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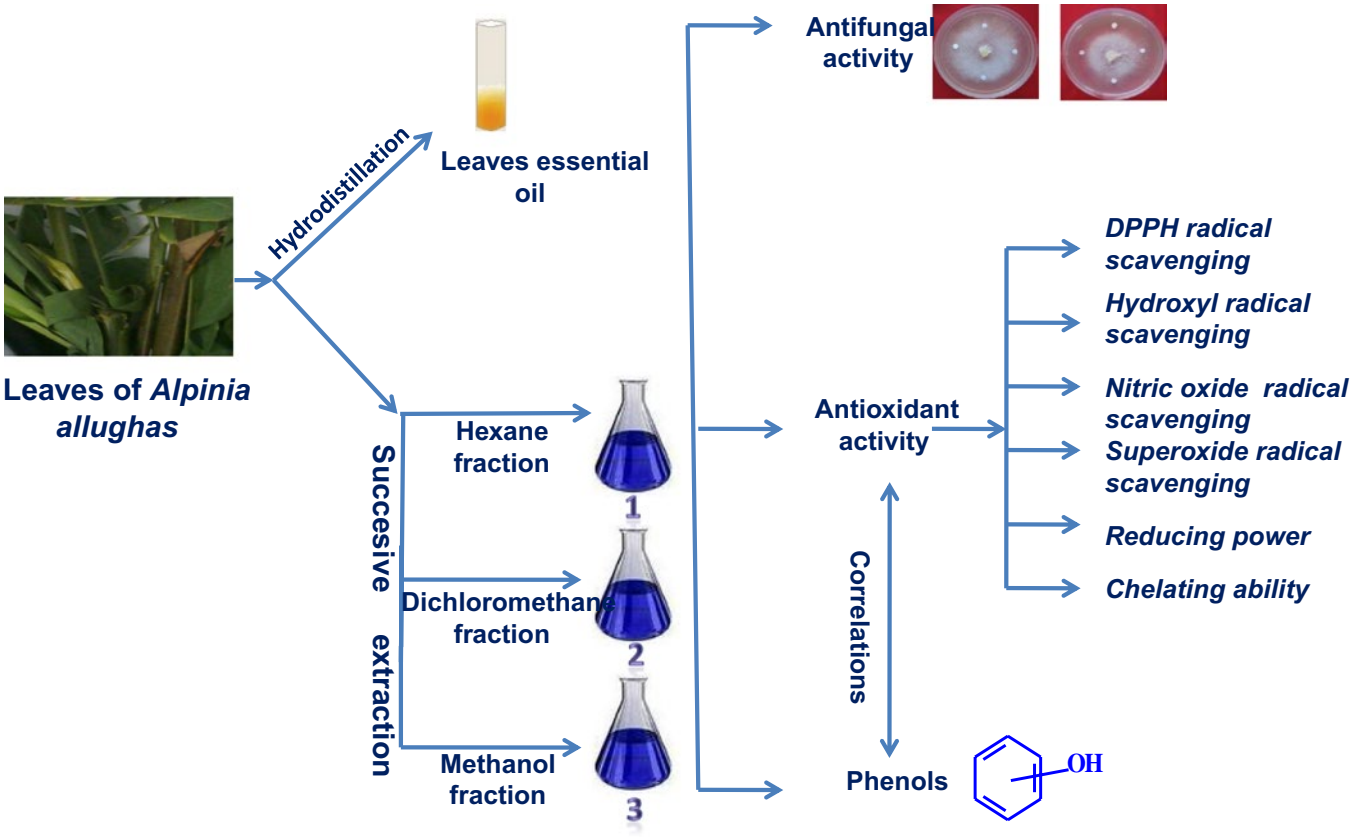

