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Research Article

IN VITRO ANTIMICROBIAL ACTIVITY AND PRELIMINARY PHYTOCHEMICAL SCREENING OF METHANOL, CHLOROFORM, AND HOT WATER EXTRACTS OF GINGER (*ZINGIBER OFFICINALE)*

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ABSTRACT

Objective: The aim of the study was to perform *in-vitro* antimicrobial activity test and preliminary phytochemical screening of methanol, chloroform and hot water extract of ginger (*Zingiber officinale*) against microbial isolates obtained from air, sewage, and soil samples.

Methods: The ginger rhizome was shade-dried and powdered using pestle and mortar. Extraction was done using methanol, chloroform, and hot water. The ginger extracts were evaluated for the presence of alkaloids, steroids, tannins, flavonoids, reducing sugars, and saponin. Preliminary antimicrobial activity of extracts was studied using agar well diffusion method on test organisms *Staphylococcus aureus, Bacillus subtilis, Escherichia coli, Klebsiella pneumoniae, Aspergillus niger, Aspergillus terreus, Trichoderma viride, Fusarium oxysporum, Saccharomyces cerevisiae,* and *Rhodotorula sp.* Evaluation of minimum inhibitory concentration against test bacterial strains was carried out.

Results: Qualitative screening for presence of phytochemicals *viz*., alkaloids, saponin, flavonoids, steroids, tannins, and reducing sugar showed the presence of all of the above phytochemicals in methanolic extract with exception of saponin while only flavonoids and steroids were present in chloroform extract. In hot water extract, with the exception of alkaloids and flavonoids all were present. Test organisms were most susceptible to methanolic extract and showed poor susceptibility to hot water extract of ginger. The present study reveals that the pattern of inhibition varied with the solvent used for extraction and the organism tested. Gram-positive bacteria were found to be more sensitive as compared to Gram-negative bacteria, and *Rhodotorula* sp. was the most sensitive among fungal test strains. Fungi also showed growth retardation, discoloration and lack of sporulation on exposure to chloroform and methanolic ginger extract, but they were not affected by aqueous extract.

Conclusion: The results of the study suggest that ginger extract contains bioactive compounds with antimicrobial activities. Further isolation and characterization of the bioactive components and evaluation of their individual effect as well as in combination on various test organisms may be done.

Keywords: *Zingiber officinale,* Phytochemicals, Antimicrobial activity, Minimum inhibitory concentration, Fourier transform infrared spectroscopy.

INTRODUCTION

Various plants and plant parts have been in use for a very long time as medicinal preparations. They are highly accepted due to their effectiveness in treating various ailments and are also considered as safe. Plant-derived products have been in use over centuries in almost every continent all over the world, and enthusiasm for this ancient form of medicine has never been greater than it is today [1]. Herbal medicines are one type of dietary supplements and are sold as tablets, capsules, powders, extracts, and fresh or dried plants. Herbs produce and contain a variety of chemical substances that act upon the body and help in maintaining and improving health [2,3].

Herbal medicines are widely used in developing and even developed countries for their safety and lesser side effects; they are also extensively used particularly in many Asian and African countries [4]. India is one of the countries that extensively use herbal medicines to meet their healthcare needs. Here, the herbal drug market is around 1 billion U.S dollars, and the export of plant-based crude drugs is around 80 million U.S dollars. However, unlike China, India has not been able to capitalize on this herbal wealth by promoting its use in the developed world despite their renewed interest in herbal medicine [5].

Ginger (*Zingiber officinale)* is a perennial herb which grows from an underground rhizome widely used as herbal medicine. Modern scientific research has revealed numerous therapeutic properties of ginger including antioxidant effects, ability to inhibit the formation of inflammatory compounds and direct anti-inflammatory effects [6]. Ancient civilizations of India and China used fresh ginger to treat nausea, asthma, cough, loss of appetite, rheumatism, fever, swelling, dysentery, heart palpitations, and sore [7]. It has been thought to stimulate circulation and improve blood flow and has been used for centuries to aid in digestion, inhibit vomiting, and prevent motion sickness and seasickness, it is also believed to relieve the common cold, flu-like symptoms, headache, and even painful menstrual period [8].

