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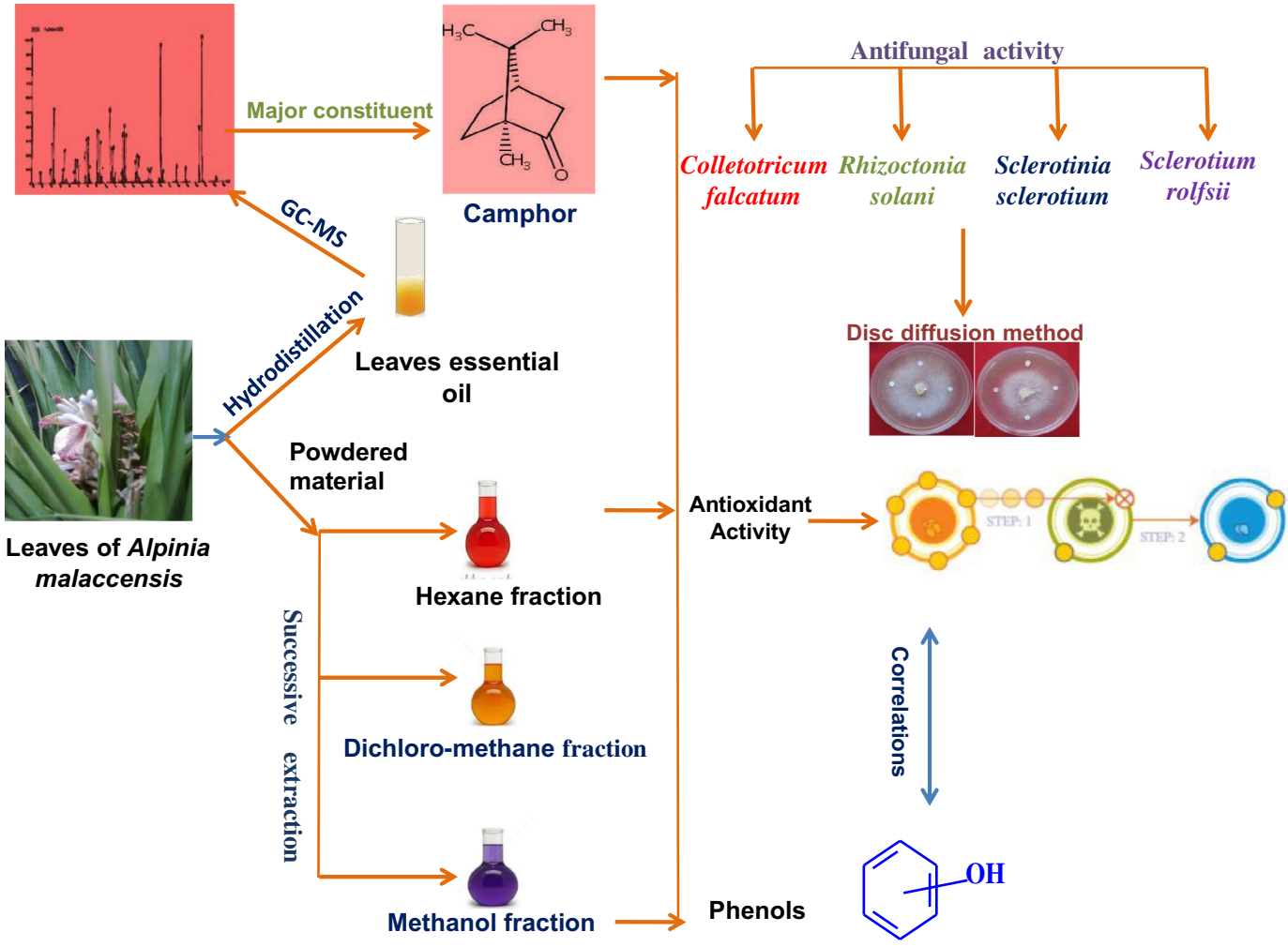

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Phytochemical analysis, antioxidant assay and antifungal activity of essential oil and various extracts of *Alpinia malaccensis* (Burm.f.) Roscoe leaves

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Abstract: The present study describes chemical composition, antifungal activities, antioxidant assays and total phenolic content of essential oil and different extracts from leaves of *Alpinia malaccensis*. Sixty-eight components were identified by gas chromatography and gas chromatography–mass spectrometry with camphor (15.2%) and eucalyptol (10.2%) as the main component. The essential oil was found to possess remarkable fungicidal action against tested pathogens like *Colletotricum falcatum*, *Rhizoctonia solani*, *Sclerotinia sclerotium* and *Sclerotium rolfsii* followed by different extracts obtained in solvents like methanol, dichloromethane and hexane, respectively. The essential oil showed higher phenolic contents (69.51 µg/ml) than the extracts. The antioxidant assay of essential oil and extracts by different methods revealed good-to-moderate antioxidant potential with different IC_{50} values viz. (174.16–230.01 µg/ml) for Fe^{3+} reducing power, (111.74–158.23 µg/ml) for Fe^{2+} metal-chelating ability, (82.64–96.66 µg/ml) for DPPH, (94.28–189.41 µg/ml) for OH radical, (76.96–135.63 µg/ml), for NO radical and (107.30–199.75 µg/ml) in superoxide anion scavenging activities, respectively, compared to the standard antioxidants. Based on obtained results, the herb *A. malaccensis* can be a good source to develop a safe and sustainable natural antioxidant.