ORGANIC CHEMISTRY | RESEARCH ARTICLE

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Essential oil composition, antioxidant assay and antifungal activity of essential oil and various extracts of *Alpinia allughas* (Retz.) 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 allughas*. Fifty-seven components were identified by gas chromatography and gas chromatography–mass spectrometry with eucalyptol (17.4%) and humulene epoxide II (14.1%) as the main component. The essential oil was found to be remarkable fungicide according to its inhibition action against tested pathogens like *Colletotricum falcatum*, *Rhizoctonia solani*, *Sclerotinia sclerotium* and *Sclerotium rolfsii* followed by different extracts obtained in hexane, dichloromethane and methanol, respectively, with their minimum inhibitory concentration values ranging from 31.25 to 500 µg/ml. The essential oil showed higher phenolic contents (82.36 µg/ml) than the extracts. The antioxidant assay evaluated in essential oil and extracts by different methods revealed good-to-moderate antioxidant potential with different IC_{50} values viz. (174.72–244.04 µg/ml) in Fe^{3+} reducing power, (124.63–135.41 µg/ml) in Fe^{2+} metal-chelating ability, (84.60–138.72 µg/ml) in DPPH, (87.06–187.44 µg/ml) in OH radical, (78.90–171.65 µg/ml), in NO radical and (97.31–143.24 µg/ml) in superoxide anion scavenging activities, respectively, in comparison to the standard antioxidants. Based on obtained results, the herb *A. allughas* can be a good source to develop a safe and sustainable natural antioxidant.

ABOUT THE AUTHOR

Sonali Sethi, a student of G.B.P.U.A&T, Pantnagar is pursuing her PhD in Agricultural Chemicals since 2010. Her thrust area for doctoral thesis was natural products and has been working on plants of family Zingiberaceae for their phytochemical make-up and biological activities viz. antioxidant, pharmacological applications, etc. She is working under the supervision of Dr Om Prakash (Professor, Chemistry, Corresponding author) who has many externally funded national projects, funded by University Grant Commission and DST, New Delhi. His work in the field can be evidenced by more than 50 papers published in National and International Journals of repute. His current research interest focuses mainly on the analysis of bioactive natural compounds and their biological activity determination. In this work, *Alpina allughas* leaves were studied for its chemical and biological activity to authenticate its traditional uses and document the data so that plant can be explored scientifically and judiciously.

PUBLIC INTEREST STATEMENT

Alpinia allughas is a Zingiberaceous herb. The plants of this family are well recognised for their uses as spice, cosmetics, flood flavour, ornamental, horticulture and also in medicine. The various synthetic antifungal and antioxidant compounds have various side effects which directly or indirectly affect the human health. The extensive research on the Zingiberaceous plant (Natural products) will explore the potency of these as an antifungal, antioxidant agent and for various medicinal purposes. The current communication reports the efficacy of *A. allughas* with antifungal and antioxidant potential so that this plant can replace synthetic antifungal and antioxidant substances and give a safe herbal food preservative.

Subjects: Environmental Studies & Management; Food Science & Technology; Health and Social Care; Medicine, Dentistry, Nursing & Allied Health

Keywords: *Alpinia allughas*; antioxidant activity; antifungal activity; total phenols

1. Introduction

Antioxidants are the substance that overcome oxidative stress by quenching of free radicals and show defensive mechanism against several reactive oxygen species and reactive nitrogen species generated in the body (Sies, 1996). Use of synthetic antioxidants like butylated hydroxyanisole and butylated hydroxytoluene (BHT) in food products is prohibited as it leads to liver damage (Valentao et al., 2002). Food contaminated with fungus leads to the production of toxins and deteriorate food products that pose a serious threat to human health (Celiktas et al., 2007). In contrast, use of synthetic fungicides causes acute toxicity, longer persistence and devastating effect on environment and human health (Pavela, 2007). Due to several side effects of synthetic compounds, there is an urgent need for intensive exploration of plant products that possess natural antimicrobials and antioxidant potential and are frequently employed in food preservative, pharmaceuticals, cosmetics, perfumes, industries, etc. (Siddhuraju & Manian, 2007).

Zingiberaceae, a well-recognised family, is 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 is represented by 24 genera and 191 species (Karthikeyan, 2000). The North-east region of India is comprised about 19 genera and about 88 species (Prakash & Mehrotra, 1995). *Alpinia allughas* Rosc. is a stout perennial herb with tuberous, aromatic roots. Its leaves are sessile, oblong and compressed, arranged in dense panicles (Kirtikar & Basu, 1987). Traditionally, *Alpinia* spp. is used in the treatment of rheumatoid inflammations, cough, asthma, stomachalgia, diabetes, cephalalgia and intermittent fevers (Warrier, Nambiar, & Ramankutty, 1993–1995). It is capable of maintaining the youthful vigour and strength (Sivarajan & Balachandran, 1994), stimulates digestion and purifies blood (Chunekar, 1982). In continuation to our research on family Zingiberaceae, the present investigation reveals the first-time reports on antifungal activity, antioxidant activity and phenolic content of essential oil of this important medicinal herb.