Thus, with respect to the long history of ginger's therapeutic values, this research work was carried out with the aim to determine the antibacterial and antifungal activities of the dried ginger rhizome extracts using methanol, chloroform, and hot water as solvents in order to help support evidence of its effectiveness in the treatment of various ailments and its use in food as additive.

METHODS

Collection of ginger plant rhizome

The rhizome of the ginger plant (*Z. officinale*) was purchased fresh from Rama Mandi market in Jalandhar, Punjab, India. The ginger rhizome collected was washed thoroughly with distilled water and then allowed to dry indoors for 4 days until it dried completely. The dried rhizome was then ground into powdered form using pestle and mortar and then sieved through a fine mesh to obtain a fine powder; this was then kept in an air-tight container.

Preparation of crude ginger extract

Hot water extraction

For hot water extraction, method described by Alo *et al.* [9] was adopted. 100 g of the powdered ginger was taken and percolated with

1 L of boiling water and kept for a period of 24 hrs. After percolation, the solution was filtered using Whatman No.1 filter paper. Filtrate was then poured into a pre-weighed beaker and evaporated to dryness in a water bath at 30°C until the solvent escaped completely leaving behind the extract. The extract in the beaker was weighed to find the percentage yield of the ginger extract [10].

Organic solvent extraction

50 g of finely ground powdered ginger was taken in a flask and percolated with 500 ml of methanol. The mixture was kept on a shaker for 1 week at room temperature. The solution was filtered using Whatman No.1 filter paper and evaporated to dryness at room temperature. The same process was carried out for chloroform extraction [11,12].

Phytochemical screening

The ginger extracts were evaluated for the presence of alkaloids, steroids, and tannins [13], flavonoids [11], reducing sugars and saponin [14].

Preparation of extract concentrations

Stock solution having 1 g/ml concentration of methanolic and chloroform extract was prepared using dimethylsulfoxide in sterile vials. For hot water extract, similar concentration was prepared using sterile distilled water as diluent. The stock solutions were further diluted to obtain concentration range of 10 mg/ml, 20 mg/ml, 30 mg/ml, 40 mg/ml, and 50 mg/ml and stored in refrigerator at 4°C [15].

Test organisms

Test organisms *Escherichia coli* and *Klebsiella pneumoniae* were isolated from sewage sample, *Staphylococcus aureus* from air and *Bacillus subtilis* from soil. These were characterized by Gram staining, study of cultural characteristics and biochemical tests [16]. The bacterial strains were grown on nutrient agar slants at 37°C for 24 hrs and stored at 4°C. The fungal test organisms were *Aspergillus niger, Aspergillus terreus, Trichoderma viride, Fusarium oxysporum, Saccharomyces cerevisiae and Rhodotorula sp*. These were isolated from soil and were identified based on macroscopic and microscopic characteristics [17]. Fungi were grown on potato dextrose agar slants at 28°C for 48 hrs and stored at 4°C. All test cultures were subcultured fortnightly. Well diffusion assay was done for detection of inhibitory activity of ginger extract against these test organisms. The minimum inhibitory concentration (MIC) test of ginger extract was conducted for the bacterial isolates.

Preparation of turbidity standard solution and standardization of inoculum

A 0.5 McFarland standard turbidity solution was prepared as described by Andrews and BSAC Working Party on Susceptibility Testing [18]. For this, 0.5 ml of 0.048 M $BaCl_2$ solution was added to 99.5 ml of 0.18 M H_2SO_4 (1% v/v) with constant stirring. The solution was mixed thoroughly to ensure an even suspension. The 0.5 McFarland standard turbidity solution corresponds to a homogenous *E. coli* suspension of 1.5×10^8 cells/ml.

For standardizing the bacterial inoculum, isolates were grown on nutrient agar plates and incubated overnight at 37°C. Colony was transferred into test tube containing 2 ml of sterile physiological saline until the turbidity of the suspension matched with the turbidity of the 0.5 McFarland standard [19].

