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In vitro antibacterial, antifungal, antioxidant and antihemolytic activities of *Alpinia galanga*

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Abstract

In the present study, fractions of methanolic extract of *A. galanga* were investigated for their antibacterial, antifungal and antioxidant potential. The fractions were first subjected to phytochemical analysis followed by evaluation of their antimicrobial potential against multi drug resistant bacteria (MDR) and fungi by agar diffusion assay and Thin Layer Chromatograpgy - Direct bioautography (TLC- DB). The antioxidant potential was measured by 1, 1-diphenyl-2-picrylhydrazyl (DPPH) scavenging potential and Ferric reducing antioxidant power (FRAP) potential. The selected fractions having high antimicrobial and antioxidant activity were further tested for their protective effect in preventing H_2O_2 induced hemolysis and lipid peroxidation in human red blood cells. The *n*-Hex fraction of *A. galanga* inhibited the growth of MDR *Escherichia coli, Staphylococcus aureus* and *Acinetobacter* in significant amounts followed by the DCM and EtOAc fractions. All the fractions of *A. galanga* also demonstrated significant antifungal activity against *Fusarium moniliforme* and *Candida albicans*. Similarly, the *n*-Hex and DCM fractions exhibited high antioxidant potential in terms of DPPH free radical scavenging and high Fe (III) reduction abilities. Maximum inhibition of hemolysis and lipid peroxidation was also observed for the *n*-Hex fraction followed by the DCM fraction. The strong antibacterial, antifungal, antioxidant and membrane stabilizing activities of *n*-Hex and DCM fractions of *A. galanga* have been demonstrated by us in the present study. These results may offer new possibilities in the therapy of pathological conditions related to development of resistance against MDR microbes and generation of free radicals.

Keywords: Alpinia galanga; Multi drug resistant (MDR); antimicrobial; antioxidant; hemolysis; lipid peroxidation.

Introduction

Alpinia galanga commonly known as Greater galanga or Kulanjan belonging to the Zingiberaceae family is widely distributed along the Western Ghats, Malabar coast and Gujarat in India and is also cultivated in other Asian countries like China, Thailand and Malaysia. It is widely used in Indian traditional medicine systems and is a significant constituent of common Ayurvedic formulations like Dasamoolarishtam, Rasnadi kashayam (Small), Rasnadi ghritam, Rasnadi choornam, Chandanasavam, Gulguluthikthakam kashayam, Aswagandharishtam, Balarishtam etc.[1]. It is used as aphrodisiac, expectorant and antiflatulant and is commonly used to treat bronchitis and heart diseases, renal disorders, colic, diarrhea and vomiting [2]. The essential oils and extracts of greater galangal rhizomes have been studied extensively and have been proven to exhibit antifungal, antigiardial, antiamoebic, antimicrobial, and antioxidant activities [3-7]. GCMS analysis of its extract has identified 1,8-cineole, β-bisaboline and β –selinene as its principle components. The rhizome is reported to contain flavonoids like kaemperol, galangin and alpinin out of which galangin is associated with many of its biological activities [8].

Even though use of plants as a source of medicine has been an ancient practice and is an important component of the health care system in India and *A. galanga* is widely used by local medical practitioners, still, in most of the cases systematic inclusive investigations involving chemists and biologists have not been organized. Though bioactivity guided fractionation is the most widely used approach but wiser approach could be to investigate each fraction methodically. Moreover, these bio-active fractions have hardly been evaluated against target specific study or *in vivo* studies. The emergence of multi-drug resistant (MDR) bacterial pathogens has become the focus of recent concern in human medicine. The increasing incidence of antibiotic resistance among bacterial pathogens necessitates medicinal plants as an alternative approach for development of novel antimicrobial agents. Similarly Oxidative stress (OS) plays a major role in the development of chronic and degenerative ailments such as arthritis, aging,

autoimmune disorders, neurodegenerative and cardiovascular disorders and cancer and plant extracts have been used as a source of natural antioxidants to maintain redox homeostatis. We have previously reported the use of extracts of *Orchis latifolia, Boswellia serrata*, *Boerhaavia diffusa* and *Centratherum anthelminticum* as antioxidants and antibacterial agents against MDR clinical isolates [9-11].Bioprospecting for novel plant based antimicrobials/antioxidants through combined efforts of ethnopharmacologists, botanists, microbiologists, and natural-products chemists can result in identification of new leads in the field of medicine.