2. Experimental

2.1. Collection of plant material

Fresh leaves of *A. allughas* were collected from Tarai region of Kumaun hills in India. The identity of the plant was confirmed by Sumer Chandra, Systematic Botany Division of Forest Research Institute, Dehradun, where herbarium specimens, Nos.: 9747 and 72265, dated 12 January 2004 were deposited.

2.2. Extraction of essential oils

Fresh leaves (1 kg) were subjected to hydrodistillation in Clevenger's apparatus for 8 h. Extraction of distillate by diethyl ether followed by drying over anhydrous Na_2SO_4 and removal of solvent yielded 0.05% of *A. allughas* leaves essential oil (AALEO).

2.3. Preparation of the extracts

The leaves of *A. allughas* 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 muslin cloths and the process was repeated till colorless solutions were obtained. The filtrates so obtained were concentrated using a rotary evaporator. Yields of different extracts viz. *A. allughas*, leaves hexane extract (AALHE), *A. allughas* leaves dichloromethane extract (AALDE) and *A. allughas*, leaves methanol extract (AALME) were observed to be 0.72, 0.68 and 0.66%, respectively. The extracts were stored at 4°C for further analysis and biological activity determinations.

2.4. GC–FID analyses

Gas chromatographic analyses were carried onto a 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 a 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 operation conditions: injection mode, split (1:1 ratio, v/v); injection volume1 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 1).

Table 1. Chemical composition of AALEO

Compound	KI	FID response (%)
α-Pinene	939	0.7
β-Pinene	980	5.3
p-Cymene	1,026	0.4
Eucalyptol	1,031	17.4
cis Para menth-2-en-1-ol	1,121	0.3
α-Fenchyl alcohol	1,120	0.2
cis-Verbenol	1,137	0.3
Iso-3-thujanol	1,138	0.2
Trans-pinocarveol	1,139	1.9
Trans-verbenol	1,144	0.3
Isopulegol	1,149	0.2
Pinocarvone	1,164	0.4
Borneol	1,169	1.0
Trans-linalool oxide	1,086	0.2
Linalool	1,096	0.4
cis-Pinocamphone	1,175	0.1
Terpinen-4-ol	1,177	0.6
α-Terpineol	1,188	4.0
Myrtenol	1,194	0.3
Myrtenal	1,195	2.2
Verbenone	1,205	0.2
Trans-carveol	1,216	0.1
Cumic alcohol	1,288	0.3
Limonene dioxide	1,294	4.4
Perilla alcohol	1,295	0.2

(*Continued*)

Table 1. (*Continued*)

Compound	KI	FID response (%)
2,3-Pinanediol	1,320	0.7
Myrtenyl acetate	1,326	0.2
Z-β-Damascone	1,387	0.9
β-Elemene	1,390	0.1
n-Tetradecane	1,400	0.4
Isocaryophyllene	1,413	0.6
Carvone hydrate	1,423	0.4
α-Cedran	1,443	1.7
Trans-*Z*-α-bisabolene epoxide	1,495	0.2
Valencene	1,496	0.4
Ledane	1,511	0.3
Spathulenol	1,578	0.2
Caryophyllene oxide	1,583	8.1
Epiglobulol	1,590	0.6
Viridiflorol	1,592	1.6
Humulene epoxide II	1,608	14.1
Germacrone	1,693	0.5
Farnesol	1,713	0.5
n-Octadecane	1,800	0.9
Phytone	1,849	0.4
Trans-phytol	1,943	0.1
Isophytol	1,947	0.2
n-Eicosane	2,000	0.4
Manool	2,057	0.6
n-Docosane	2,200	0.3
n-Tricosane	2,300	0.03
n-Tetracosane	2,400	0.5
n-Pentacosane	2,500	0.6
n-Hexacosane	2,600	1.1
n-Octacosane	2,800	0.8
n-Nonacosane	2,900	1.1
n-Triacontane	3,000	1.2
% Identified		**81.33**
MH		6.4
OM		36.5
SH		2.5
SO		26.4
DH		0.4
DO		0.9
OT		8.23