Sonali Sethi

ABOUT THE AUTHORS

Sonali Sethi, the first author, obtained her PhD from G. B. Pant University of Agriculture and Technology, Pantnagar in 2015. Her research focuses on the plants of family Zingiberaceae for several biological activities. She had pursued her PhD under the supervision of Om Prakash.

Prakash has 16 years of experience in the field of natural product and he has handled many national projects funded by University Grants Commission and DST, New Delhi. His work in the field can be evidenced by many published national and international papers in referred journals. His current research interest focuses mainly on analysis of several natural compounds by chromatographic techniques such as GC and HPLC, mass spectrometry, FTIR, NMR, and determination of various biological activities. The present study deals with chemical analysis and biological activity of *Alpinia malaccensis* leaves. The study will be helpful in the preparation of database, so that the herb can be explored scientifically and judiciously.

PUBLIC INTEREST STATEMENT

The family Zingiberaceae, particularly known as Ginger family, is cosmopolitan in distribution. Herbs of this family are frequently used for various purposes like spices, dyes, perfumes, cosmetics, food flavour, ornamental, horticulture, etc. including traditional system of medicine. In the present scenario, there is an increasing demand for nutraceutical and natural food preservative to reduce the oxidative deterioration of food and food products over the synthetic compounds which may have devastating effect on human health and environment. The voluminous research on the Zingiberaceous plant is needed to explore its potential as antifungal and antioxidant agent so that it can be used for curing several diseases and for medicinal purposes. The present investigation reports the efficacy of *A. malaccensis* with antifungal and antioxidant potential so that this plant can be used as a safe and herbal food preservative because of its antifungal and antioxidant activity.

Subjects: Bioscience; Environment & Agriculture; Food Science & Technology; Medicine, Dentistry, Nursing & Allied Health

Keywords: *Alpinia malaccensis*; leaves; antioxidant activity; antifungal activity; total phenols

1. Introduction

Presently, the plants and plant-derived compounds are frequently employed in industries, food preservatives, pharmaceutical, cosmetics, perfumes, etc. because of their antifungal, antibacterial, antioxidant and bioregulatory properties (Ahmadi, Sadeghi, Modarresi, Abiri, & Mikaeli, 2010). They also possess a variety of phenolics, alkaloids and flavonoids that are often used to treat various diseases (Siddhuraju & Manian, 2007).

Synthetic compounds are used as antimicrobial agent in crop protection for the control of plant pathogen but have certain threats to environmental quality and human health (Tian et al., 2011). Antioxidants quench reactive oxygen species (ROS) and reactive nitrogen species (RNS) generated in the body, thereby reducing oxidative stress (Al-Tawaha, Al-Karaki, & Massadeh, 2013). Though synthetic antioxidants like butylated hydroxyanisole (BHA) and butylated hydroxytoluene (BHT) are employed in food products, they lead to liver damage (Valentão et al., 2002). Due to several limitations of synthetic compounds there is an urgent need for exploration and development of new, safe natural antifungal and antioxidants products (Pavela, Sajfrtová, Sovová, Bárnet, & Karban, 2010).

Zingiberaceae or the Ginger family is being explored worldwide due to its high medicinal value and versatile nature. It comprises 52 genera and about 1,587 species distributed throughout tropical Asia (The Plant List, 2013). India is rich in the diversity of Zingiberaceae and family being represented by 24 genera and 191 species (Karthikeyan, 2000). The zone of greatest concentration of this family is the north-east region of India comprising 19 genera and about 88 species (Prakash & Mehrotra, 1995).

A. malaccensis (Jangali adrak) is a perennial plant found abundantly and widely in the tropical and subtropical regions. It is a tall herb growing in forests whose rhizomes are fibrous in nature and cultivated as an ornamental plant (Nuntawong & Suksamrarn, 2008). Tuberpaste of *A. malaccensis* is applied as curative on sores (Abhyankar & Upadhyay, 2011). Fruits of this herb are applied on gastralgia (Bhuiyan, Chowdhury, Begum, & Nandi, 2010). There is no published report regarding the antioxidant activity and antifungal activity of the non polar extracts of *Alpinia malaccensis* leaves. In continuation to our research programme on Zingiberaceae, the present investigation was carried out to evaluate GC–MS analysis and evaluation of its antifungal and antioxidant activity. The total phenolic content of essential oils and extracts of leaves *A. malaccensis* were also evaluated.

