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Article

Phytochemical Analysis, Antioxidant and Antifungal Activity of Essential oil and Extracts of *Alpinia malaccensis* (Burm.f.) Roscoe flowers

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The present study describes chemical composition, phytochemicals, antifungal activities, antioxidant assays and total phenolic content of essential oil and varied polarity solvent extract from flowers of *Alpinia malaccensis* (Burm.f.). Total 27 components were identified in essential oil by GC-MS with terpinen-4-ol (28.6%) and α- terpineol (12.8%) as the main constituent. The essential oil was found to have maximal levels of phenolic content (64.60 µg/mL) as compared to the other extracts. The antioxidant assay evaluated in extracts and essential oil by different methods revealed good-to-moderate antioxidant potential with different IC^{50} values viz. (188.02 –250.25 µg/mL) in Fe^{3+} reducing power, (153.15–201.59 µg/mL) in Fe^{2+} metal-chelating ability, (130.39–181.12 µg/mL) in DPPH, (88.29–187.32 µg/mL) in OH radical, (79.04–156.79 µg/mL), in NO radical and (138.72–233.00 µg/mL) in superoxide anion scavenging activities, respectively. The methanolic extract display remarkable fungicidal activity against the tested pathogens followed by dichloromethane extract, essential oil, hexane extract and petroleum ether extract respectively, with MIC values ranging from 31.25 to 500 µg/mL. Based on results, it can be inferred that the flower of *A. malaccensis* if explored further for its medicinal properties, might be a good source to develop a safe and sustainable natural food preservative.

Keywords: *Alpinia malaccensis*. Antifungal activity. Antioxidant activity. Flower. Total phenol.

INTRODUCTION

The medicinal properties of the plants have been explored extensively throughout the world with a key focus for identifying several phytochemicals that possess potent therapeutic effects (Okwu, 2001). Alkaloids, flavonoids, tannins, phenols, proteins and carbohydrates are some of the vital phytochemicals that are found excessively in plants (Merculieff *et al*., 2014). Phytochemicals being extracted from various parts of the plants such as leaves, stems, flowers, roots, seeds etc. possess valuable antioxidant potential and antimicrobial activity that form their basis for their usage in perfumery, cosmetics, food safety, agricultural and pharmaceutical sectors (Ahmadi *et al*., 2010). The phytochemicals are used as curative for several degenerative diseases such as Diabetes, Parkinson disease, Alzheimer's disease etc. (Gulcin, 2012).

The interest in essential oils/extract and their application in food preservation have been amplified in recent years due to consumer's negative perception about synthetic preservatives (Tripathi, Dubey, 2004). In present scenario, consumers demand natural products, over synthetic one due to their effectiveness, safe and environmentally benign (Pavela, 2007). Among them, essential oils/extracts may be natural alternatives

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of synthetic food preservatives against food borne pathogens, food spoiling bacteria etc. (Siddhuraju, Manian, 2007).

Alpinia malaccensis (Burm.f.) is a perennial herb belonging to family Zingiberaceae. It is found in tropical as well as subtropical regions. It is a tall herb growing widely in forests and is being cultivated as an ornamental plant. Its rhizomes are fibrous in nature and are applied on sores for its cure (Nuntawong, Suksamrarn, 2008; Abhyankar, Upadhyay, 2011). Flowers are broad, large, ovate lip, yellow in colour with red patches at the base and edges (Nor Azah *et al.,* 2005). Fruits of this important herb are applied on gastralgia and for bathing feverish people (Bhuiyan *et al.,* 2010). However, to the best of our knowledge there is no published report regarding the phytochemical analysis, antioxidant activity, and antifungal activity of flowers of *A. malaccensis*. In continuation to our research on the family Zingiberaceae the present investigation was performed to evaluate phytochemicals, antifungal and antioxidant activity along with total phenolic content of extracts of flowers of *A. malaccensis* in order to develop a safe natural antioxidant and antifungal source.

MATERIAL AND METHODS

Collection of plant material

Fresh flowers of *A. malaccensis* were collected from the Tarai region of Kumaun hills in India. The plant was identified and authenticated by Dr. D.S. Rawat (Plant taxonomist), Department of Biological Science, G.B. Pant University of Agriculture and Technology, Pantnagar, India. The voucher specimen has been deposited in the department of Botany, for future reference.

Extraction of essential oils

Fresh flowers (1kg) were subjected to hydro distillation in Clevenger's apparatus for 3-4 hours. Extraction of distillate by diethyl ether followed by drying over anhydrous Na_2SO_4 and removal of solvent yielded 0.03% of *A. malaccensis* flower essential oil (AMFEO).

Preparation of the extracts

The flowers of *A. malaccensis* were cut into small pieces and shade-dried at room temperature. The material was then ground to fine powder. About 2 kg of the material was extracted through cold percolation method by successive soaking for 7 days each in different solvents of varying polarity like petroleum ether, hexane, dichloromethane and methanol. The extracts were filtered using muslin cloths and the process was repeated till colorless solutions were obtained. The filtrates so obtained were concentrated using a vacuum rotary evaporator. Yields of different extracts viz. *A. malaccensis*, flower petroleum ether extract (AMFPE), *A. malaccensis*, flowers hexane extract (AMFHE), *A. malaccensis* flower dichloromethane extract (AMFDE) and *A. malaccensis*, flower methanol extract (AMFME) were observed to be 0.84, 0.79, 0.74 and 0.70%, respectively. The extracts were stored at 4°C for further analysis and biological activity determinations.