In the present study fractions of *A. galanga* were investigated for their antibacterial, antifungal and antioxidant potential. The methanolic (MeOH) extract of *A. galanga* was first partitioned with various solvents to obtain fractions. The fractions were then subjected to phytochemical analysis followed by evaluation of their antimicrobial potential by agar diffusion assay and Thin Layer Chromatograpgy - Direct bioautography (TLC- DB). The antioxidant potential was measured by 1, 1-diphenyl-2-picrylhydrazyl (DPPH) scavenging potential and Ferric reducing antioxidant power (FRAP) potential. Erythrocytes have been the subject of wide range of investigation involving oxidative stress due to high content of poly unsaturated fatty acids (PUFA). Oxidative damage to erythrocytes is manifested as increased hemolysis and lipid peroxidation. It has been suggested by various researchers that phytocompounds can protect erythrocytes or can even increase their resistance towards artificially generated oxidative stress as in exposure to H2O2 or exposure to chemotherapeutic drugs used during treatment of cancer [9]. The selected fractions of *A. galanga* were hence further tested for their protective effect in preventing H_2O_2 induced hemolysis and lipid peroxidation in human red blood cells.

Materials and methods

Plant material collection and extraction

The plant material *A. galanga* was procured from authorized vendor of Delhi and was authenticated by Dr. M.P. Sharma, Department of Botany, Hamdard University, New Delhi. The voucher specimen was deposited in the herbarium of Chemical Biology Lab of Amity Institute of Biotechnology, Amity University-Uttar Pradesh, Noida, India. 500 g of the plant material was extracted with MeOH:Water (9:1) at room temperature over a period of 5 days. The methanolic extract was pooled, concentrated and subsequently partitioned into *n*-hexane (*n*-Hex), dichloromethane (DCM), ethylacetate (EtOAc) and aqueous (Aq) fractions as described by Priyanka *et al.* [12]. The fractions were evaporated under reduced pressure and dried using rotary evaporator below 50^0C. The fractions were first analyzed for their phytochemical constituents and then evaluated for various biological activities.

Phytochemical analysis of the fractions

Detailed phytochemical analysis was performed with *n*-Hex, DCM, EtOAc and Aq fractions of methanolic extract of *A. galanga* for the presence of various phytochemicals as described by Rajesh *et al.* [13]. Flavanoids, steroids, alkaloids and tannins were detected by NaOH/HCl test, Salkowsi's reaction, Dragendroff's reaction and ferric chloride test respectively. Additional tests were carried out to check the presence of reducing sugars, cardiac glycosides, anthraquinones, triterpenoids and phlobatannins.

Evaluation of antimicrobial activity

Bacterial strains used and growth condition

The MDR clinical isolates *Escherichia coli, Staphylococcus aureus, Enterococcus sp., Acinetobacter sp.* and *Serratia sp.* along with their respective antibiotic resistance profiles (Table 1) were provided by Rajiv Gandhi Cancer Research Institute, New Delhi, India. All bacterial strains were maintained on nutrient agar slants at 4 C, and sub-cultured on to nutrient broth for 24 h prior to antimicrobial testing.

Table 1-Antibiotic resistance profiles of MDR clinical isolates

Antibiotics	*E. coli*	*S. aureus*	*Enterrococcus* sp.	*Serratia* sp.	*Acinetobacter* sp.
Amikacin	S	S	R	S	R
Ampicillin	R	-	-	R	-
Ciprofloxacin	R	S	R	R	R
Ceftriaxone	R	S	R	R	-
Chloramphenicol	R	-	-	R	-
Gentamicin	S	S	R	R	R
Imepenem	S	S	R	S	R
Levofloxacin	R	S	R	R	-
Meropenem	S	S	R	S	R
Nalidixic acid	R	-	-	-	-
Nitrofurantoin	S	-	-	-	-
Norfloxacin	R	-	-	-	-
Ofloxacin	R	S	R	R	-
Piperacillin	R	S	R	S	R
Vancomycin	-	S	R	-	-
Tobramycin	R	-	-	R	R

(R): Resistant; (S): Sensitive

Fungal strains used and growth condition

Standard fungal strains *Aspergillus niger* (MTCC 872), *Cladosporium herbarum* (MTCC 2143) and *Fusarium moniliforme* (MTCC 2088) were procured from IMTECH, Chandigarh and revived and maintained on their specific media for antifungal assays. Similarly standard human opportunistic pathogen *Candida albicans* (MTCC 227) was also obtained from IMTECH, Chandigarh, India and revived and maintained on Sabarouds-Dextrose broth for anticandidal assays.

Determination of antibacterial activity

Antibacterial activity of plant fractions was determined by agar well diffusion method as described by Jhon J Rojas *et al* [14]. Nutrient agar plates were inoculated with 0.1 ml of each organism (1 10^8 CFU/ml) and was evenly spread with sterile glass spreader. Subsequently, wells of 7 mm size were bored into the agar set plates containing the bacterial culture and filled with 50µL of the plant extract prepared from a stock solution of 5 mg/mL of DMSO and water, where the proportion of DMSO was not more than 2 %. Sterilized distilled water was taken as the negative control where as standard antibiotic disc of gentamicin (30 µg) was used as positive control. The plates were incubated at 37ºC for 24 h. All tests were performed in triplicate and the antibacterial activity was expressed as the mean diameter of inhibition zones (mm) with standard deviation produced by the tested fractions.