2. Experimental

2.1. Collection of plant material

Fresh leaves 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.

2.2. Extraction of essential oils

Fresh leaves (1 kg) were subjected to hydrodistillation in Clevenger's apparatus for 8 h. Extraction of distillate with diethyl ether followed by drying over anhydrous Na_2SO_4 and removal of solvent yielded 0.08% of *A. malaccensis* leaves essential oil (AMLEO).

2.3. Preparation of the extracts

The leaves 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 hexane, dichloromethane and methanol. The extracts were filtered using filter paper and muslin cloths. The filtrates so obtained were concentrated using a rotary evaporator. Yields of different extracts viz. *A. malaccensis*, leaves hexane extract (AMLHE), *A. malaccensis* leaves dichloromethane extract (AMLDE) and *A. malaccensis*, leaves methanol extract (AMLME) were found to be 0.82, 0.77 and 0.75%, respectively. The extracts were stored at 4°C for further analysis and evaluation of biological activities.

2.4. GC–FID analyses

Gas chromatographic analyses were carried using Thermoseries 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). Gas chromatography operation conditions: injection mode, split (40:1, v/v); injection volume1 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 (Table 1).

2.5. GC–MS analyses

Gas chromatography–mass spectrometry (GC–MS) analyses were carried out on Hewlett Packard 6890 gas chromatograph (Agilent Technologies, USA), fitted with an HP-5 (5% phenyl methylpolysiloxane, 30 m × 0.32 mm id., 0.25 µm film thickness) capillary column coupled with a model 5973 mass detector. GC–MS, 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 comparison of KI values and mass spectra of constituents with Adams database (Adams, 2007) (Table 1).

2.6. Antioxidant activity

The antioxidant activity of essential oil and extracts were studied using different methods.

2.6.1. DPPH radical scavenging activity

To evaluate scavenging ability of the antioxidants by this method, a standard protocol was followed (Sethi, Prakash, & Pant, 2015a). In brief, different amounts of the tested sample (50–250 µg/ml) were added to 5 ml of 0.004% methanol solution of DPPH. Finally, the absorbance was read against a blank at 515 nm after 30 min of incubation in the dark. All the observations were taken in triplicate. BHT, catechin and gallic acid were used as the standard antioxidant. Inhibition of free radical by DPPH in per cent (IC%) was calculated 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. Per cent inhibition was plotted against concentrations and the equation for the line was used to obtain the IC_{50} value.

2.6.2. Hydroxyl radical scavenging activity

This activity was evaluated using the method described earlier (Olabinri et al., 2010). Sixty microlitres of $FeSO_4 \cdot 7H_2O$ (1 mM) was added to 90 µl of aqueous 1, 10 phenanthroline (1 mM), 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 (0.17 mM) and 1.5 ml of different concentrations of the sample (50–250 µg/ml). The absorbance of the mixture was read at 560 nm against blank after 5min. Ascorbic acid was used as 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. Lower IC_{50} value indicates greater hydroxyl radical scavenging ability.

2.6.3. NO radical scavenging activity

The nitric oxide scavenging activity of oil and extracts was determined using the previously reported method (Naskar et al., 2010). Two millilitres of sodium nitroprusside (SNP) (10 mM) in phosphate buffer saline (PBS) pH 7.4 were mixed with different concentration of sample (50–250 µg/ml) and