Qualitative Phytochemical Screening

Phytochemical screening for the presence of major types of compounds present in the different solvent extracts of *A. malaccensis* flower was done by standard methods reported earlier (Shaik, 2011).

GC–FID analyses

Gas chromatographic analyses were carried onto a Thermo series CERES 800 plus gas chromatograph with FID fitted detector using DB-5 capillary column (non-polar, 30 m × 0.32 mm id., 0.25 µm film thickness). GC operation conditions: injection mode, split (40:1, v/v); injection volume 1 µL, injector temperature: 220°C; detector temperature: 250°C; oven temperature programme: 60-246°C (3°C/min); carrier gas: helium (1.0 mL/min). Percentages of the individual components in the oil were obtained from the GC–FID peak area-% reports.

GC–MS analysis

Gas chromatography–mass spectrometry (GC–MS) analyses were carried out on a Hewlett-Packard 6890 gas chromatograph (Agilent Technologies, USA), fitted with an HP-5 (5% phenyl methylpolysiloxane, 30 m × 0.32 mm i.d., 0.25 µm film thickness) capillary column coupled with a model 5973 mass detector. GC–MS operation conditions: injection mode, split (1:1 ratio, v/v); injection volume 1 µl, injector temperature: 220°C; transfer line temperature: 240°C; oven temperature programme: 60– 246°C (3°C/min); carrier gas: helium (1 mL/min); detector temperature: 250°C; mass spectra, electron impact (EI) mode, 70 eV; ion source temperature: 240°C. Individual components were identified by Wiley or NIST database matching, comparison of retention times and mass spectra of constituents with Adams database (Adams, 2007) (Table II).

Antioxidant activity

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

In order to evaluate scavenging ability of the antioxidants by this method, a standard protocol was followed (Sethi *et al.,* 2015). In brief, different amounts of the test sample (50-250 µg/mL) were added to 5mL of a 0.004% methanol solution of DPPH. Finally, the absorbance was read against a blank at 515nm after 30 min of incubation in the dark. All the observations were taken in triplicate. Butylated hydroxyl toluene (BHT), catechin and gallic acid were used as the standard antioxidant. Inhibition of free radical by DPPH in percent (IC %) was calculated by using the equation, IC % = $(A_0 – A_t/A_0) \times 100$, where A_0 and A_t are the absorbance values of the control and test sample respectively. Percent inhibition was plotted against concentrations and the equation for the line was used to obtain the IC_{50} value.

Hydroxyl radical (·OH) scavenging activity

This activity was evaluated using the method as described earlier (Olabinri *et al*., 2010). Approximately 60µl of $FeSO_4.7H_2O$ (1 mM) was added to 90µl of aqueous 1, 10 phenanthroline (1 mM) and 2.4 mL of 0.2 M phosphate buffer (pH 7.8) was added to the above mixture, followed by addition of 150 µl of hydrogen peroxide (0.17mM) and 1.5mL of different concentrations of sample (50-250 µg/mL). The absorbance of the mixture was read at 560 nm against blank after 5min. Ascorbic acid was used as the standard. The % inhibition was calculated as, % hydroxyl radical scavenging capacity (IC %) = $[(A_0 - A_t)/A_0] \times 100$, where A_0 and A_t are the absorbance values of the control and the test sample respectively. The lower IC_{50} value indicates greater hydroxyl radical scavenging ability.

Nitric oxide ($NO^{\cdot}$) radical scavenging activity

The nitric oxide scavenging activity of oils and extracts was determined by using a previously reported method (Naskar *et al*., 2010). About 2mL of sodium nitroprusside (SNP) (10mM) in phosphate buffer saline (PBS) pH 7.4 was mixed with different concentration of sample (50-250 µg/mL) and incubated at 25°C for two and a half hours. To the above sample 1mL of Griess reagent (1% sulphanilamide, 0.1% naphthylethylenediamine dichloride and 2mL orthophosphoric acid) was added. As a result, pink colour was obtained and the absorbance was read at 546nm. Ascorbic acid was used as the standard. The % inhibition was calculated by the equation. % Nitric oxide scavenging capacity (IC %) = $(A_0 – A_t/A_0) \times 100$ where A_0 and A_t are the absorbance values of the control sample and the test sample respectively. The percent inhibition was plotted against concentration, and the equation for the line was used to obtain the IC_{50} value.

Superoxide anion scavenging activity

Superoxide anion scavenging activity of oils and extracts was determined according to the reported method with slight modifications (Nishimiki, Rao, Yagi, 1972). In brief 1 mL of nitroblue tetrazolium (NBT) solution (100 µM of NBT in 100 mmol/L phosphate buffer, pH 7.4), 1 mL of NADH (468 µmol in 100 mM/L phosphate buffer, pH 7.4) solution and varying concentration of test samples (50-250 µg/mL) were mixed well. The reaction was started by the addition of 100 µl of Phenazine methosulfate solution (PMS) (60 mM of 100 mM/l phosphate buffer, pH 7.4). The reaction mixture was incubated at 30 °C for 15 min. The absorbance was measured at 560 nm

in a spectrophotometer. All the readings were taken in triplicate and ascorbic acid was used as the standard. The % inhibition was calculated by the equation. % superoxide scavenging capacity (IC %) = $(A_0 – A_t/A_0) \times 100$ where A_0 and A_t are the absorbance values of the control sample and the test sample respectively. Percent inhibition was plotted against concentration, and the equation for the line was used to obtain the IC_{50} value.