Antimicrobial assay of ginger extract

Antimicrobial activity of ginger extracts was done using well diffusion technique [20]. Mueller-Hinton agar plates were prepared and inoculated with a standardized test organism using sterile swab sticks [21]. The plates were allowed to stand for 30 minutes after which wells were made using 6 mm diameter cork borer. With the help of sterile pipettes, 100 µl each of the five extract concentrations i.e., 50 mg/ml, 40 mg/ml, 30 mg/ml, 20 mg/ml, and 10 mg/ml were introduced into each of the wells. The plates were allowed to stand for a pre-diffusion time of 15 minutes after which they were placed in an incubator at 37°C±1°C for 24 hrs. For the fungal isolates, potato dextrose agar plates were used, and the surface inoculated with fungal spore suspension. After incubation at 28°C±1°C for 48 hrs, zone of inhibition (mm) was measured. The solvents were used as a negative control in the test while chloramphenicol and nystatin were used as positive controls. The experiments were performed in triplicates, and antibacterial activity was expressed as mean diameter of the zone of inhibition.

Determination of MIC

The MIC of ginger extracts was determined by macrotube dilution assay as described by Acharya [22] and European Committee for antimicrobial susceptibility test [23]. Chloroform extract concentrations of 5 mg/ml, 2.5 mg/ml, 1.25 mg/ml, 0.625 mg/ml, and 0.316 mg/ml, were prepared by serial dilution in nutrient broth and 0.1 ml of test organism suspension was added to each tube. For methanol and hot water extract, the concentration range of 8-0.316 mg/ml was taken. All the tubes were incubated at 37°C for 24 hrs. The lowest extract concentration that inhibited the test bacterium was recorded as MIC.

Fourier transform infrared (FTIR) spectroscopy

The ginger extracts were subjected to FTIR spectroscopy analysis [24].

RESULTS

Characteristics of ginger extract

The result of the extraction process showed a yield of 2.183 g from 50 g of ginger with chloroform, 3.439 g from 50 g of sample with methanol and 9.034 g from 100 g of ginger powder sample with hot water. The physical characteristic of the extract is shown on Table 1, and the phytochemical composition of the extract is presented in Table 2.

The three extracts showed varying degree of inhibitory activity against all test organisms. However, it was observed that hot water extract showed activity against only two fungal test organisms and no activity against all four test bacterial strains. Both organic solvent extracts showed remarkable activity against most of the test organisms. Between the two organic solvents used, chloroform extract showed zone of inhibition against all bacterial strains, whereas methanolic extract showed no action against *K. pneumoniae*. Moreover, chloroform extract inhibited *A. niger, F. oxysporum,* and *Rhodotrula sp.* while methanolic extract showed inhibition against *A. niger, A. terreus* and *Rhodotrula sp.*

Sporulation of fungal culture *T. viride* was affected by methanolic and hot water extract. The data revealed that amongst the test bacterial strain *K. pneumonia* was most resistant and most susceptible was *S. aureus* and *B. subtilis*. Among the molds, *A. niger* was most susceptible to all the extracts. In the case of yeasts test strains *Rhodotorula* was more susceptible to organic solvent extracts. The effectiveness of the extracts against the various test organisms was compared with antimicrobial responses shown by chloramphenicol and nystatin. The inhibition of the bacterial strain by antibiotic chloramphenicol is comparable to that shown by extracts. Thus, the results of the study indicate that ginger extracts exhibit appreciable activity against most of the test organisms (Table 3).

The MIC tested against the bacterial isolates using the chloroform extract showed that a concentration of 1.25, 2.5, 2.5, and 1.25 mg/ml is sufficient to inhibit the growth of *B. subtilis, E. coli, K. pneumoniae, and S. aureus,* respectively, as determined by the optical density readings obtained from spectrophotometric analysis (Table 4). With methanolic extract, the MIC recorded was 2.5 mg/ml for both *K. pneumoniae and*

Table 1: Percentage yield and physical characteristics of the extracts

Extract	Percentage yield (%)	Organoleptic properties		
		Colour	Odour	Texture
Chloroform	4.366	Brown	Pungent	Soft, gummy
Methanolic	6.878	Brown	Pungent	Soft, oily
Hot water	9.034	Dark brown	Pungent	Hard

Table 2: Phytochemical composition of the extracts

Extract	Alkaloids	Flavonoids	Red sugar	Saponin	Steroids	Tannins
Chloroform	-	+	-	-	+	-
Methanolic	+	+	+	-	+	+
Hot water	-	-	+	+	+	+