Table 1. Chemical composition of AMLEO

Compounds	KI	FID response (%)	Method of identification (GC/MS)
β-pinene	980	0.6	M^+ = 136, *m/e* = 121,93,79,69,53,41
Dehydro-1,8 cineole	991	0.1	M^+ = 152, *m/e* = 109,94,79,69,43,41
p-cymene	1,026	0.2	M^+ = 134, *m/e* = 119,91,77,65,51,41
Eucalyptol	1,031	10.2	M^+ = 154, *m/e* = 139,125,108,84,69
Linalool oxide	1,072	0.1	M^+ = 170, *m/e* = 155,137,111,94,59
Linalool	1,096	0.5	M^+ = 154, *m/e* = 136,121,107,93,71
Cis pinene hydrate	1,103	0.1	M^+ = 154, *m/e* = 139,121,111,93,43
Fenchyl alcohol	1,116	0.1	M^+ = 154, *m/e* = 139,121,111,69,43
Nopinone	1,137	0.5	M^+ = 138, *m/e* = 123,109,95,83,69
Camphor	1,146	15.2	M^+ = 152, *m/e* = 119,108,95,81,41
Camphene hydrate	1,148	0.5	M^+ = 154, *m/e* = 136,121,96,71,41
Trans-pinocamphone	1,158	0.1	M^+ = 152, *m/e* = 136,95,83,69,55,41
Pinocarvone	1,160	0.5	M^+ = 150, *m/e* = 135,122,108,81,53
Borneol	1,165	2.8	M^+ = 154, *m/e* = 139,95,71,67,55
4-terpineol	1,177	0.9	M^+ = 154, *m/e* = 136,111,93,71,41
α-terpineol	1,186	6.6	M^+ = 154, *m/e* = 136,121,93,81,59
Myrtenal	1,195	1.3	M^+ = 150, *m/e* = 121,107,91,79,66
p-cymen-8-ol	1,196	0.9	M^+ = 150, *m/e* = 135,117,105,91,65
Trans-p-mentha-1(7),8-diene-2-ol	1,196	0.1	M^+ = 152, *m/e* = 134,119,109,91,79
Verbenone	1,205	0.6	M^+ = 150, *m/e* = 135,122,107,91,80
Trans-carveol	1,216	0.3	M^+ = 152, *m/e* = 134,119,109,84,41
Cis chrysanthenol acetate	1,265	0.1	M^+ = 194, *m/e* = 134,119,109,69,43
Cis-pulegone oxide	1,275	0.1	M^+ = 168, *m/e* = 150,126,108,69,41
Bornyl acetate	1,285	2.2	M^+ = 196, *m/e* = 154,136,121,80,67
Methyl-myrtenate	1,293	0.1	M^+ = 180, *m/e* = 165,137,105,93,77
Perilla alcohol	1,295	0.2	M^+ = 152, *m/e* = 134,121,93,79,68
2,3 pinanediol	1,318	0.8	M^+ = 170, *m/e* = 155,126,108,71,43
Myrtenyl acetate	1,326	0.1	M^+ = 194, *m/e* = 134,119,91,79,43
Methyl (*E*)-cinnamate	1,378	7.7	M^+ = 162, *m/e* = 131,103,77,51,49
Daucene	1,380	0.8	M^+ = 204, *m/e* = 189,161,133,121,105
β-elemene	1,390	0.3	M^+ = 204, *m/e* = 189,121,107,81,53
n-tetradecane	1,400	0.1	M^+ = 198, *m/e* = 169,113,85,71,57
(*Z*)-caryophyllene	1,408	0.2	M^+ = 204, *m/e* = 189,161,133,93,79
α-*trans*-bergamotene	1,434	0.2	M^+ = 204, *m/e* = 119,107,93,79,69
ε-muurolene	1,446	0.1	M^+ = 204, *m/e* = 189,161,148,105,91
Trans-geranylacetone	1,453	0.1	M^+ = 194, *m/e* = 176,151,107,69,43
Bicyclogermacrene	1,494	0.2	M^+ = 204, *m/e* = 189,121,93,79,67
γ-cadinene	1,513	0.2	M^+ = 204, *m/e* = 161,133,119,105,91
α-copaen-11-ol	1,541	0.3	M^+ = 220, *m/e* = 162,147,105,91,59
α-elemol	1,548	0.3	M^+ = 222, *m/e* = 161,147,121,93,59
Hedycaryol	1,548	0.7	M^+ = 222, *m/e* = 204,161,107,93,59
(*E*)-nerolidol	1,561	0.4	M^+ = 222, *m/e* = 161,136,93,69,43

(*Continued*)

Table 1. (*Continued*)

Compounds	KI	FID response (%)	Method of identification (GC/MS)
Caryophyllene oxide	1,582	2.1	M^+ = 220, *m/e* = 177,121,93,79,69
α-thujopsan-2-ol	1,584	1.2	M^+ = 222, *m/e* = 205,123,109,95,67
Epiglobulol	1,588	0.1	M^+ = 222, *m/e* = 161,109,93,82,43
Carotol	1,594	7.9	M^+ = 222, *m/e* = 204,161,119,97,81
Widdrol	1,597	1.7	M^+ = 222, *m/e* = 151,121,109,95,81
Fokienol	1,599	1.2	M^+ = 220, *m/e* = 159,134,119,107,71
Humulene epoxide II	1,608	0.2	M^+ = 220, *m/e* = 138,123,109,96,67
Cadin-4-en-10-ol	1,627	0.8	M^+ = 222, *m/e* = 204,161,121,95,81
Daucol	1,641	2.6	M^+ = 238, *m/e* = 220,151,133,93,43
β-eudesmol	1,650	1.5	M^+ = 222, *m/e* = 204,149,108,93,59
α-bisabolol	1,683	0.4	M^+ = 222, *m/e* = 204,119,109,69,43
α-(Z)-*trans*-bergamotol	1,690	0.1	M^+ = 220, *m/e* = 187,119,79,68,55
Exo-2-hydroxycineole acetate	1,716	0.2	M^+ = 212, *m/e* = 152,108,93,71,43
n-hexadecanol	1,874	0.1	M^+ = 242, *m/e* = 196,125,111,97,41
Butyl phthalate	1,922	0.2	M^+ = 278, *m/e* = 223,149,132,104,27
Phytol	1,942	0.2	M^+ = 296, *m/e* = 279,197,140,71,43
Manool	2,057	0.7	M^+ = 257, *m/e* = 204,137,123,95,81
n-octadecanol	2,077	0.1	M^+ = 270, *m/e* = 252,140,111,97,83
n-tricosane	2,300	0.3	M^+ = 324, *m/e* = 267,155,113,85,71
n-tetracosane	2,400	0.2	M^+ = 338, *m/e* = 211,141,99,71,57
n-pentacosane	2,500	0.5	M^+ = 352, *m/e* = 281,183,113,85,57
Isooctyl phthalate	2,519	0.2	M^+ = 390, *m/e* = 279,167,149,71,57
n-hexacosane	2,600	0.4	M^+ = 366, *m/e* = 281,239,197,85,57
n-nonacosane	2,900	0.5	M^+ = 408, *m/e* = 267,169,99,71,57
Dotriacontane	3,200	0.1	M^+ = 450, *m/e* = 211,155,99,71,57
Tetratriacontane	3,400	0.1	M^+ = 478, *m/e* = 211,183,127,71,57
% identified		81.0	