Reducing power activity

The reducing power of essential oils and various extracts was determined by the method reported earlier (Sethi *et al*., 2015). Varying concentrations of tested sample (50- 250 µg/mL) were mixed with 2.5 mL of the phosphate buffer (200 mM, pH 6.6) and 2.5 mL of 1% potassium ferricyanide $K_3Fe(CN)_6$. The mixtures were incubated at 50^0C for 20 minutes. After incubation, 2.5mL of 10% trichloroacetic acid was added to the mixtures, followed by centrifugation at 650g for 10 min. The upper layer (5 mL) was mixed with 5mL of distilled H_2O and 1mL of 0.1% ferric chloride and absorbance of the resulting solution were measured at 700 nm using spectrophotometer. All the readings were taken in triplicate and BHT (Butylated hydroxyl toluene), catechin and gallic acid were taken as the standard. The reducing power of samples was calculated by the following formula: RP (%) = $(A_0 – A_t) \times 100$; where: A_0 and A_t are the absorbance values of the control sample and the test sample respectively. Percent inhibition was plotted against concentration, and the equation for the line was used to obtain the RP_{50} value.

Metal chelating ability

The chelation of Fe^{2+} by oils and extracts were evaluated by using the method developed earlier (Sethi *et al*., 2015). Different concentrations of tested sample (50- 250 µg/mL) were first mixed with 1mL of methanol and 3.7 mL of deionized water. The resulting mixture was allowed to react with 0.1mL of $FeCl_2$ (2 mM) and 4.2mL of ferrozine (5 mM) for 10 min at room temperature. The absorbance was measured at 562 nm. All the readings were taken in triplicate and Na_2EDTA (0.01 mM), citric acid (0.025 M) were taken as standard. The metal-chelating ability of the tested sample, expressed as percentage were calculated according to the formula IC (%) = $[(A_0 – A_t/A_0] \times 100$, where A_0 and A_t are the absorbance values of the control sample and the test sample respectively. The percent of chelating ability was plotted against concentration, and the equation for the line was used to obtain the IC_{50} value.

Total Phenols

The total phenolic content of oil and extracts was estimated by following the standard protocol (Sethi *et al*., 2015; Sethi *et al*., 2016). An aliquot of 0.5 mL of the oil or extract solutions were mixed with 1.0 mL of Folin–Ciocalteu reagent, 1.0 mL of aqueous solution of 7% sodium carbonate and 5mL of distilled water. The reaction mixture was mixed thoroughly and was allowed to stand for 30 min. The absorbance was read at 765 nm. The same procedure was also applied to the standard solutions of gallic acid. The calibration equation for gallic acid obtained was $y = 0.011x + 0.031$ ($R^2 = 0.998$) where y is the absorbance and x is the concentration of gallic acid in µg/mL. All tests were carried out in triplicate and the obtained results were the mean values and the standard deviation.

Microorganism

The pathogenic fungi viz; *Colletotricum falcatum, Rhizoctonia solani, Sclerotinia sclerotium* and *Sclerotium rolfsii* were obtained from Department of Pathology, G.B. Pant University of Agriculture and Technology, Pantnagar, India, and were maintained on potato dextrose agar (PDA).

Antifungal activity of extracts of *A. malaccensis*

The antifungal activity was determined by using the disc diffusion method (Murray *et al*., 1995). Initially, each sample was diluted with acetone: water (1:1) to obtain the final concentrations of 1000, 500, 750, 250 µg/mL respectively. The pathogenic fungi were cultured on potato dextrose agar (PDA) media and incubated at 30ºC for 3-4 days. A plug of 1-weekold fungal culture (5 mm diameter) was placed on the centre of the sterilized plates containing potato dextrose agar. About 10µL of each concentration was injected to the sterile disc papers

(6mm diam.). Then the prepared discs were placed on the culture medium. Negative control was prepared using the same solvent employed to dissolve the extracts. The plates were then incubated at 30ºC for 3-4 days in which fungal growth was monitored. The growth inhibition of each fungal strain was calculated as the percentage of inhibition of a radial growth relative to the control as: -

Percentage of inhibition (%) = 100 × [(1-radial growth of treatment (mm)/radical growth of control (mm)]. All experiments were performed in triplicate.

Determination of Minimum Inhibitory (MIC) by broth microdilution

The minimum inhibitory concentrations (MICs) of oil and extracts were determined by broth micro dilution method with slight modifications (Gulluce *et al*., 2004). Plant extracts were re-suspended in 50% acetone (which has no activity against test microorganisms) to make 1000 µg/mL final concentration and then two-fold serially diluted to final concentrations of 0.48, 0.97, 1.95, 3.90, 7.81, 15.62, 31.25, 62.5, 125, 250, 500, 1,000 µg/mL. A volume of 1mL of each different concentration along with a 10 µL inoculum of each test strain was placed in the test tubes containing PDB medium and incubated for 2-7 days at 28ºC. The control tubes comprised of PDB medium with 50% acetone and fungus inoculum. MICs were described as the lowest concentration of each extract resulting in the slight reduction of the inoculum.

Statistical analysis

Experiments were performed in triplicate and data analyzed were mean ± SE subjected to one-way ANOVA by using SPSS 16 (Statistical Package for the Social Science). Means were separated by the Tukey's multiple range test when analysis of variance (ANOVA) was significant ($p<0.05$). Pearson correlation test was used to assess correlations between the means.

