii* is the most vulnerable.

Total Phenolic Content

The total phenol content of the essential oil of *A. ciniformis* was determined to be 206.20 ± 4.58 μg gallic acid equivalent/mg sample (GAE/mg). The phenolic assay involving an electron-transfer reaction was evaluated by using Folin-Ciocalteu reagent. The results showed that the essential oil of *A. ciniformis* was rich in phenolic compounds. TPC measures both types of antioxidants, hydrophobic and hydrophilic form complexes with Fe^{2+} . Phenols and flavonoids are known to inhibit lipid peroxidation by quenching lipid peroxy

Table 3: Antioxidant activity of the leaf essential oil of *A. ciniformis* Krasch. & Popov ex Poljakov.

<i>A. ciniformis</i> oil (mg)	6	8	10	12	IC ₅₀ (mg/ml)
DPPH radical scavenging	33.55 ± 3.95	39.96 ± 2.67	49.05 ± 0.98	53.36 ± 1.92	10.75
Standard	1mM (or 0.22 mg/mL)		BHT	38.26 ± 0.54	
	1mM (or 0.18 mg/mL)		BHA	49.14 ± 0.75	
	1mM (or 0.25 mg/mL)		Trolox	34.17 ± 0.53	

sample		concentration	% β-Carotene - linoleic acid
<i>A. ciniformis</i> oil		0.625 (mg/ml)	86.39 ± 2.53
Standard	BHT	1mM	86.21 ± 2.24
	BHA	1mM	80.88 ± 2.36

DPPH IC ₅₀ (mg/mL)	DPPH radical scavenging (%)	FRAP (Gallic acid equivalent) (mg/g)	β-Carotene-linoleic acid assay (%)	TPC (μg/mg GAE)
	Oil (mg)		Oil (mg/ml)	
10.75	53.36 ± 1.92	0.315±0.08	86.39 ± 2.53	206.20 ± 4.58
	12 mg		0.625 (mg/ml)	

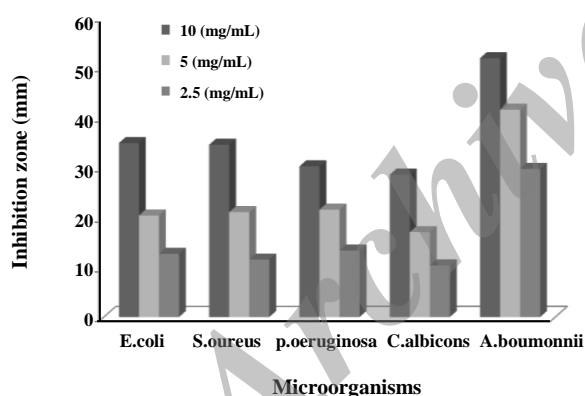


Fig. 2: Comparison of antimicrobial activity by disk diffusion method.

radicals and reduce or chelate iron in lipoxygenase enzyme and thus prevent initiation of lipid peroxidation reaction (Torel *et al.*, 1986 [17]).

Antioxidant activity

Antioxidative properties of the essential oil from the leaves of *A. ciniformis* were determined by three methods: The Ferric-Reducing Antioxidant Power (FRAP),

Radical-scavenging capacity of the essential oil or bleaching of 2,20-diphenylpicrylhydrazyl (DPPH) and β-carotene-linoleic acid assay. The antioxidant capacities of the essential oil as assessed by different assay methods are summarized in Table 3.

The Ferric-Reducing Antioxidant Power (FRAP) was expressed as gallic acid equivalent or known Fe(II) concentration for the essential oil of *A. ciniformis*. The FRAP of *A. ciniformis* essential oil was determined 0.315 ± 0.08 gallic acid equivalent (mg/g).

The essential oil of *A. ciniformis* has shown $53.36 \pm 1.92\%$ (12 mg of essential oil) inhibition of DPPH activity with an IC₅₀ = 10.75 mg/mL. The radical scavenging activity of the essential oil of *A. ciniformis* was performed in the presence of BHT, BHA and Trolox as standards. As shown in Table 3, the radical scavenging activity of *A. ciniformis* essential oil at the concentration of 12 mg/mL of essential oil, was found to be 1.39 times more potent than the standard BHT, 1.08 times more potent than the standard BHA and 1.56 times greater than Trolox.

Many different methods have been established for evaluating the antioxidant capacity of certain biological samples, with such methods being classified, roughly,

Table 4: Nitric oxide and superoxide anion radical scavenging activity of the leaf essential oil of *A. ciniformis* Krasch. & Popov ex Poljakov.