+=Present, -=Absent

Table 3: Agar well diffusion assay studies result on the test organisms

Test organisms	Inhibition zone (mm)							
	Solvent used for extraction			Positive control		Negative control		
	Chloroform	Methanol	Water	Chloramphenicol	Nystatin	Chloroform	Methanol	Water
S. aureus	18±0.7	15±0	NI	21	NT	NI	NI	NI
B. subtilis	14±1	17±0	NI	20	NT	NI	NI	NI
E. coli	12±0	15±0	NI	22	NT	NI	NI	NI
K. pneumoniae	10±0.5	NI	NI	21	NT	NI	NI	NI
A. niger	11	11	10	NT	17≤	NI	NI	NI
A. terreus	NI	10	NI	NT	15≤	NI	NI	NI
T. viride	NI	LZ	LZ	NT	13≤	NI	NI	NI
F. oxysporum	11	NI	NI	NT	17≤	NI	NI	NI
S. cerevisiae	NI	NI	NI	NT	10≤	NI	NI	NI
Rhodotorula sp.	18	20	NI	NT	16≤	NI	NI	NI

NI: Not inhibited, NT: Not tested, LZ: Light zone around well, *S. aureus: Staphylococcus aureus, B. subtilis: Bacillus subtilis, E. coli: Escherichia coli, K. pneumonia: Klebsiella pneumoniae, A. niger: Aspergillus niger, A. terreus: Aspergillus terreus, T. viride: Trichoderma viride, F. oxysporum: Fusarium oxysporum, S. cerevisiae: Saccharomyces cerevisiae*

Table 4: MIC of ginger extract against test bacterial strains

Test organisms	Concentration (mg/ml)								
	8	7	6	5	2.5	1.25	0.625	0.316	M+T.O
OD (600 nm) of methanolic extract treatment									
Control (M+E)	1.070	0.930	0.857	0.759	0.463	0.264	0.131	0.095	-
B. subtilis	-	-	-	0.758	0.461	0.264*	0.339	0.425	0.833
S. aureus	-	-	-	0.753	0.461*	0.288	0.311	0.443	0.605
E. coli	1.071	0.928*	0.879	0.803	0.774	0.901	0.943	0.918	0.937
K. pneumoniae	-	-	-	0.760	0.462*	0.302	0.391	0.416	0.646
OD (600 nm) of chloroform extract treatment									
Control (M+E)	-	-	-	1.030	0.555	0.228	0.207	0.128	-
B. subtilis	-	-	-	1.031	0.554	0.227*	0.345	0.531	0.836
S. aureus	-	-	-	1.029	0.556	0.229*	0.541	0.791	1.023
E. coli	-	-	-	1.024	0.555*	0.511	0.604	0.650	0.736
K. pneumoniae	-	-	-	1.030	0.554*	0.234	0.318	0.562	0.557
OD (600 nm) of hot water extract treatment									
Control (M+E)	0.992	0.891	0.884	0.879	0.563	0.394	0.231	0.147	-
B. subtilis	-	-	-	0.882*	1.051	1.057	1.277	1.243	1.118
S. aureus	-	-	-	0.878*	0.983	1.323	1.195	1.023	0.878
E. coli	0.990	0.891	0.883*	0.942	0.942	0.934	0.974	0.911	0.762
K. pneumoniae	0.991*	0.901	0.557	0.942	1.203	1.109	0.941	0.866	0.718

*MIC, M+E=Media+extract (control), M+T.O=Media+test organism (control), MIC: Minimum inhibitory concentration, *S. aureus: Staphylococcus aureus, B. subtilis: Bacillus subtilis, E. coli: Escherichia coli, K. pneumonia: Klebsiella pneumonia*, OD: Optical density

S. aureus while for *B. subtilis,* a lower concentration of 1.25 mg/ml was sufficient to inhibit its growth. For *E. coli,* it was found that the organism resist both the concentrations used. However, upon increasing the concentration, the organism was found to be inhibited at a concentration of 7 mg/ml.

For the hot water extract, the MIC was registered at 5 mg/ml on *S.aureus* and *B. subtilis* while the other Gram-negative isolates *i.e. E. coli* and *K. pneumoniae* were found to resist the highest concentration of 5 mg/ml, however, when tested at high concentration, the organisms *E. coli* and *K. pneumoniae* were found to be inhibited at a concentration of 6 mg/ml and 8 mg/ml, respectively.