Notes: M^+ = molecular mass and *m/e* = major mass fragments.

incubated at 25°C for two and half hours. To the above sample, 1 ml of Griess reagent (1% sulphanilamide, 0.1% naphthylethylenediamine dichloride and 2 ml orthophosphoric acid) was added. As a result pink colour was obtained and the absorbance was read at 546 nm. Ascorbic acid was used as 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 per cent inhibition was plotted against concentration, and the equation for the line was used to obtain the IC_{50} value.

2.6.4. Superoxide anion scavenging activity

Superoxide anion scavenging activity of the oil and extracts was determined according to the reported method with slight modifications (Nishikimi, 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 by 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. Per cent inhibition was plotted against concentration, and the equation for the line was used to obtain the IC_{50} value.

2.6.5. Reducing power

The reducing power of essential oil and extracts was determined by the method reported earlier (Sethi et al., 2015a). 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°C for 20 min. After incubation, 2.5 ml of 10% trichloroacetic acid was added to the mixtures, followed by centrifugation at 650 rpm for 10 min. The upper layer (5 ml) was mixed with 5 ml distilled H_2O and 1 ml 0.1% ferric chloride and absorbance of the resulting solution was 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. Per cent inhibition was plotted against concentration, and the equation for the line was used to obtain the RP_{50} value.

2.6.6. Metal chelating ability

The chelation of Fe^{2+} by essential oil and extracts was evaluated using the method developed earlier (Sethi et al., 2015a). Different concentrations of tested sample (50–250 µg/ml) were first mixed with 1 ml methanol and 3.7 ml deionized water. The resulting mixture was allowed to react with 0.1 ml of $FeCl_2$ (2 mM) and 4.2 ml 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 EDTA (0.01 mM), citric acid (0.025 M) were taken as standard. The metal-chelating ability of the tested sample, expressed as percentage was 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 per cent of chelating ability was plotted against concentration, and the equation for the line was used to obtain the IC_{50} value.

2.7. Total phenols

The total phenolic content of oil and extracts was estimated by the method reported earlier (Singleton & Rossi, 1965). About 0.5 ml of the extract solutions was mixed with 1.0 ml of Folin–Ciocalteu reagent, 1.0 ml aqueous solution of 7% sodium carbonate and 5 ml 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 adopted for 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.

2.8. Micro-organism

The pathogenic fungi viz. *Colletotricum falcatum*, *Rhizoctonia solani*, *Sclerotinia sclerotium* and *Sclerotium rolfsii* were maintained on potato dextrose agar (PDA).

2.9. Antifungal activity of AMLEO and extracts

The antifungal activity was determined using the disc diffusion method (Murray, Baron, Pfaller, Tenover, & Yolke, 1995). Initially, each sample was diluted with acetone: water (1:1) to obtain the final concentrations of 1,000, 500, 750, and 250 µg/ml, respectively. The pathogenic fungi were cultured on PDA media and incubated at 30°C for 3–4 days. A plug of one-week-old fungal culture (5 mm diameter) was placed on the centre of the sterilized plates containing PDA. About 10 µl of each concentration was injected to the sterile disc papers (6 mm diam.). Then the prepared discs were placed on the culture medium. Negative control was prepared using the same solvent employed to dissolve the oil and the extracts. The plates were then incubated at 30°C 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 by the following equation:

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

3. Results and discussion

3.1. GC–MS of AMLEO

Sixty-nine components comprising over 81.0% were identified in AMLEO. The oil was dominated by 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%), respectively (Table 1). Among the identified components, 0.8% were monoterpene hydrocarbons, 44.5% oxygenated monoterpene. Among sesquiterpenoids 2% were hydrocarbons, and 21.5% were oxygenated sesquiterpenes, respectively. In the oil, 0.9% were oxygenated diterpenes and 11.3% aliphatic compounds could be identified.