Determination of antifungal activity

The antifungal assay was performed using agar well diffusion technique as described by Najafi [15]. SDA media plates were prepared and inoculated with 100 µL of fungal culture suspension and spread with a sterilised glass spreader. The plates were allowed to dry and a 7 mm sterile cork borer was used to bore wells in the agar medium. The wells were filled with 50µL of 5 mg/mL of each fraction. Sterilized distilled water was taken as the negative control where as standard antibiotic disc of griseofulvin (30 µg) was used as positive control. The plates were incubated at 28 C for 24 h and observed for the presence or absence of zone of inhibition around the wells. The experiments were performed in duplicate and the antifungal activity was expressed as mean diameter of zone of inhibition (in mm) with standard deviation.

Evaluation of Antioxidant activity

DPPH free radical scavenging assay

The antioxidant activity of the selected extracts was measured in terms of hydrogen donating ability 1, 1-diphenyl-2-picrylhydrazyl (DPPH) radical assay [17] in a reaction mixture containing different concentrations of extracts (2, 10, 20 and 50 µg/ml) and 1 mM methanolic solution of DPPH. Subsequently, the mixture was shaken vigorously and left to stand for 30 min in the dark. Disappearance of the purple colour was monitored at 517 nm using spectrophotometer. Test samples and positive control ascorbic acid were tested in triplicate over the same range of sample concentrations. The radical scavenging activity (RSA) was calculated as

% RSA= $100(1-A_E/A_D)$,

Where A_E is the absorbance of solution containing antioxidant extract and A_D is the absorbance of the methanolic DPPH solution. The antioxidant activity of each test sample and ascorbic acid was expressed in terms of concentration required to inhibit 50% methanolic DPPH radical formation (IC_{50} µg/ml) and calculated from the graph of % RSA and plant extract concentrations.

Ferric reducing antioxidant power (FRAP) assay

FRAP assay was performed according to the modified protocol of Benzie and Strain as adapted by Krishnaraju *et al.* [18]. The assay was based on the ability of the antioxidant to reduce ferric to ferrous ions in the presence of 2, 4, 6-tri (2-pyridyl)-S-triazine(TPTZ), forming an intense blue ferrous –TPTZ complex with absorption maxima at 593nm. An assay mixture containing 2.5 ml of 30mM acetate buffer(pH 3.6), 0.25 ml of 10 mM TPTZ in HCl, 0.25 ml of 20 mM ferric chloride and different concentration of plant extract was incubated for 30 min at room temperature and the absorbance of the coloured product (ferrous tripyridyltriazine complex) was read at 593 nm. A standard graph for ferrous sulphate in methanol at different concentrations was prepared. FRAP values of the fractions were expressed as mM of Fe (II)/ g of extract.

Inhibition of erythrocyte hemolysis

Erythrocytes were obtained from healthy donors and processed by protocol as described by Battistelli *et al.* [19]. After the removal of plasma and buffy coat, the erythrocytes were washed thrice with phosphate buffer saline (PBS) and resuspended in same buffer.

For hemolysis, modified protocol as described by Okoko *et al.* [20], was used. The reaction mixture contained 200 µL of erythrocyte suspension and 10 µL of the tested plant fraction. The mixture was incubated for 30 min at 37^0 C. Hemolysis was induced by addition of 100 µL of 100 µM of H_2O_2 followed by incubation at 37^0C for 3 hours. 200 µL of supernatant was diluted with 1.4mL of PBS and the samples were centrifuged at 3000 rpm for 10 min and absorbance of the content was measured at 540nm. For this experiment, the absorbance obtained from H_2O_2 alone without the plant extract was taken as 100% hemolysis. Hence, the absorbance values obtained at 540 nm were expressed as % hemolysis inhibition.

Inhibition of lipid peroxidation

The most widely used method for measurement of lipid peroxidation is thio barbituric acid reactive substances (TBARS) method. The TBARS method is based on the principle that malondialdehyde forms a 1:2 red colored adduct with thiobarbituric acid which can be quantitatively estimated either spectrophotometrically (532 nm) or by fluorimetry. RBCs were processed as described in previously and after incubation for 3h, the proteins were precipitated by the addition of 10% TCA followed by centrifugation at 3000 rpm for 5 min. To 1 mL of the obtained supernatant 0.67% of TBA reagent was added. The reaction mixture was boiled for 20 min and the absorbance was read at 532 nm. The results were expressed as % decrease in lipid peroxidation [21].