Notes: MH, monoterpene hydrocarbons; OM, oxygenated monoterpenes; SH, sesquiterpene hydrocarbons; OS, oxygenated sesquiterpene; DH, diterpenes hydrocarbons; OD, oxygenated diterpenes; OT, others.

2.6. Antioxidant activity

2.6.1. Radical scavenging activity

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

2.6.1.1. DPPH radical scavenging activity. In order to evaluate the scavenging ability of the antioxidants by this method, a standard protocol generally practised was followed (Sethi, Prakash, Pant, Batra, & Kumar, 2015). In brief, different amounts of the tested sample (50–250 µg/ml) were added to 5 ml of a 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 as triplicate. BHT, catechin and gallic acid were used as the standard antioxidants. Inhibition of free radicals 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.

2.6.1.2 Hydroxyl radical scavenging activity. This activity was evaluated using the method described earlier (Olabinri et al., 2010). Sixty microliters 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 sample (50–250 µg/ml). The absorbance of the mixture was read at 560 nm against blank after 5 min. 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.

2.6.1.3. NO radical scavenging activity. The nitric oxide scavenging activity of oil and extracts was determined using a previously reported method (Naskar et al., 2010). Two millilitres of sodium nitroprusside (10 mM) in phosphate buffer saline pH 7.4 was mixed with different concentrations of sample (50–250 µg/ml) and incubated at 25°C for two-and-a-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. The % inhibition was calculated by the equation. Percent 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.

2.6.1.4. Superoxide anion scavenging activity. Superoxide anion scavenging activity of oil 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 concentrations of test samples (50–250 µg/ml) were mixed well. The reaction was started by the addition of 100 µl of phenazine methosulfate solution (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. Percent 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.

2.6.2. Reducing power

The reducing power of essential oil 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°C for 20 min. After incubation, 2.5 ml of 10% trichloroacetic acid was added to the

mixtures, followed by centrifugation at 650×*g* for 10 min. The upper layer (5 ml) was mixed with 5 ml of distilled H_2O and 1 ml 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, 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.

2.6.3. Metal-chelating ability

The chelation of Fe^{2+} by essential oil and extracts was evaluated using the method developed earlier and which is in practice (Sethi et al., 2015). Different concentrations of tested sample (50–250 µg/ml) were first mixed with 1 ml of methanol and 3.7 ml of deionised 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. Then 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 the oil and extracts was estimated by following the method of Singleton and Rossi (1965). 0.5 ml of the oil or the extract solutions were mixed with 1.0 ml of Folin–Ciocalteu reagent, 1.0 ml of aqueous solution of 7% sodium carbonate and 5 ml 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.

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 AALEO 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, 250 µg/ml, respectively. The pathogenic fungi were cultured on PDA media and incubated at 30°C for 3–4 days. A plug of 1-week-old fungal culture (5 mm diameter) was placed on the centre of the sterilised plates containing PDA. About 10 µl of each concentration was injected to the sterile disc papers (6 mm diameter). 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 the 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.

2.10. Determination of minimum inhibitory (MIC) by broth micro dilution

The minimum inhibitory concentrations (MICs) of AALEO and extracts were determined by broth micro dilution method with slight modifications (Gulluce et al., 2004). Plant extracts and AALEO were re-suspended in 50% acetone (which has no activity against test micro-organisms) to make 1,000 µg/ml

final concentration and then twofold 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. One millilitre 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 28°C. The control tubes comprised of PDB medium with 50% acetone and fungus inoculum. MICs were described as the lowest concentration of AMLEO resulting in the slight reduction of the inoculum.