Comparing the present investigation the report with Sahoo, Singh, and Nayak (2014) (21) from Southern part of India, it was observed that there was a vast chemical diversity in the qualitative and quantitative make-up of essential oil constituents in leaves of *A. malaccensis*. The oil from Southern part revealed the presence of α-phellandrene (43.9%), β-cymene (31.7%), β-pinene (4.6%), β-caryophyllene (3.3%), caryophyllene oxide (1.7%), α-pinene (1.5%) as the major compound. Muchtaridi et al. (2014) (12) from Indonesia reported α-pinene (30.57%) as the major compound in oil. The analysis of oils reported from Indian habitat by us and Sahoo et al. (2014) (21) were different in qualitative and quantitative make-up beside some common molecules in all the oils viz. methyl (*E*)-cinnamate, trans carveol, α-terpineol, pinocarvone, linalool oxide, linalool reported by us and Indonesian oils were missing in the oil reported from Southern India. In Indian oils, the compounds reported by us and missing in the oil reported by Sahoo et al. (2014) (21) were carotol (7.9%), daucol (2.6%), β-eudesmol (1.5%), fokienol (1.2%), widdrol (1.7%), α-thujopsan-2-ol (1.2%), myrtenal (1.3%), borneol (2.8%), eucalyptol (10.2%), etc. Similarly, the compounds reported by Sahoo et al. (2014) (21) and missing in our findings were δ-cadinene (0.8%), β-caryophyllene (3.3%), α-phellandrene (43.9%), β-cymene (31.7%), pinocarveol (2.2%) and α-selinene (0.7%). In other study Bhuiyan et al. (2010) (5) from Bangladesh reported α-phellandrene (31.80%), eucalyptol (13.76%), o-cymene (11.45%), β-pinene (11.34%) and limonene (6.44%) in *A. malaccensis* leaves essential oil. From this study, it can be concluded that the essential oils from different origins have different molecular diversities in terms of terpenoids, in spite of having same morphological and taxonomical characters, hence are different chemotypes of *A. malaccensis*. This molecular diversity of chemotypes could be possibly due to the climatic, altitudinal and edaphic factors.

3.2. Antioxidant activity

AMLEO and the extracts exhibited strong to good DPPH radical scavenging activity (Table 2). The radical scavenging potential in the form of their IC_{50} values revealed the order AMLEO (IC_{50} = 82.64 µg/ml) > AMLME (IC_{50} = 83.28 µg/ml) > AMLDE (IC_{50} = 86.39 µg/ml) > AMLHE (IC_{50} = 96.66 µg/ml). The order of IC_{50} for standard antioxidant was ascorbic acid (IC_{50} = 32.46 µg/ml) > BHT (IC_{50} = 33.81 µg/ml) > catechin (IC_{50} = 42.99 µg/ml) > gallic acid (IC_{50} = 49.27 µg/ml). DPPH exhibiting absorbance at 517 nm disappeared after accepting a hydrogen radical from an antioxidant compound (Matthäus, 2002). The antioxidant power of AMLEO and extracts might be attributed to their hydrogen-donating ability to DPPH free radical.

AMLEO and extracts scavenge OH free radical as function of amounts in a selected dose levels in comparison to the standard antioxidant ascorbic acid. The results obtained for their good antioxidant potential in terms of IC_{50} (µg/ml) values were 189.41, 162.22, 135.46 and 94.28, for AMLHE, AMLDE, AMLME and AMLEO, respectively. IC_{50} of ascorbic acid was obtained as 44.36 µg/ml.

Table 2. Antioxidant potential in term of IC_{50} values for AMLEO and different extracts of *A. malaccensis* along with their total phenolic content

Sample/ Standard	Antioxidant assay						
	DPPH scavenging activity/IC_{50} (µg/ml)	$^{\cdot}OH$ 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)
AMLHE	96.66 ± 1.77^{f}	189.41 ± 2.29^{e}	135.63 ± 1.36^{e}	199.75 ± 3.25^{e}	230.01 ± 1.51^{h}	158.23 ± 2.28^{e}	31.51 ± 0.37^{a}
AMLDE	86.39 ± 0.96^{e}	162.22 ± 1.37^{d}	108.44 ± 0.90^{d}	173.87 ± 3.12^{d}	225.17 ± 0.71^{g}	144.77 ± 1.56^{d}	48.48 ± 0.10^{b}
AMLME	83.28 ± 0.38^{de}	135.46 ± 2.72^{c}	86.74 ± 1.52^{c}	74.20 ± 2.37^{b}	194.46 ± 0.95^{f}	110.37 ± 0.61^{c}	56.84 ± 0.37^{c}
AMLEO	82.64 ± 0.75^{d}	94.28 ± 1.62^{b}	76.96 ± 0.14^{b}	107.30 ± 3.76^{b}	174.16 ± 0.86^{e}	111.74 ± 1.32^{c}	69.51 ± 0.27^{d}
BHT	33.81 ± 0.41^{a}	–	–	–	117.88 ± 0.33^{b}	–	–
Catechin	42.99 ± 2.70^{b}	–	–	–	143.91 ± 0.61^{c}	–	–
Gallic acid	49.27 ± 1.11^{c}	–	–	–	151.47 ± 0.64^{d}	–	–
Ascorbic acid	32.46 ± 0.28^{a}	44.36 ± 4.54^{a}	62.03 ± 0.31^{a}	32.28 ± 1.17^{a}	115.63 ± 0.29^{a}	–	–
EDTA	–	–	–	–	–	47.72 ± 2.35^{a}	–
Citric acid	–	–	–	–	–	61.76 ± 0.36^{b}	–

Notes: – = 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 ($p < 0.05$).