Thin Layer Chromatography and Direct Bioautography (TLC-DB)

TLC provides semi quantitative information about the various phytocontituents [1]. The *n*-Hex and DCM fractions of *A. galanga* exhibiting maximum antibacterial potential against *E.coli* were further analyzed using TLC-DB. TLC profiling was performed in accordance with the methods described by Das Talukdar *et al* [16]. 10 µL of 1mg/mL of these fractions were loaded on pre-coated silica gel plates (TLC-grade; Merck India; 60 F254). The plates were developed with 30:70 EtOAc: *n*-Hex solvent system in four sets. TLC chromatogram A (lane 1) for *n*-Hex and TLC chromatogram A (lane 2) for DCM fractions were visualized in UV light at 254 nm and the fluorescent bands were marked. TLC chromatograms B was subjected to p-anisaldehyde-sulphuric acid

spray reagent for detection of steroids and glycosides. The bacterial suspension of *E.coli* was sprayed on TLC chromatogram C until wet and was incubated at 37^0C for 24 h in a humid environment for the bacteria to multiply on the plate. Subsequently, the plate was sprayed with 2.5 mg/mL 2, 3, 5 – triphenyl tetrazolium chloride (TTC) and further incubated at 37^0C again for 4-5 h. White zones against pink background indicated the presence of antibacterial compounds in the particular zone of the chromatogram C. The *Rf* of the inhibition zones on plate C were compared with the *Rf* of reference chromatograms (plates A and B). The TLC chromatogram D was sprayed with methanolic 1, 1-diphenyl-2-picrylhydrazyl (DPPH) to screen for antioxidant activity. Appearance of yellow spots against dark purple background was indicative of the free radical scavenging potential of the separated spots.

Statistical analysis

All experiments were carried out in triplicates and the results were expressed as mean ± SD values wherever applicable.

Results and Discussion

Phytochemical analysis

The qualitative phytochemical analysis of the methanolic extract and fractions of the *A. galanga* revealed extensive presence of flavonoids and cardiac glycosides in all the fractions of the methanolic extract. Steroids and triterpenoides were detected in DCM and EtOAc fraction whereas alkaloids were found in *n*-Hex and DCM fractions when tested with appropriate detecting reagents (Table 2). Various plant secondary metabolites like flavonoids, saponins, cardiac glycosides, tannins, triterpenes and alkaloids have been reported to possess biological activities and observed wide range of antimicrobial and antioxidant properties for the methanol extract and fractions can be explained by the presence of various groups of potentially active classes of these secondary metabolites. The results are in agreement with previous studies by Chitra and Thoppil in which alkaloids, steroids and terpenoids have been detected when the *A. galanga* rhizome was extracted with various solvents [1].

Table 2-Phytochemical screening of methanol extract and fractions of the plant *Alpinia galanga* as described by Rajesh *et al* [7].

Phytoconstituents	*n*-Hex	DCM	EtOAc	MeOH	Aq.
Flavonoids	+	+	+	+	+
Alkaloids	+	++	+	+	-
Reducing Sugars	-	-	-	+	+
Steroids	-	+	+	+	+
Tannins	-	-	+	++	+
Anthraquinones	-	-	-	+	+
Cardiac Glycosides	+	++	++	++	+
Triterpenoids	+	++	++	++	+
Phlobatanins	-	-	-	-	-
Saponins	-	-	-	-	-

(+): Presence; (-): Absence

Antibacterial Activity

The results of antibacterial activity of various fractions of methanolic extract of *A. galanga* are presented in Table 3. The maximum antibacterial effect was demonstrated by the *n*-Hex fraction of *A. galanga* which could inhibit the growth of *E. coli, S. aureus* and *Acinetobacter* in significant amount. DCM and EtOAc fractions also effectively inhibited the growth of these MDR bacterial strains. None of the fractions however had antibacterial activity against *Enterococcus* and *Serratia* sp. The negative control

plate containing sterilized distilled water did not exhibit inhibition on the tested bacteria where as standard gentamicin antibiotic discs produced significantly larger inhibition zones.

Table 3-Antibacterial activity of the methanolic extract and plant fractions of *Alpinia galanga* expressed as zone of inhibition in mm (Mean ± SD of three assays)

Bacterial Strains	*n*-Hex	DCM	EtOAc	Aqueous	Gentamicin
Enterococcus sp.	-	-	-	-	10± 0.9
E. coli	25± 0.9	21± 0.8	15± 0.3	8± 0.4	28± 0.4
Acinetobacter sp.	10± 0.5	16± 0.2	1.6± 0.6	8± 0.3	-
S. aureus	19± 0.3	18 ± 0.2	8± 0.7	8± 0.5	27± 0.9
***Serratia* sp.**	-	-	-	-	-

- Inhibition zone in mm includes diameter of the borer (7mm).
- 50 µL of 1 mg/mL of the extracts were poured into 7 mm diameter agar wells and zone of inhibition diameter was noted after incubation at 37^0C for 24 hours.
- (-): No inhibition