2.11. Statistical analysis

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

3. Results and discussion

3.1. GC–MS of AALEO

Fifty-seven components comprising over 81% were identified in AALEO. The results revealed that the oil was dominated by eucalyptol (17.4%), trans-pinocarveol (1.9%), borneol (1.0%), myrtenal (2.2%), α-terpineol (4.0%), limonene dioxide (4.4%), α-cedran (1.7%), viridiflorol (1.6%), caryophyllene oxide (8.1%), humulene epoxide II (14.1%), n-hexacosane (1.1%), n-nonacosane (1.1%) and n-triacontane (1.2%), respectively, besides the minor constituents (Table 1). Among the identified components, three components comprising 6.4% were monoterpene hydrocarbons, oxygenated monoterpene were represented by twenty-three components (36.5%), three components comprising 2.5% were sesquiterpene hydrocarbons, nine components comprising 26.4% were oxygenated sesquiterpenes, one component comprising 0.4% was diterpene hydrocarbon, three components comprising 0.9% were oxygenated diterpenes and twelve components comprising 8.23% were aliphatic compounds. The analysis of AALEO has been compared with the earlier reports by Prakash, Joshi, Pant, Chanotiya, and Mathela (2007), who collected the plant in the month of July, while in present study, the collection was done in the month of October from the same place. The results reveal that there is a qualitative and quantitative difference in the essential oil composition of AALEO. In the previous study, β-pinene (25.5%) as the major constituent was reported by Prakash et al. **(**2007) which contribute to 5.3% of the total oil. While reinvestigating the oil in present investigation, eucalyptol (1,8 cineole) (17.4%) has been identified as the major constituent. Many of the compounds were totally missing in the earlier reports and identified in the present study, which include fenchyl alcohol, *trans* pinocarveol, pinocarvone, myrtenal and limonene dioxide etc. The variation in the qualitative and the quantitative make-up of the constituents in the present investigation with earlier report might be possibly due to the seasonal variation and climatic factors.

3.2. Antioxidant activity

AALEO and all the extracts exhibited strong-to-good DPPH radical scavenging activity in a dose-dependent manner, minimum at lower and maximum at higher dose levels (Table 2). The radical scavenging potential of AALEO and extracts in the form of their IC_{50} values revealed the order AALEO (IC_{50} = 84.60 µg/ml) > AALME (IC_{50} = 95.67 µg/ml) > AALDE (IC_{50} = 121.43 µg/ml) > AALHE (IC_{50} = 138.72 µg/ml). The order of IC_{50} for standard were 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). The antioxidant power of AALEO and extracts might be attributed to their hydrogen-donating ability to DPPH free radical.

AALEO and all extracts scavenge OH-free radical as function of amounts in selected dose levels in comparison to the standard antioxidant ascorbic acid. The results obtained in AALEO and extract for their good antioxidant potential in terms of IC_{50} (µg/ml) values 187.44, 176.86, 78.54 and 87.06 for AALHE, AALDE, AALME and AALEO, respectively. IC_{50} of ascorbic acid was obtained, 44.36 µg/ml.

Through their nitric oxide scavenging ability, AALEO and all the extracts exhibited different degrees of antioxidant activity as indicated by the IC_{50} values obtained from these samples. However, the order of nitric oxide scavenging activity was AALEO (IC_{50} = 78.90 µg/ml) > AALME

Table 2. Antioxidant potential in term of IC_{50} values for AALEO and different extracts of *A. allughas* 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)
AALHE	138.72 ± 0.3^{g}	187.44 ± 4.426^{d}	171.65 ± 0.6^{e}	143.24 ± 2.9^{d}	244.04 ± 1.4^{h}	135.41 ± 0.9^{e}	43.70 ± 0.3^{a}
AALDE	121.43 ± 1.3^{f}	176.86 ± 1.376^{c}	130.06 ± 0.5^{d}	130.62 ± 2.7^{c}	207.94 ± 0.8^{g}	142.59 ± 0.6^{f}	64.06 ± 0.3^{b}
AALME	95.67 ± 0.4^{e}	78.54 ± 5.467^{b}	108.32 ± 0.1^{c}	127.20 ± 2.5^{c}	202.87 ± 0.7^{f}	118.94 ± 1.8^{c}	69.63 ± 0.4^{c}
AALEO	84.60 ± 0.6^{d}	87.06 ± 1.281^{b}	78.90 ± 0.2^{b}	97.31 ± 0.9^{b}	174.72 ± 0.7^{e}	124.63 ± 2.1^{d}	82.36 ± 0.5^{d}
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 ± 0.3^{a}	44.36 ± 4.5^{a}	62.03 ± 0.3^{a}	32.28 ± 1.2^{a}	115.63 ± 0.3^{a}	–	–
EDTA	–	–	–	–	–	47.72 ± 2.4^{a}	–
Citric acid	–	–	–	–	–	61.76 ± 0.4^{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 ($p < 0.05$).