Through their nitric oxide scavenging ability, AMLEO and all the extracts exhibited varying degrees of antioxidant potential as indicated by the IC_{50} values obtained from these samples. However, the order of nitric oxide scavenging activity was AMLEO (IC_{50} = 76.96 µg/ml) > AMLME (IC_{50} = 86.74 µg/ml) > AMLDE (IC_{50} = 108.44 µg/ml) > AMLHE (IC_{50} = 135.63 µg/ml), whereas the IC_{50} of ascorbic acid was 62.03 µg/ml lower than the samples.

AMLEO and different extracts were found to scavenge the superoxides with different rates in terms of different IC_{50} values. AMLHE, AMLDE, AMLME and AMLEO were able to scavenge the superoxide anion with an IC_{50} of 199.75, 173.87, 74.20 and 107.30 µg/ml, respectively. IC_{50} ascorbic acid, the standard antioxidant was obtained as 32.28 µg/ml.

Reducing power acts as an important indicator of testing antioxidant potential. In this assay, ferric ions (Fe^{3+}) are reduced to ferrous ions (Fe^{2+}) with change in colour whose intensity depends on the reducing potential of the compounds available in the reaction medium, subsequently, related to the antioxidant activity (Siddhuraju, Mohan, & Beaker, 2002). In the present study, the reducing activity of Fe^{3+} to Fe^{2+} to exhibit the antioxidant ability in terms of their RP_{50} values was obtained in the order of AMLEO (RP_{50} = 174.16 µg/ml) > AMLME (RP_{50} = 194.46 µg/ml) > AMLDE (RP_{50} = 225.17 µg/ml) > AMLHE (RP_{50} = 230.01 µg/ml). However the RP_{50} of the entire standard was obtained as 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 forms complex with ferrozine in the presence of competitor complexing agent the equilibrium between Fe^{2+} ion and ferrozine is disturbed and indicated by decrease in colour intensity, thus showing its antioxidant activity. The IC_{50} values for AMLEO and various extracts for their antioxidant potentiality in terms of chelating ability were obtained as AMLEO (IC_{50} = 111.74 µg/ml) > AMLME (IC_{50} = 110.37 µg/ml) > AMLDE (IC_{50} = 144.77 µg/ml) > AMLHE (IC_{50} = 158.23 µg/ml). However, the IC_{50} for standard viz. EDTA and citric acid was obtained IC_{50} = 47.72 and 61.76 µg/ml, respectively. The IC_{50} values of AMLEO and all the extracts assayed for the antioxidant activity by various methods along with their phenolic content are reported in Table 2.

Table 3. Correlation of total phenols with IC_{50} values

Phenols	Correlation coefficient (*R*)						
	Total phenols	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	−0.921**	−0.981**	−0.983**	−0.805**	−0.929**	−0.907**

**Significant at $\alpha = 0.01$.

3.3. Total phenols

The total phenols in AMLEO and all the extracts were obtained in the range of 31.51–69.51 µg/ml. The highest phenolic content was observed in AMLEO the followed by AMLME, AMLDE and AMLHE. The antioxidant activity of AMLEO and different extracts might be possibly due to the presence of phenols in them. A direct correlation has been observed between phenols and antioxidant activity (Sethi et al., 2015a). The total phenols were correlated with IC_{50}/RP_{50} values of different methods used for the determination of antioxidant assay in essential oil and extracts. The results obtained showed negative correlation with DPPH radical scavenging, metal-chelating ability, superoxide anion scavenging activity, ·OH radical scavenging activity and nitric oxide radical scavenging activity and at $\alpha = 0.01$ or 0.05 (level of significance) (Table 3).

3.4. Antifungal activity of AMLEO and extracts

Antifungal activity of AMLEO and all the extracts exhibited inhibitory effect on the growth of all fungi in a dose-dependent manner (Table 4). *S. rolfsii* was most suppressed as its growth was mostly reduced by all tested doses followed by *S. sclerotium*, *R. solani* and *C. falcatum*. AMLEO exhibited strong inhibitory effect against fungus *R. solani* (77.50%), *S. sclerotium* (83.33%) and *S. rolfsii* (90.83%) even at a lower concentration of 250 µg/ml. However, it could not suppress the *C. falcatum* at this concentration.