RESULTS AND DISCUSSION

Qualitative Phytochemical Screening

Preliminary phytochemical screening of the various solvent extracts of flowers of *A. malaccensis* revealed the presence of various bioactive components which include glycosides, alkaloids, tannin, saponin, phytosterol, phenol and flavonoid etc. The present investigation showed that AMFME and AMFDE were certainly much better than the other extracts. This might be possibly due to the better solubility of the active compound in organic solvents of greatest polarity (Table I).

TABLE I - Phytochemical specifications of different extracts of flowers of *A. malaccensis*

Phytoconstituent	AMFPE	AMFHE	AMFDE	AMFME
Alkaloids	+	+	+	+
Carbohydrates	-	-	+	+
Glycosides	+	+	+	+
Saponins	+	+	+	+
Phytosterols	-	+	+	+
Fats & Oils	+	+	+	+
Resins	-	-	-	-
Phenols	+	+	+	+
Tannins	+	+	+	+
Flavonoids	-	+	-	+
Diterpenes	-	-	+	-

+ = presence, - =absence

GC-MS Analysis

The flower essential oil from *Alpinia malaccensis* was analysed by GC-MS analysis which revealed more than 70 compounds, out of which 27 compounds were identified which contributed 91.6% of the total oil. The major compounds identified were terpinen-4-ol (28.6%), α-terpineol (12.8%), *E*-methyl- cinnamate (12.4%) α-caryophyllene oxide (5.3%), octadecane (6.9%), docosane (4.5%), humuladienone (3.1%), β-caryophyllene (2.3%), α-cadinol (1.6%), *trans*-linaloloxide, α-caryophylene (1.9%), *E*- nerolidol (1.7%), tetracosane (1.0%) besides other compounds like linalool, *cis*- β- terpineol, camphor, linalyl formate, *cis* -piperitol, β- methyl cinnamate, α-copaene, *E*-ethyl cinnamate, α-muurolene, *cis*- calamenene, farnesol etc. which contributed less than 1.0% of the total oil. The detailed chemical composition of essential oil of flower of *A. malaccensis* has been reported in Table II. We already have reported leaf essential oil of *A. malaccensis* with camphor (15.2%), eucalyptol (10.2%), carotol (7.9%), methyl (*E*)-cinnamate (7.7%), α-terpineol (6.6%), borneol (2.8%), daucol (2.6%), bornyl acetate (2.2%), caryophyllene oxide (2.1%), widdrol (1.7%), β-eudesmol (1.5%), myrtenal (1.3%), fokienol (1.2%) and α-thujopsan-2-ol (1.2%) as major compounds with antioxidant and antifungal activity of the essential oil (Sethi *et al*., 2014; Sethi *et al*., 2016). It has been observed that both oils have different qualitative and quantitative make up.

TABLE II - Chemical composition of AMFEO

SN	Compound Name	KI	%Conc.	Mol. formula	Method of identification Major fragments
1	Linalool	1095	0.5	$C_{10}H1_8O$	**M**$^+$ 154, **M/z:**, 71,43,41
2	cis-β-terpineol	1140	0.8	$C_{10}H_{18}O$	**M**$^+$ 154, **M/z:,** 43,71,41
3	Camphor	1146	0.6	$C_{10}H_16O$	**M**$^+$ 152, **M/z:,** 95,41,81
4	**terpinen-4-ol (1)**	**1168**	**28.6**	$\mathbf{C_{10}H_{18}O}$	**M**$^+$ 154, **M/z:,** 71,93,111
5	**α-terpineol (2)**	**1188**	**12.8**	$\mathbf{C_{10}H_{18}O}$	**M**$^+$ 154, **M/z:,** 59,43,93
6	linalyl formate	1200	0.3	$C_{11}H_{18}O_2$	**M**$^+$ 182, **M/z:,** 93,41,43
7	*cis*- piperitol (**3**)	1203	1.0	$C_{10}H_{18}O$	**M**$^+$ 154, **M/z:,** 84,83,41
8	β.-citronellol	1225	0.1	$C_{10}H_{20}O$	**M**$^+$ 154, **M/z:,** 41,69,55
9	Methylheptenone	1303	0.2	C_8H1_4O	**M**$^+$ 126, **M**/z:, 43,41,69
10	*Z*- methyl -cinnamate	1299	0.3	$C_{10}H_{10}O_2$	**M**$^+$ 162, **M/z:,** 131,103,77
11	α.-copaene	1376	0.5	C15H24	**M**$^+$ 154, **M/z:**, 204,119,41
12	***E*- methyl-cinnamate (4)**	**1378**	**12.4**	$\mathbf{C_{10}H_{10}O_2}$	**M**$^+$ 154, **M/z:,** 162,103,77
13	β.-caryophyllen (**5**)	1408	2.3	$C_{15}H_{24}$	**M**$^+$ 204, **M/z:,** 41,69,93
14	*trans*- linaloloxide (6)	1409	1.9	$C_{10}H_{18}O2$	**M**$^+$ 170, **M/z:,** 41,82,69
15	α-caryophyllene (**7**)	1419	1.9	$C_{15}H_{24}$	**M**$^+$ 204, **M/z:,** 93,80,41
16	*E* -ethyl-cinnamate	1467	0.1	$C_{11}H_{12}O_2$	**M**$^+$ 176, **M/z:,** 131,103,77
17	α-muurolene	1500	0.1	$C_{15}H_{24}$	**M**$^+$ 204, **M/z:,** 105,41,91
18	*cis*-calamenene	1529	0.5	$C_{15}H_{22}$	**M**$^+$ 202, **M/z:,** 159,41,131
19	*E* –nerolidol **(8)**	1563	1.7	$C_{15}H_{26}O$	**M**$^+$ 222, **M/z:,** 69,41,43