(IC_{50} = 108.32 µg/ml) > AALDE (IC_{50} = 130.06 µg/ml) > AALHE (IC_{50} = 171.65 µg/ml), whereas the IC_{50} of ascorbic acid was 62.03 µg/ml lower than the samples.

AALEO and different extracts from leaves of *A. allughas* were found to scavenge the superoxides with different rates in terms of different IC_{50} values. AALHE, AALDE, AALME and AALEO were able to scavenge the superoxide anion with an IC_{50} of 143.24, 130.62, 127.20 and 97.31 µg/ml, respectively. The IC_{50} ascorbic acid, the standard antioxidant, was obtained 32.28 µg/ml.

In this assay, ferric ions (Fe^{3+}) are reduced to ferrous ions (Fe^{2+}) with change in colour whose intensity depends upon the reducing potential of compounds present in the reaction medium (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 AALEO and different extracts was obtained in the order of AALEO (RP_{50} = 174.72 µg/ml) > AALME (RP_{50} = 202.87 µg/ml) > AALDE (RP_{50} = 207.94 µg/ml) > AALHE (RP_{50} = 244.04 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).

When 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, which thus shows its antioxidant activity. The IC_{50} values for AALEO and extracts for their antioxidant potentiality in terms of chelating ability was obtained as AALME (IC_{50} = 118.94 µg/ml) > AALEO (IC_{50} = 124.63 µg/ml) > AALDE (IC_{50} = 142.59 µg/ml) > AALHE (IC_{50} = 158.23 µg/ml). However, the IC_{50} for standard viz. EDTA and citric acid were obtained IC_{50} = 47.72 and 61.76 µg/ml, respectively.

3.3. Total phenols

The total phenols in all the extracts were obtained in the range 43.70–82.36 µg/ml. The highest phenolic content was observed in the AALEO followed by the AALME, AALDE and AALHE. The antioxidant activity of AALEO 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., 2015). 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, superoxides anion scavenging activity, ·OH radical scavenging activity, nitric oxide radical scavenging activity and reducing power ability at α = 0.01 or 0.05 (level of significance) (Table 3).

3.4. Antifungal activity of AALEO and extracts

Antifungal activity of AALEO and all the extracts of *A. allughas* exhibited inhibitory effect on the growth of all fungi in a dose-dependent manner (Table 4). *S. sclerotium* was most suppressed as its growth was mostly reduced by all tested doses followed by *S. rolfsii, C. falcatum* and *R. solani*. AALEO exhibited strong inhibitory effect against fungus *S. rolfsii* (43.33%), *S. sclerotium* (38.33%), *C. falcatum* (23.33%) and *R. solani* (15.83%), even at a lower concentration of 250 µg/ml.

AALEO and all the extracts of *A. allughas* were fungicidal (100% inhibition) towards *R. solani* at a concentration of 1,000 µg/ml. AALEO and AALME exhibited strongest fungicidal action by completely suppressing all the fungus at a concentration of 1,000 µg/ml. At the same concentration, AALDE and AALHE exhibited moderate antifungal activity against all the plant pathogenic fungi tested ranging from 91.66 to 100% and 85 to 100%, respectively. The MIC defined as the lowest concentrations of each extract/oil resulting in the reduction of the inoculum demonstrated in Table 4. The MIC values

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	1	−0.953**	−0.807**	−0.995**	−0.915**	−0.996**	−0.527

**Significant at α = 0.01.

Table 4. Antifungal activity with MIC values of AALEO and different extracts of *A. allughas*