<i>A. ciniformis</i> oil (μg)	1	2	4	8	16	IC ₅₀ (μg)
NORC percent	27.58 ± 5.97	30.44 ± 9.84	37.35 ± 8.02	46.45 ± 4.30	60.31 ± 4.29	10.63
<i>A. ciniformis</i> oil (μg)	2.78	5.56	11.12	22.24	IC ₅₀ (μg)	
SARC percent	18.38 ± 3.40	27.83 ± 3.72	40.30 ± 2.26	60.20 ± 4.29	16.81	

into 1 of 2 categories based upon the nature of the reaction that the method involved (Huang *et al.*, 2005 [18]). The methods involving an electron-transfer reaction include the DPPH radical-scavenging, ferric-reducing antioxidant power and β-carotene-linoleic acid assay. *A. ciniformis* essential oil exhibited a dose-dependent scavenging of DPPH radicals. The DPPH radical scavenging is a sensitive antioxidant assay and is independent of substrate polarity (Yamaguchi *et al.*, 1998 [19]). DPPH is a stable free radical that can accept an electron or hydrogen radical to become a stable diamagnetic molecule (Ho *et al.*, 2008 [20]). A significant correlation was shown to exist between the phenolic content and with DPPH scavenging capacity.

In β-carotene-linoleic acid test system, oxidation of linoleic acid was effectively inhibited by *A. ciniformis* essential oil (86.39 ± 2.53%, 0.625 mg/mL essential oil). The β-carotene-linoleic acid assay of the leaf essential oil of *A. ciniformis* was performed in the presence of BHT and BHA as standards (Table 3). In Lipid Peroxidation Inhibition (LPI) activity, oxidation of linoleic acid was effectively inhibited by *A. ciniformis* essential oil. Results such as the relative abundance of phenolic compounds, and the significant correlations that existed between phenolic content and antioxidant capacity, as measured by β-carotene or DPPH scavenging methods, would appear to be highly consistent with corresponding results reported by previous researches (Dragland *et al.*, 2003 [21]; Shan *et al.*, 2005 [22]; Mammadov *et al.*, 2010 [23]; Mammadov *et al.*, 2011 [24]).

Radical-scavenging activity

Nitric oxide radical-scavenging activity

The nitric oxide radical-scavenging activity of the essential oil of *A. ciniformis* is reported in Table 4. The essential oil of *A. ciniformis* has shown 60.31 ± 4.29% NO[•] scavenging activity at 16 μg. Nitric oxide radical

scavenging activity of the leaf essential oil of *A. ciniformis* at 50% concentration (IC₅₀) was 10.63 μg.

According to the results, the percentage of nitric oxide radical scavenging activity was also increased with increasing concentration of the *A. ciniformis* essential oil. Increased levels of nitric oxide can be found in certain spasmodic conditions, for example, allergic rhinitis, adult respiratory distress syndrome and asthma immediate and late phase (Ashutosh, 2000 [25]). In addition to reactive oxygen species, nitric oxide is also implicated in inflammation, cancer and other pathological conditions (Nabavi *et al.*, 2008 [26]; Nabavi *et al.*, 2008b [27]). No similar reports were found in the literature regarding radical scavenging activities of the leaf essential oil of *A. ciniformis*.

Superoxide anion radical scavenging activity

The superoxide anion radical scavenging of the essential oil of *A. ciniformis* is presented in Table 4. The maximum percentage of superoxide radical scavenging activity of the essential oil was 60.20 ± 4.29% at 22.24 μg. *A. ciniformis* essential oil exhibited a dose-dependent scavenging of superoxide anion radicals and 16.81 μg of the *A. ciniformis* essential oil was sufficient to scavenge 50% of superoxide anion. Superoxide radical is produced in human body by various oxidative enzymes in the form of one electron reduction of molecular oxygen. Xanthine oxidase is one of the major oxidative enzymes to produce superoxide radical as a result in tissue injury (Haraguchi *et al.*, 1998 [28]). *In vitro* superoxide radical was generated by xanthine oxidase during the reaction; NBT undergoes oxidation and leads to water-soluble blue formazan (Gulcin *et al.*, 2004 [29]). The decrease in blue color formation after adding the solvent fractions in the reaction mixture was measured as superoxide radical scavenging. In this study, percentage of superoxide radical scavenging activity and inhibition of uric acid formation were increased with increasing concentration of *A. ciniformis* essential oil.

Table 5: Ferrous-ion chelating (FIC) ability of the leaf essential oil of *A. ciniformis* Krasch. & Popov ex Poljakov.

<i>A. ciniformis</i> oil	Oil (μg)	31.25	62.5	125	250	IC ₅₀ (μg)
	Chelating percent	17.02 \pm 6.58	25.88 \pm 8.41	34.88 \pm 7.88	54.25 \pm 5.34	220.90
EDTA	EDTA (μg)	0.2	0.4	0.8	1.6	IC ₅₀ (μg)
	Chelating percent	37.10 \pm 6.01	41.56 \pm 7.43	49.41 \pm 3.40	60.17 \pm 2.04	0.931

Table 6: Tyrosinase inhibition of the leaf essential oil of *A. ciniformis* Krasch. & Popov ex Poljakov.