Table 4. Antifungal activity of AMLEO and different extracts of *A. malaccensis*

Essential oil/ extracts	Concentration (µg/ml)	Antifungal activities (% of inhibition)			
		C. falcatum	*R. solani*	*S. sclerotium*	*S. rolfsii*
AMLHE	250	0.00 ± 0.00^{a}	0.00 ± 0.00^{a}	0.00 ± 0.00^{a}	2.50 ± 1.44^{a}
	500	15.00 ± 2.88^{bc}	42.50 ± 1.44^{b}	34.16 ± 3.00^{cd}	41.66 ± 0.83^{c}
	750	47.50 ± 1.44^{de}	55.00 ± 1.44^{c}	56.66 ± 1.66^{efg}	55.00 ± 1.44^{d}
	1,000	100.00 ± 0.00^{h}	100.00 ± 0.00^{e}	85.00 ± 2.5^{fgh}	80.83 ± 1.66^{e}
AMLDE	250	21.66 ± 2.20^{c}	00.00 ± 0.00^{a}	0.00 ± 0.00^{a}	2.50 ± 1.44^{a}
	500	45.83 ± 1.66^{de}	44.16 ± 3.00^{b}	49.16 ± 3.00^{b}	23.33 ± 0.83^{b}
	750	60.00 ± 1.44^{f}	80.83 ± 0.83^{d}	87.50 ± 1.44^{d}	55.00 ± 1.44^{d}
	1,000	100.00 ± 0.00^{h}	100.00 ± 0.00^{e}	91.66 ± 0.83^{ef}	100.00 ± 0.00^{g}
AMLME	250	53.33 ± 2.20^{ef}	46.66 ± 1.66^{b}	45.83 ± 0.83^{c}	40.83 ± 0.83^{c}
	500	60.83 ± 0.83^{f}	56.66 ± 0.83^{c}	82.50 ± 2.50^{e}	89.16 ± 1.66^{f}
	750	80.83 ± 0.83^{g}	100.00 ± 0.00^{e}	90.83 ± 1.66^{efg}	100.00 ± 0.00^{g}
	1,000	100.00 ± 0.00^{h}	100.00 ± 0.00^{e}	100.00 ± 0.00^{h}	100.00 ± 0.00^{g}
AMLEO	250	00.00 ± 0.00^{a}	77.50 ± 2.50^{d}	83.33 ± 0.88^{ef}	90.83 ± 0.83^{f}
	500	05.83 ± 2.20^{a}	100.00 ± 0.00^{e}	95.00 ± 1.44^{gh}	100.00 ± 0.00^{g}
	750	07.50 ± 1.44^{ab}	100.00 ± 0.00^{e}	100.00 ± 0.00^{h}	100.00 ± 0.00^{g}
	1,000	43.33 ± 2.20^{d}	100.00 ± 0.00^{e}	100.00 ± 0.00^{h}	100.00 ± 0.00^{h}

Notes: 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 and AMLEO were fungicidal (100% inhibition) towards *C. falcatum* and *R. solani* at a concentration of 750 µg/ml or 1,000 µg/ml. AMLME exhibited strongest fungicidal action by completely suppressing all the fungus at a concentration of 1,000 µg/ml. At the same concentration, AMLEO and AMLDE exhibited strong antifungal activity against all the plant pathogenic fungi by suppressing them completely. However, the suppression of AMLEO towards *C. falcatum* was found to be weak (43.33%). AMLHE exhibited moderate antifungal activity against all the plant pathogenic fungi ranging from 80.83 to 100%. The present study indicated that AMLME, AMLDE and AMLEO 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.

The fungal toxicity of AMLEO might be possibly due to the presence of mono and sesquiterpenoids or might also be due to the synergistic and antagonist effects of the compounds (Cakir, Kordali, Zengin, Izumi, & Hirata, 2004; Deba, Xuan, Yasuda, & Tawata, 2008). Camphor (15.2%), the major constituent beside eucalyptol, α-terpineol, methyl (*E*)-cinnamate, and carotol in AMLEO might be responsible for the antifungal activity of this plant, as the antifungal activity of these compounds have already been reported (Carson & Riley, 1995; Gupta & Saxena, 2010; Huang et al., 2009; Misiak et al., 2004; Morcia, Malnati, & Terzi, 2012). The antifungal activity of extracts might be due to the presence of diverse group of phytoconstituents. The properties and antifungal action of flavonoids, phenolics have been reported earlier (Orhan, Özçelik, Özgen, & Ergun, 2010; Winkelhausen, Pospiech, & Laufenberg, 2005). In our previous investigation, we have reported the antifungal activity of AMLEO (Sethi, Prakash, & Pant, 2015b). The fungicidal action of AMLME might be due to high polarity of methanol which can extract polar compounds, thus inhibiting the fungus. Thus, it can be concluded that AMLEO and AMLME of this traditional herb can be developed as alternative natural fungicides, thereby replacing synthetic fungicide after proper screening. However, further clinical studies are needed to obtain proper information regarding the practical effectiveness.

Supplementary material
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