TABLE II - Chemical composition of AMFEO

SN	Compound Name	KI	%Conc.	Mol. formula	Method of identification Major fragments
20	**α-caryophyllene oxide (9)**	**1580**	**5.3**	**$C_{15}H_{24}O$**	**M^+** 220, **M/z:,** 43,41,79
21	humuladienone (**10**)	1580	3.1	$C_{15}H_{24}O$	**M^+** 220, **M/z:,** 43,41,67
22	α-cadinol **(11)**	1654	1.6	$C_{15}H_{26}O$	**M^+** 222, **M/z:,** 43,41,85
23	farnesol	1698	0.5	$C_{15}H_{26}O$	**M^+** 222, **M/z:,** 69,41,81
24	**octadecane (12)**	**1800**	**6.9**	**$C_{18}H_{38}$**	**M^+** 254, **M/z:,** 57,43,71
25	palmitinic acid **(13)**	1960	2.1	$C_{16}H_{32}O_2$	**M^+** 256, **M/z:,** 43,60,73
26	docosane **(14)**	2200	**4.5**	$C_{22}H_{46}$	**M^+** 310, **M/z:,** 57,43,71
27	tetracosane (**15**)	2400	1.0	$C_{24}H_{50}$	**M^+** 338, **M/z:,** 57,43,71
	Total		**91.6**		

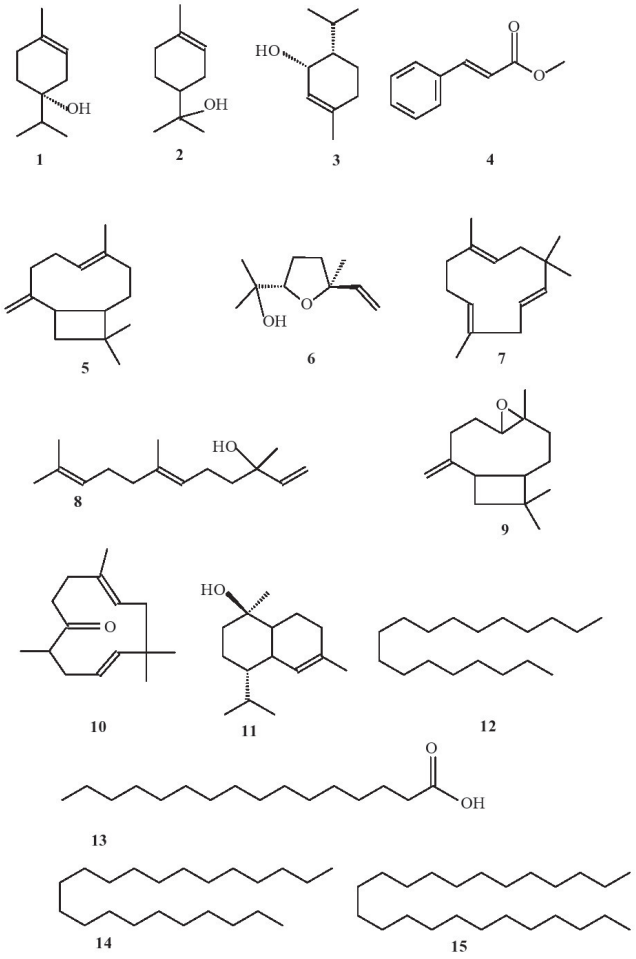

FIGURE 1 - Strectures of major compounds identified in AMFEO.

Methyl cinnamate, one of the major compound in our study has been used as fragrance and flavoring agent and frequently investigated for its effect on food browning in mushrooms (Huang *et al*, 2009). It has been reported earlier that terpinen-4-ol possess anti inflammatory activity by suppressing pro-inflammatory mediator production by activated human monocytes. Anticonvulsant activity and significant activity against *Demodex* mites, the most common ectoparasite found in the human skin extending to the eye. However, it has been reported that the activity is reduced by antagonistic effect of α-terpineol (Tighe, Gao, Tseng, 2013). The other biologically active molecules caryophyllene, caryophyllene oxide which contribute more than 7% in our oil are reported to possess anti-inflammatory, antinociceptive, anxiolytic, antidepressant, anti-alcoholism and antioxidant activities (Katsuyama *et al*., 2013*;* Shamma *et al*., 2014; Hayate *et al*., 2016). Similarly, docosane present 4.5% in our oil has been reported to possess antibacterial activity (Uma, Parvathavarthini, 2010).

Antioxidant activity

All the extracts of flower of *A. malaccensis* exhibited strong to good DPPH radical scavenging activity in a dose dependent manner, minimum at

lower and maximum at higher dose levels (Table III). The radical scavenging potential of extracts in the form of their IC_{50} values revealed the order AMFEO (IC_{50}= 130.39 µg/mL) > AMFME (IC_{50}= 138.20 µg/mL) > AMFDE (IC_{50}= 154.09 µg/mL) > AMFHE (IC_{50} = 169.47 µg/mL) > AMFPE (IC_{50} = 181.12 µg/mL). The order of IC_{50} for standard were ascorbic acid (IC_{50}= 32.46 µg/mL) > BHT (IC_{50}= 33.81µg/mL) > catechin (IC_{50}= 42.99 µg/mL) > gallic acid (IC_{50}= 49.27 µg/mL). DPPH free radical method is a fast and rapid antioxidant assay based on electron-transfer that produces a violet solution in methanol. This stable free radical at room temperature is reduced in the presence of an antioxidant to give colorless methanol solution. Thus, various products can be assessed at a particular time spectrometrically (Huang, Ou, Prior, 2005).