Our results identify potentially bioactive fractions of *A. galanga* which can be used as therapeutic agents against MDR bacteria. The results are in conformation with previous studies carried out by Hsu *et al.,* which reported broad spectrum antibacterial activity of *A. galanga* flowers against *Salmonella, Escherichia coli* O157:H7, *Listeria monocytogenes, Staphylococcus aureus,* and *Shigella* [22]. In similar study essential oils of *A. galanga* exhibited antibacterial activity against *S. aureus, Klebsiella pneumonia, E. coli,* and *Pseudomonas aeruginosa* [23]. Further, the antibacterial activity demonstrated by agar well diffusion method was found to be line with the TLC-DB results.

Antifungal/ Anticandidal Activity

Results of antifungal and anticandidal activity of fractions of *A. galanga* are presented in Table 4. All the fractions of *A. galanga* showed significant antifungal activity against *F. moniliforme* and *C. albicans.* The EtOAc and aqueous fractions also displayed antifungal activity against *A. niger.* None of the fractions however had significant antifungal activity against *C. herbarum.* Ficker *etal.,* have previously reported inhibition of pathogenic fungi by members of *Zingiberaceae* family[24].Similarly, activity against *C. albicans*, *Trichophyton, Colleotrichum, Fusarium* and *Rhizopus* by Alpinia has also been previously described by other workers[25,26]. However none of the fractions had a significant activity when compared to Griseofulvin.

Table 4-Antifungal activity of the methanolic extract and plant fractions of *Alpinia galanga* expressed as zone of inhibition in mm (Mean ± SD of three assays)

Fungal Strains	*n*-Hex	DCM	EtOAc	Aqueous	Griseofulvin
Aspergillus niger (MTCC 872)	-	-	13.9±0.3	12.8±0.7	18.8 ±0.7
Cladosporium herbarum(MTCC 2143)	-	-	-	-	14.6 ±0.6
Fusarium moniliforme (MTCC 2088)	15.2± 0.3	14.8 ±0.6	16.8± 0.5	12.9±0.5	24.8 ±0.4
Candida albicans (MTCC 227)	14.7± 0.3	16.8± 0.7	13.8± 0.4	13.9±0.3	24.8 ±0.5

- Inhibition zone in mm includes diameter of the borer (7mm).
- 50 µL of 1 mg/mL of the extracts were poured into 7 mm diameter agar wells and zone of inhibition diameter was noted after incubation at 37I C for 24 hours.
- (-): No inhibition

Various plant secondary metabolites have been reported to act as antifungal agents through various mechanisms. Flavonoids have been reported to inhibit cytoplasmic membrane function and DNA gyrase and β-hydroxyacyl-acyl carrier protein dehydratase activities [27, 28]. Terpenes have been reported to promote membrane disruption and tannins bind to enzymes and other proteins causing their inactivation [29, 30]. Phytochemical investigations of the various factions have revealed the presence of flavonoids, triterpenoids, cardiac glycosides and tannins and the observed antimicrobial activities can be attributed to the presence of these secondary metabolites in the fractions. We are reporting for the first time the antimicrobial activity of *A. galanga* fractions against MDR clinical isolates. Isolation and characterisation of active principles from this plant would contribute significantly towards development of new antibiotics against drug resistance pathogens.

DPPH free radical scavenging assay

DPPH, a highly stable free radical has been widely used to assess antioxidant potential of many natural products. IC_{50} in µg/mL was calculated for each fraction as amount of antioxidant present in the sample necessary to decrease the initial DPPH concentration by 50%. The lower the IC_{50} value, the higher is the antioxidant activity. The data for IC_{50} for each fraction of *A. galanga* is presented in Table 5 and Fig.1. Lowest IC_{50} values were found to be for *n*-Hex and DCM fractions (38.82 ± 0.3 and 38.38 ± 0.4 µg/mL respectively) which were comparable to the standard antioxidant Vitamin C (35.4 ± 0.2 µg/ mL) signifying the high proton donating and free radical scavenging potential of these fractions. The aqueous fraction did not show any appreciable antioxidant activity.