Essential oil/ extracts	Concentration (µg/ ml)	Antifungal activities (% of inhibition) (MIC)			
		C. falcatum	*R. solani*	*S. sclerotium*	*S. rolfsii*
AALHE	250	2.5 ± 1.4^{a}	0 ± 0^{a}	0 ± 0^{a}	0 ± 0^{a}
	500	16.66 ± 1.6^{b}(125)	2.5 ± 1.4^{a}(500)	3.33 ± 2.2^{a}(500)	39.16 ± 0.8^{b}(500)
	750	54.16 ± 1.6^{d}	32.5 ± 2.5^{d}	78.33 ± 0.8^{d}	55 ± 1.4^{c}
	1,000	85 ± 2.5^{f}	100 ± 0^{h}	95.83 ± 0.8^{g}	$89.16 \pm 1.6^{e,f}$
AALDE	250	25 ± 1.4^{a}	0 ± 0^{a}	4.16 ± 2.2^{a}	6.66 ± 3.0^{a}
	500	24.16 ± 0.8^{b}(250)	$4.16 \pm 2.2^{a,b}$(500)	45.83 ± 0.8^{b}(250)	40.83 ± 0.8^{b}(125)
	750	63.33 ± 2.2^{e}	41.66 ± 1.6^{e}	$85.83 \pm 2.2^{d,e,f}$	86.66 ± 0.8^{e}
	1,000	91.66 ± 0.8^{f}	100 ± 0^{h}	100 ± 0^{g}	$95.83 \pm 0.8^{f,g}$
AALME	250	5 ± 1.4^{a}	$11.66 \pm 0.8^{b,c}$	4.16 ± 2.2^{a}	42.50 ± 3.8^{b}
	500	39.16 ± 2.2^{c}(250)	32.5 ± 1.4^{d}(125)	57.5 ± 1.4^{c}(250)	54.16 ± 1.6^{c}(31.25)
	750	68.33 ± 0.8^{e}	54.16 ± 1.6^{f}	$86.66 \pm 1.6^{e,f}$	$87.5 \pm 1.4^{e,f}$
	1,000	100 ± 0^{g}	100 ± 0^{h}	100 ± 0^{g}	100 ± 0^{g}
AALEO	250	23.33 ± 0.8^{b}	15.83 ± 2.2^{c}	38.33 ± 2.2^{b}	43.33 ± 3.0^{b}
	500	43.33 ± 1.6^{c}(125)	$39.16 \pm 3.0^{d,e}$(125)	$81.66 \pm 0.8^{d,e}$(62.5)	44.16 ± 1.4^{d}(31.25)
	750	100 ± 0^{g}	62.5 ± 1.4^{g}	$93.33 \pm 2.2^{f,g}$	$93.33 \pm 2.2^{e,f,g}$
	1,000	100 ± 0^{g}	100 ± 0^{h}	100 ± 0^{g}	100 ± 0^{g}

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 ($p < 0.05$).

of AALHE, AALME, AALDE, AALEO ranged from 125 to 500, 31.25 to 250, 125 to 500 and 31.25 to 125 µg/ml, respectively. The present study indicated that AALEO, AALME 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 fungitoxicity of AALEO might be due to the presence of mono and sesquiterpenoids or due to the presence of major and the minor components. There exist so many evidences for the fungitoxic effects of the oil (Souza, Sales, & Martins, 2009). Eucalyptol (17.4%), the major constituent, obtained in AALEO might be possibly responsible for the antifungal activity of this plant. The antifungal activity of eucalyptol and their role as the antifungal agent have already been reported earlier (Hendry, Worthington, Conway, & Lambert, 2009; Marei, Rasoul, & Abdelgaleil, 2012). The antifungal activity of extracts might be due to the presence of various phytoconstituents. The antifungal/microbial activity of flavonoids, phenolics have been reported (Orhan, Ozçelik, Ozgen, & Ergun, 2010; Winkelhausen, Pospiech, & Laufenberg, 2005). The fungicidal action of AALME might be possibly due to high polarity of methanol which can extract polar compounds that exhibit fungal inhibition. Thus, it can be concluded that AALEO and AALME could be considered as an alternative natural fungicides, thereby replacing synthetic fungicide after proper screening. However, further clinical studies are needed to be done to obtain proper information regarding the practical effectiveness.

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