TABLE III - Antioxidant potential in term of IC_{50} values for different extracts of *A. malaccensis* along with their total phenolic content

Sample/ Standard	Antioxidant assay						
	DPPH Scavenging activity/ IC_{50}(µg/ml)	·OH scavenging activity/ IC_{50} (µg/ml)	Nitric oxide radical scavenging activity/ IC_{50}(µg/ml)	Superoxide anion scavenging activity/ IC_{50}(µg/ml)	Reducing power ability/ RP_{50}(µg/ml)	Metal chelating ability/ IC_{50}(µg/ml)	Total phenols(µg/ml)
AMFPE	181.12±1.3 ^{h}	187.32±1.3^{f}	156.79±0.0^{f}	233.00±0.4^{f}	250.25±0.8^{i}	201.59±2.9^{f}	15.03±0.2^{a}
AMFHE	169.47±2.1^{g}	164.45±0.6^{e}	130.38±1.1^{e}	214.83±1.1^{e}	221.00±0.9^{h}	184.79±2.6^{e}	33.09±0.1^{b}
AMFDE	154.09±1.1^{f}	142.81±1.2^{d}	114.61±0.7^{d}	206.98±2.3^{d}	202.24±0.4^{g}	177.17±2.0^{d}	46.66±0.2^{c}
AMFME	138.20±1.7^{e}	88.29±2.0^{b}	79.04±1.5^{b}	166.51±4.4^{c}	188.02±0.1^{e}	156.88±3.0^{c}	51.75±0.2^{d}
AMFEO	130.39±0.196^{d}	102.25±0.5^{c}	83.87±0.5^{c}	138.72±0.4^{b}	190.71±0.9^{f}	153.15±0.4^{c}	64.60±0.1^{e}
BHT	33.81±0.4^{a}	-	-	-	117.88±0.3^{b}	-	-
CATECHIN	42.99±2.7^{b}	-	-	-	143.91±0.6^{c}	-	-
GALLIC ACID	49.27±1.1^{c}	-	-	-	151.47±0.6^{d}	-	-
ASCORBIC ACID	32.46±0.2^{a}	44.36±4.5^{a}	62.03±0.3^{a}	32.28±1.1^{a}	115.63±0.2^{a}	-	-
EDTA	-	-	-	-	-	47.72±2.3^{a}	-
CITRIC ACID	-	-	-	-	-	61.76±0.3^{b}	-

- = *Not applicable,* Values are means of three replicates ± SD. Within a column, mean values followed by the same letter are not significantly different according to Tukey's test ($p<0.05$).

All the extracts of flowers of *A. malaccensis* scavenge OH free radical as function of amounts in a selected dose levels in comparison to the standard antioxidant ascorbic acid. The minimum IC_{50} values indicated higher scavenging activity thus revealing good antioxidant activity of the samples under investigation. The results obtained in extracts for their good antioxidant potential in terms of IC_{50} (µg/mL) values 187.32, 164.45, 142.8, 88.29, 102.25 for AMFPE, AMFHE, AMFDE, AMFME and AMFEO respectively. IC_{50} of ascorbic acid was obtained 44.36 µg/mL. During endogenous oxidative metabolism

Hydrogen peroxide is an intermediate which mediates the formation of OH radical which is used to scavenge antioxidants in biological system.

Hydrogen peroxide is an intermediate during endogenous oxidative metabolism and mediates radical oxygen formation such as OH, which may be used to predict the scavenging capability of antioxidants in biological systems (Juntachote, Berghofer, 2005). H_2O_2 has less activity for lipid peroxidation, but it is highly active when produces highly reactive hydroxyl radical through the Fenton reaction (Hussein, 2011).

Through their nitric oxide scavenging ability, all the extracts exhibited varying degree of antioxidant potential as indicated by the IC_{50} values obtained from these samples. However, the order of nitric oxide scavenging activity was AMFME (IC_{50}= 79.04 µg/mL) > AMFEO (IC_{50}= 83.87 µg/mL) > AMFDE (IC_{50}= 114.61 µg/mL) > AMFHE (IC_{50} = 130.38 µg/mL) > AMFPE (IC_{50}= 156.79 µg/mL) whereas the IC_{50} of ascorbic acid was 62.03 µg/mL lower than the samples.

Superoxide radical, being very harmful to cellular components contributes to tissue damage and various diseases. Biologically, its toxic effect can be eliminated by superoxide dismutase (Chung *et al.,* 2005). Some oxidative enzymes are responsible for the generation of superoxide ion by using molecular oxygen (Sreedhar *et al.,* 2010). Different extracts from flowers of *A. malaccensis* were found to scavenge the superoxides with different rates in terms of different IC_{50} values. AMFPE, AMFHE, AMFDE, AMFME and AMFEO were able to scavenge the superoxide anion with an IC_{50} of 233.00, 214.83, 206.98, 166.51 and 138.72 µg/mL respectively. The IC_{50} ascorbic acid, the standard antioxidant was obtained 32.28 µg/mL.