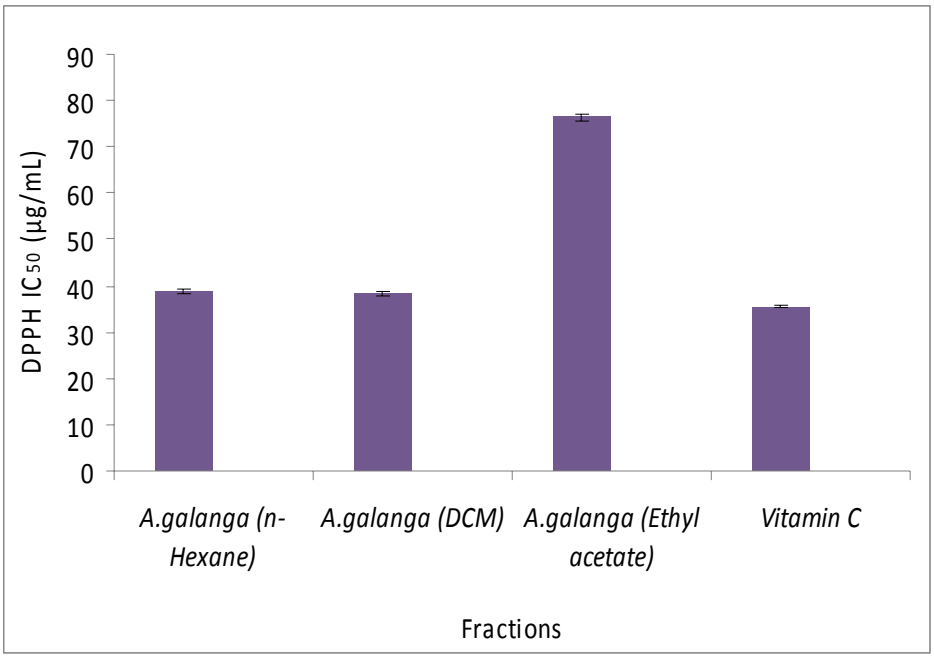

Figure1: IC_{50} values for DPPH free radical scavenging of various fractions of *A. galanga* expressed as µg/ml required to scavenge 50% of free radicals.

FRAP assay

The FRAP assay is a simple and inexpensive procedure that measures the total antioxidant levels in a sample. The method measures the reducing ability of antioxidants against oxidative effects of ROS. Among all the tested fractions, *n*-Hex fraction of *A. galanga* exhibited highest FRAP Value (1500 ± 0.95 mM of Fe (II) /g) followed by the DCM fraction (1200 ± 0.82 mM of Fe(II) /g). In contrast the standard antioxidant Vitamin C had a FRAP value of 1600 ± 0.56 mM of Fe(II) /g. Zaeoung *et al.,* have previously reported DPPH scavenging activity in aqueous and methanolic extracts of *A. galanga* [31]. The results of FRAP assay are depicted in Table 5 and Figure 2. Flavonoids and other polyphenols due to their redox properties play an active role in quenching of free radicals. The results of the present study indicate that *n*-Hex and DCM fractions of *A. galanga* may be useful for treating free radical related pathological damage.

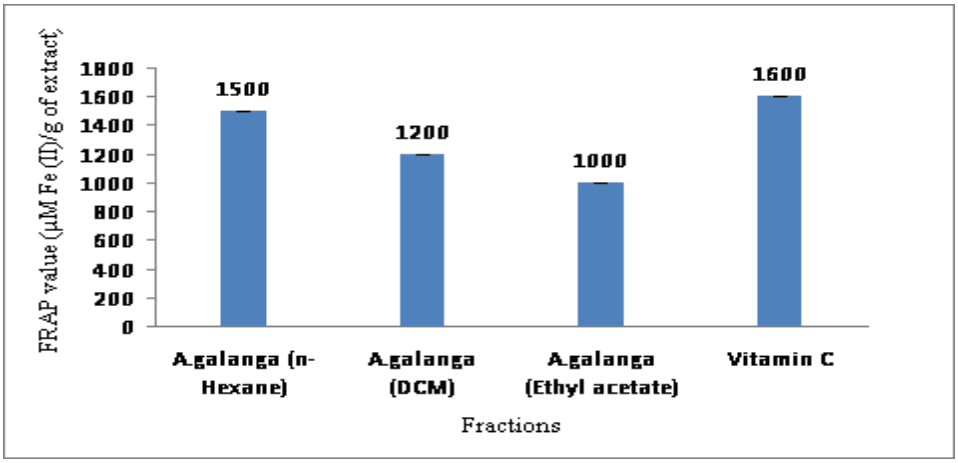

Figure 2: FRAP antioxidant capacity of various fractions of *A. galanga* expressed as mM of Fe (II) formed /g of extract

Table 5-Antioxidant potential of various fractions of *A.galanga*

Fractions	DPPH IC50 (µg/mL)	FRAP value ± S.D.(µM Fe (II)/g of extract)
n-Hexane	38.82 ± 0.3	1500 ± 0.95
DCM	38.38 ± 0.4	1200 ± 0.82
Ethyl acetate	76.4 ± 0.8	1000± 0.82
Aqueous	>100	-
Vitamin C	35.4 ± 0.2	1600 ± 0.56

- DPPH radical scavenging activity expressed as IC50 in µg/mL extract required to scavenge 50 % of free radicals
- FRAP value expressed as mM of Fe (II) formed /g of extract