<i>A. ciniformis</i> oil (mg)			2.85	3.58	4.28	5	IC ₅₀ (mg)
Anti tyrosinase activity (%)			18.95 ± 0.74	23.09 ± 0.58	30.89 ± 4.66	36.95 ± 4.77	6.53
Quercetin equivalent (mg)			0.27	0.44	0.76	1.72	
standard	Quercetin (mg)	0.025	0.05	0.1	0.5	1	2
	Anti tyrosinase activity (%)	15.51 ± 0.51	17.01 ± 0.88	18.56 ± 0.21	23.15 ± 0.17	28.36 ± 0.42	40.05 ± 4.30

Ferrous-ion chelating assay

The strongest iron chelating activity was noticed at a concentration of 250 μg (54.25 \pm 5.34%), while the concentration of 31.25 μg exhibited the lowest activity (17.02 \pm 6.58%) (Table 5).

The ferrous ion chelating activity increased with the increasing concentration. EDTA which serves as the positive control shows the highest percentage of the chelating effect (60.17 \pm 2.04%) at the concentration of 1.6 μg . IC₅₀ of *A. ciniformis* essential oil was 220.90 μg , while the IC₅₀ of EDTA was 0.931 μg . FIC assay is a common test used to determine the secondary antioxidant activity by observing the reducing purple colour of the reaction solution. The assay mechanism is based on the decrease in the absorbance of iron (II)-ferrozine complex. Meanwhile, secondary antioxidants are also known as the peroxide decomposers, where it inhibits polypropylene oxidation by decomposing hydroperoxide (Lim *et al.*, 2006 [2]). Iron-ferrozine complex has the maximum absorbance at 562 nm and large decrease in absorbance indicates strong chelating power. By forming a stable iron (II) chelate, an extract with a high chelating power reduces free ferrous ion concentration, which leads to decrease the extent of Fenton reaction that are implicated in many diseases (Lim *et al.*, 2006 [2]). Iron is known to generate free radicals through the Fenton and Haber-Weiss reaction. Fenton Weiss reaction is a reaction between ferrous ion and hydrogen peroxide which produces highly reactive hydroxyl radicals implicated in many diseases (Lloyd *et al.*, 1997 [30]). Metal ion-chelating capacity plays a significant role in antioxidant

mechanism since it reduces the concentration of the catalysing transition metal in lipid oxidation (Che Othman *et al.*, 2011 [31]). In this study, the ferrous-ion chelating activity of the essential oil was lower than EDTA. EDTA showed an excellent chelating ability. There was no correlation between ferrous ion chelating activity and total phenolic content implying that the essential oil contain no chelating ligands but radical scavenging properties of *A. ciniformis* essential oil is associated with the presence of phenolic compounds possessing the ability to donate hydrogen to the radical.

Tyrosinase inhibition

The maximum percentage of anti-tyrosinase activity of this essential oil was 36.95 \pm 4.77% at 5 mg. Anti-tyrosinase activity of *A. ciniformis* essential oil at 50% concentration (IC₅₀) was 6.53 mg (Table 6).

Quercetin was used as positive control. The maximum percentage of tyrosinase inhibition of quercetin was 40.05 \pm 4.30% at the concentration of 2 mg. Tyrosinase is a copper containing enzyme hence any substance which reduces this metal ion was considered as an effective tyrosinase inhibitor (Amin *et al.*, 2010 [32]). Essential oils are found to be rich in compounds consisting of hydrophobic part which would have acted as competitive inhibitors on the enzyme tyrosinase and thereby on melanin synthesis. Hence, the determination of tyrosinase inhibitory potential of the present essential oils may lead to develop skin whitening agents (Montaz *et al.*, 2008 [33]). In this study, activity of quercetin was much more pronounced than the essential oil of *A. ciniformis*.

The reducing power reported might be due to phyto constituents such as phenolics, carbonyl compounds and also other constituents which are present in the essential oils. The possible mechanism underlying behind the tyrosinase inhibitory ability might be chelation of copper ion present in tyrosinase enzyme by phyto constituents and thereby suppression of tautomerisation to dopochrome by the essential oils, thereby the essential oils act as reducing agents on melanin intermediates by blocking oxidation chain reaction at various points from tyrosinase/DOPA to melanin and hence causing reduction of skin pigmentation (Slominski *et al.*, 2004 [34]). The effects of antioxidant phytochemicals in the biological systems are defense on their ability to scavenge radicals, chelating metals, activate the antioxidant enzymes and to inhibit the oxidases (Kulkarni *et al.*, 2004 [35]).

CONCLUSIONS

From the previously mentioned results, it can be concluded that the leaf essential oil of *A. ciniformis* exhibited antimicrobial activity against the tested microorganisms and it could be a natural radical scavenger agent. This study suggests that *A. ciniformis* with a high phenolic content and good antioxidant activity can act as antioxidant and anti-tyrosinase agent.

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