Reducing power activity is one of the crucial indicators of antioxidant activity of several compounds. Basically, ferric ions (Fe^{3+}) are converted to ferrous ions (Fe^{2+}) in this assay which can be estimated by a change in color that can be read spectrophotometrically (Siddhuraju, Mohan, Beaker, 2002). In the present study the Fe^{3+} to Fe^{2+} reducing activity to exhibit the antioxidant ability in terms of their RP_{50} values of various extracts of *A. malaccensis* was obtained in the order of AMFME (RP_{50}= 188.02 µg/mL) > AMFEO (RP_{50}= 190.71 µg/mL) >AMFDE (RP_{50}= 202.24 µg/mL) > AMFHE (RP_{50}= 221.00 µg/mL) >AMFPE (RP_{50} = 250.25 µg/mL). However, the RP_{50} of the entire standard was obtained as Ascorbic acid (RP_{50}= 115.63 µg/mL) > BHT (RP_{50}= 117.88 µg/mL) > catechin (RP_{50}= 143.91 µg/mL) > gallic acid (RP_{50}= 151.47 µg/mL).

Fe^{2+} ion form complex with ferrozine in the presence of complexing agent and equilibrium in between Fe^{2+} ion and ferrozine is disturbed which is indicated by decrease in intensity of colour. Different extracts and essential oil of flower of *A. malaccensis* demonstrated varying degree of binding capacity for Fe^{2+} ion that expressed their ability as a per oxidation protector. The IC_{50} values for various extracts and essential oil for their antioxidant potentiality in terms of chelating ability was obtained as AMFEO (IC_{50}= 153.15 µg/mL) > AMFME (IC_{50}= 156.88 µg/mL) > AMFDE (IC_{50}= 177.77 µg/mL) > AMFHE (IC_{50} = 184.79 µg/mL) > AMFPE (IC_{50} = 201.59 µg/mL). However, the IC_{50} for standard viz; EDTA and citric acid were obtained IC_{50}= 47.72 µg/mL and 61.76 µg/mL respectively. The IC_{50} values of all the extracts assayed for the antioxidant activity by various methods along with their phenolic content have been reported in Table III while the correlation of phenols with IC_{50} has been recorded in Table IV.

Total Phenols

The total phenols in all the extracts were obtained in the range of 15.03 to 64.60 µg/mL. The highest phenolic content was observed in AMFME the followed by AMFDE, AMFHE and AMFPE. The presence of phenol in various extracts of flowers of *A. malaccensis* may be responsible for its antioxidant potential of the plant. A direct correlation between phenols and antioxidant activity has already been reported earlier (Sethi *et al.,* 2015). The total phenols were correlated with IC_{50} /RP_{50} values of different methods used for the determination of antioxidant assay in extracts. The results obtained showed negative correlation with DPPH radical scavenging, metal chelating ability, superoxides anion scavenging activity, $^{\bullet}OH$ radical scavenging activity and nitric oxide radical scavenging activity and at α=0.01 or 0.05 (level of significance) (Table IV)

TABLE IV - Correlation of total phenols with IC_{50} values

Phenols	Total phenols	Correlation coefficient (R)					
		IC_{50}(µg/ml)					RP_{50}(µg/ml)
		DPPH Scavenging	OH scavenging	Nitric oxide radical scavenging activity/	Super oxide anion scavenging activity	Metal chelating ability	Reducing power ability
Total phenols	01	-0.975**	-0.901*	-0.942*	-0.924*	-0.959*	-0.963*

* Significant at α=0.05, ** Significant at α=0.01

Antifungal activity of essential oil and extracts of flowers of *A. malaccensis*

Antifungal activity of all the extracts of flowers of *A. malaccensis* exhibited inhibitory effect on the growth of all fungi in a dose dependent manner (Table V). *S. sclerotium* was most suppressed as its growth was mostly reduced by all tested doses followed by *S. rolfsii*, *R. solani* and *C. falcatum.* AMFME exhibited strong inhibitory effect against fungus *S. sclerotium* (90.83%), *R. solani* (48.33%), *S. rolfsii* (55%) and *C. falcatum* (21.66%) even at a lower concentration of 250 µg/mL. At the same concentration AMFEO exhibited good inhibitory effect against *S. solani* by inhibiting its fungal mycelial growth upto 49.16%. However, towards the other three fungi, it exhibited slight or no inhibitory effects.

TABLE V - Antifungal activity with MIC values of AMFEO and different extracts of *A. malaccensis*