Inhibition of H_2O_2 induced erythrocyte hemolysis and lipid peroxidation

All the organic fractions exhibiting high antioxidant potential were further selected for *in vitro* determination of antioxidant activity on H_2O_2 induced erythrocyte hemolysis and lipid peroxidation. The ability of various fractions of *A.galanga* to inhibit H_2O_2 induced erythrocyte hemolysis and lipid peroxidation is presented in Figure 3 and 4. Hydrogen peroxide is one of the most important ROS formed from the superoxide. It has the ability to form potentially toxic hydroxyl radical which can react with many macromolecules including proteins and DNA. Hydrogen peroxide can also damage cells via direct oxidation of cellular components ultimately leading to cell death via mitochondrial driven apoptosis [32]. High concentrations of polyunsaturated fatty acids coupled with inhibition of active oxygen transport contribute towards hemolysis of RBCs. Various plant extracts with antioxidant activity have been found to protect the erythrocytes from oxidative stress or increase their resistance to damage caused by oxidising agents like H_2O_2. In our present study we found that the plant derived fractions of *A. galanga* could restore or reduce the effects of oxidative stress induced by exposure to H_2O_2 as manifested by decrease in % hemolysis and lipid peroxidation. Maximum inhibition of hemolysis and lipid peroxidation was observed for the *n*-Hex fraction (83.2 ± 0.3 and 78.2 ± 0.2 % respectively) followed by the DCM fraction (81.2 ± 0.4 and 78.2± 0.3 respectively). Even EtOAc fraction of *A. galanga* also exhibited some inhibition of hemolysis and lipid peroxidation (64.3 ±0.3 and 61.3 ± 0.2 % respectively). A similar study conducted by us has reported up to 90% inhibition in H_2O_2 induced hemolysis by *Centratherum antehelmenticum* and *Orchis latifolia* [9]. It has been previously reported that phenolics and flavonoids can enter the hydrophobic core of the membrane

thereby altering the lipid packing. Another possible mechanism for the observed membrane stabilizing activity may be that these bioactive fractions might be altering the influx of calcium that is required to maintain membrane integrity [33]. Hence, we can conclude that *A. galanga* exhibited protective effect against oxidative stress induced erythrocyte damage.

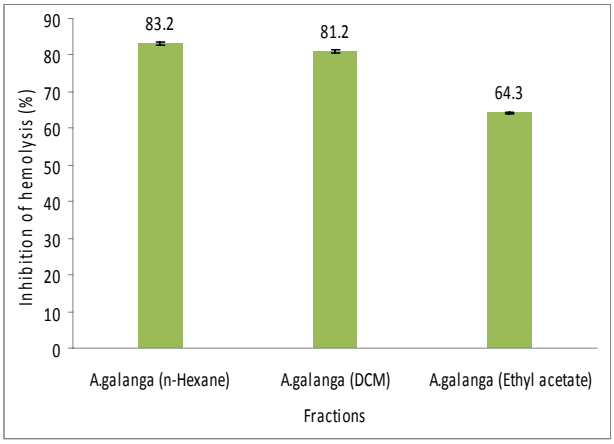

Figure 3

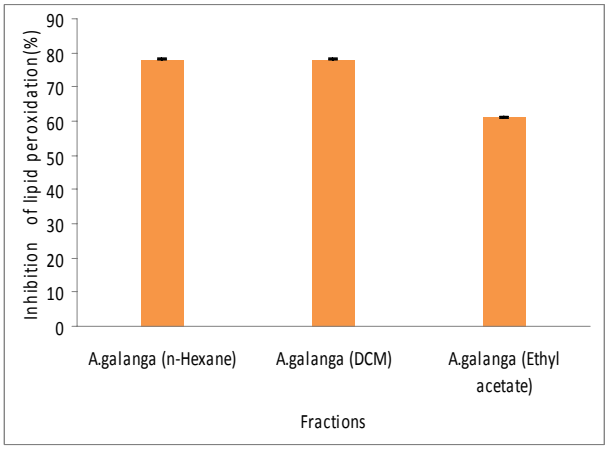

Figure 4

Figure 3 and 4: Inhibition of H2O2 induced hemolysis (Figure3) and lipid peroxidation (Figure 4) by bioactive fractions of *A. galangal*. Inhibition expressed as percentage decrease in released hemoglobin/MDA

Thin Layer Chromatography and Direct Bioautography (TLC-DB)