Extracts	Concentration (µg/ml)	Antifungal activities (% of inhibition) (MIC)			
		C. falcatum	*R.solani*	*S. sclerotium*	*S. rolfsii*
AMFPE	250	$0±0^{a}$	$2.5±1.4^{a}$	$0±0^{a}$	$0±0^{a}$
	500	$5±1.4^{ab}$	$3.33±2.2^{a}$	$34.16±3.0^{b}$	$6.66±2.2^{a}$
	750	$29.16±2.2^{d}$(500)	$32.5±1.4^{c}$(250)	$78.33±1.6^{e}$(500)	$42.5±1.4^{c}$(500)
	1000	$61.66±0.8^{f}$	$44.16±1.6^{d}$	$86.66±0.8^{fg}$	$79.16±0.8^{e}$
AMFHE	250	$0±0^{a}$	$3.33±2.2^{a}$	$0±0^{a}$	$5±1.4^{a}$
	500	$10±2.5^{bc}$(500)	$20.83±0.8^{b}$(250)	$42.5±1.4^{cd}$(500)	$41.66±3.0^{c}$(250)
	750	$49.16±0.8^{e}$	$45±2.5^{d}$	$83.33±2.2^{ef}$	$46.66±2.2^{cd}$
	1000	$100±0^{g}$	$100±0^{g}$	$93.33±0.8^{gh}$	$81.66±0.8^{ef}$
AMFDE	250	$5±2.5^{ab}$	$31.66±1.6^{c}$	$40.83±2.2^{bc}$	$43.33±2.2^{c}$
	500	$43.33±1.6^{e}$(250)	$45±1.4^{d}$(250)	$87.5±1.4^{fg}$(125)	$55±1.4^{d}$(125)
	750	$60.83±0.8^{f}$	$59.16±0.8^{e}$	$91.66±0.8^{g}$	$86.66±0.8^{ef}$
	1000	$100±0^{g}$	$100±0^{g}$	$100±0^{h}$	$100±0^{g}$
AMFME	250	$21.66±1.6^{d}$	$48.33±0.8^{d}$	$90.83±0.8^{fg}$	$55±1.4^{d}$
	500	$47.5±2.5^{e}$(250)	$69.16±1.6^{f}$(125)	$93.33±1.6^{gh}$(31.25)	$90.83±3.0^{g}$(62.5)
	750	$65.83±2.2^{f}$	$100±0^{g}$	$100±0^{h}$	$100±0^{g}$
	1000	$100±0^{g}$	$100±0^{g}$	$100±0^{h}$	$100±0^{g}$

TABLE V - Antifungal activity with MIC values of AMFEO and different extracts of *A. malaccensis*

Extracts	Concentration (µg/ml)	Antifungal activities (% of inhibition) (MIC)			
		C. falcatum	***R.solani***	***S. sclerotium***	***S. rolfsii***
AMFEO	250	0 ± 0^{a}	1.66 ± 1.6^{a}	49.16 ± 1.6^{d}	0 ± 0^{a}
	500	13.33 ± 1.6^{c}(500)	5 ± 1.4^{a}(250)	90 ± 2.8^{fg}(125)	5 ± 1.4^{a}(500)
	750	46.66 ± 0.8^{e}	41.66 ± 0.8^{d}	91.66 ± 0.8^{g}	23.33 ± 3.3^{b}
	1000	100 ± 0^{g}	100 ± 0^{g}	100 ± 0^{h}	46.66 ± 2.2^{cd}

Values are means of three replicates± SE. Within a column, mean values followed by the same letter are not significantly different according to Tukey's test ($p<0.05$).

Almost all the extracts (except AMFPE) and essential oil of *A. malaccensis* were fungicidal (100% inhibition) towards *C. falcatum* and *R. solani* at a concentration of 750µg/mL or 1000 µg/mL. AMFME and AMFDE exhibited strongest fungicidal action by completely suppressing all the fungus at a concentration of 1000 µg/mL. At the same concentration AMFEO exhibited strong antifungal activity against all the plant pathogenic by suppressing them completely. However, the suppression of AMFEO towards *S. rolfsii* was found to be weak. AMFHE exhibited moderate antifungal activity against all the plant pathogenic fungi ranging from 81.66% to 100%. AMFPE exhibited weak antifungal activity against all the plant pathogenic fungi ranging from 44.16% to 86.66%. The minimum inhibitory concentration (MIC) defined as the lowest concentrations of each extract resulting in the reduction of the inoculum demonstrated in Table V. The MIC values of AMFPE, AMFHE, AMFDE, AMFME, AMFEO ranged from 250 to 500, 250 to 500, 125 to 250, and 31.25 to 250 µg/mL, 125 to 500 µg/mL respectively. The present study indicated that AMFME and AMLDE were found to be the significant, effective and remarkable fungicides in this study in accordance to their inhibition action against all tested pathogenic fungi followed by other extracts. Several oils are reported to possess strong fungitoxic effects. (Sethi *et al.,* 2016). The fungitoxicity of AMFEO might be possibly due to presence of several mono and sesquiterpenoids. (Souza, Sales, Martins, 2009). Terpinen-4-ol (28.6%), the major constituent, obtained in AMFEO might be possibly responsible for the antifungal activity of this plant. The antifungal activity of terpinen-4-ol and its role as antifungal agent had already been reported (Morcia, Malnati, Terzi, 2012)

The antifungal activity of extracts and oils is indicative of the presence of several phytoconstituents such as flavonoids, phenolics whose antifungal activity has already been reported in earlier studies (Winkelhausen, Pospiech, Laufenberg, 2005, Orhan *et al.,* 2010). The fungicidal action of AMFME and AMFDE might be possibly due to high polarity index of methanol and DCM that can extract polar compounds, thereby inhibiting fungus effectively.

CONCLUSION

The essential oil was dominated by terpinen-4-ol (28.6%) and α- terpineol (12.8%) as the main constituent while the extracted from *Alpinia malaccensis* (Burm.f.) Roscoe flowers possess high phenolic content and exhibits marked antioxidant properties in terms of Fe^{3+} reducing power, metal chelating ability, OH and NO radical and superoxide anion scavenging activities vis-à-vis standard antioxidant. Moreover, most of extracts and essential oil of *A. malaccensis* exerts antifungal activities. The result is significant as fungal strains are known to be stubborn in terms of their pathogenesis and there are only few antifungal drugs available. If further explored properly, bioprospecting of *A. malaccensis* can lead to utilization of plant as cost effective potent antioxidant and antifungal agent for pharma and related industries.

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