TLC profiling of plant fractions gives an idea about the presence of various phytochemicals present in that particular fraction. Since strong antimicrobial activity was demonstrated by the *n*-Hex and DCM fractions of the plant, these fractions were subjected to TLC analysis followed by bioautography. TLC bioautographic methods combine chromatographic separation and *in situ* activity determination facilitating the localization and target-directed isolation of active constituents in a mixture [10]. Plate A is the chromatogram showing separation of the components of *n*-Hex and DCM fractions of *A.galanga* developed with 30:70 EtOAc: *n*-Hex visualized under UV at 254 nm. The components of both these fractions separated as few major spots and several minor spots. Plate B is the chromatogram sprayed with *p*-anisaldehyde – sulphuric acid reagent for the detection of steroids, glycosides and phenolic compounds. All of the spots stained positive for phenolic compounds. During TLC-DB against MDR *E.coli* (Plate C), both the *n*-Hex and the DCM fractions showed the presence of prominent clear zones corresponding to the spots on Plate B which was sprayed with *p*-anisaldehyde-sulphuric acid reagent. The appearance of white areas against a red-pink background on the chromatograms denotes inhibition of growth of the bacteria due to presence of compound(s) that inhibit their growth. Actively growing micro organisms have the ability to reduce TTC to a pink-red colour. In the presence of active plant compounds on the chromatograms, the growth of the organism is inhibited. Hence it can be suggested that the antibacterial activity of these fractions against MDR *E.coli* must be due to the presence of phenolics that are present in these fractions. The observed results are consistent with agar well diffusion test results and phytochemical tests performed on the fraction (Table 2 and 3). In Plate D the yellow zones against purple background denoted the antioxidant potential of the separated compounds. It is interesting to note that there are some separated spots which have activity against MDR *E.coli* and as well as antioxidant activities. The same spots also test positive for phenolics with *p*-anisaldehyde-sulphuric acid reagent again reinforcing the fact that the observed biological activities of the separated fractions are due to the presence of phenolic compounds. Some spots, particularly in the DCM fraction, however, showing antibacterial activity, do not exhibit antioxidant property.

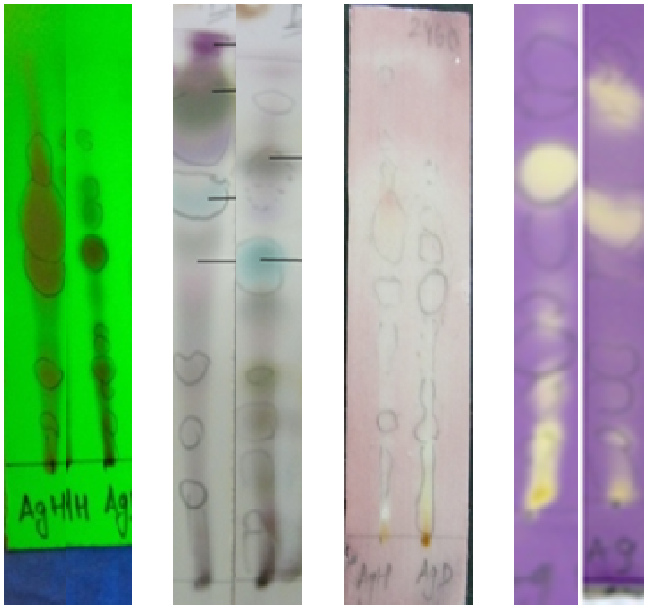

Plate A Plate B Plate C Plate D

Figure 5: TLC chromatogram as visualized under UV (Plate A), TLC chromatogram sprayed with *p*-anisaldehyde- sulphuric acid (Plate B), Bioautograms for *n*-Hex and DCM fraction against MDR *E. coli* (Plate C) and sprayed with DPPH (Plate D). 10µL of sample was loaded on TLC-grade; Merck India; 60 F_{254} and developed with 70:30 *n*-Hex:EtOAc solvent system. White zones against pink background in Plate C indicated the presence of antibacterial compounds in the particular zone of the chromatogram. Yellow zones against purple background in Plate D indicated the presence of antioxidants in the particular zone of the chromatogram.

Conclusion

The strong antibacterial, antifungal, antioxidant and membrane stabilizing activituies of *n*-Hex and DCM fractions of *A. galanga* has been demonstrated by zone of inhibition assay against MDR *E. coli* and *S. aureus*. The same fractions demonstrated antifungal activities. Further high phenolic and flavonoid content and strong free radical scavenging activity of the fractions of *A. galanga* has been observed. The fractions also protected membrane integrity resulting in a reduction of RBC hemolysis and lipid peroxidation during oxidative stress. The results provide scientific evidence to support the utilization of *A. galanga* for the treatment of various ailments. These results may offer new possibilities in the therapy of pathological conditions related to development of resistance against MDR microbes and generation of free radicals.

Authors' contributions

ASA carried out the extraction, fractionation and phytochemical profiling, MB carried out the evaluation of antimicrobial activity of the fractions, SJ carried out the evaluation of antioxidant activities. ASA performed the TLC-DB and drafted the manuscript. SG conceived the study, and participated in its design and coordination and helped to draft the manuscript. All authors read and approved the final manuscript.

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