FTIR analysis

The FTIR spectroscopy result of the chloroform extract showed peak 653.89 which indicates the presence of alkyl halide functional group having bond C-Cl. The peaks 4-6 (676.07, 707.9, 952.87) indicate the presence of alkene group with bond =C-H as the peaks are bending type in nature. The peaks numbered 7-10 (1021.34, 1124.54, 1153.47, and 1272.1) are characterized by being stretch with two or more bands thus indicate the presence of anhydride functional group with a bond C=O while the 11th peak (1316.46) indicates the presence of an acid group, with a bond C=O. The 12-14th peaks show the presence of aromatic functional group with a bond C=C while the 15th peak (1660.77) indicates that there is a presence of the amide group with a bond C=O. The 17th and 18th peaks (2853.78 and 2922.25) indicates the presence of alkane group with bond C-H while the peak 19 showed the presence of primary amine with a bond N-H (Fig. 1).

For the methanolic extract, the peak 706.93 falls within the range of 600-800 absorption range and is characterized by being stretch and broad thus showed the presence of alkyl halide group with bond C-Cl,

the peak 953.83 is within the range of 675-1000 and characterized by bending vibration type shows the presence of alkene group with a bond =C-H, the peak 1022.31 falls between 1000 and 14,000, indicate the presence of alkyl halide group with a bond C-F while the peak of 1316.46 falls within the range of 1210-1320 and indicates the presence of acid group with C-O bond which is stretched having strong intensity. The peaks 1406.15, 1437.02, and 1512.24 falls within the range of 1400-1600 absorption range and are characterized by being stretch, with medium to weak intensity thus defines the presence of aromatic functional group with C=C bond. The peak 1658.84 is found within the range of 1620-1680 and is variable and stretched thus shows the presence of alkene group with C=C bond. The peaks 2854.74, 2920.32, and 3004.23 are stretched and thus represent the presence of alkane group with a bond C-H. The peak 3419.9 falls within the range of IR spectra of 3400-3500 and is stretched, very strong, and thus shows the presence of primary amine with the bond N-H (Fig. 2).

For the hot water extract (Fig. 3), the same readings were true as that of the methanolic extract but with minor variations within the peaks but yet within the same range. The peaks 1437.02, 2332.98, 3419.8 were exactly the same between the hot water extract and that of methanol while in others, there are few minor differences which still makes no change to the type of functional groups present [25,26].

Thus, the various peaks recorded showed that the solvents used in the extraction have different ability to dissolve the essential bioactive components of the ginger, hence, explaining the variability in activity. In comparison with the peak obtained for the FTIR on chloroform and methanol only, the different peaks recorded with the chloroform and methanol further proves that the activity registered on the test organisms was solely due to extract not the solvent used for extracting it.

DISCUSSION

The overall inhibitory activity of the extracts against the bacterial isolates was higher with methanolic extract than with chloroform extract or hot water extract with exception on *S. aureus* where the chloroform extract exhibited higher activity than the methanolic extract. The higher activity of the methanolic extract could however be attributed to the presence of more phytochemicals than in the chloroform extract. The studies also showed that the extract activity was more pronounced on the Gram-positive isolates than on the Gram-negative isolates. This is in accordance with the findings that showed that most antibacterial medicinal plants attack Gram-positive strains while few are active against Gram-negative bacteria [27-29]. The overall antibacterial activities are attributed to the presence of phytochemicals in plants which protect them against pathogens [30]. Kaushik *et al.* had reported that an important characteristic of essential oils and their constituents is their hydrophobicity, which enables them to permeate in the lipids of bacterial cell membranes and mitochondria, thus disturbing the structures and rendering them more permeable [31].

The inhibitory activity shown on the fungal isolates was growth retardation, discolouration due to lack of sporulation which was recorded on *A. niger, T. viride,* and *F. oxysporum*. The inhibition activity shown on *Rhodotorula spp* was remarkable because the organism was highly sensitive to the organic solvent extracts. According to Wirth and Goldani [32], *Rhodotorula* spp. are reliably resistant to fluconazole and echinocandins. In general, the resistance shown by other fungal isolates could be due to the fact that fungi are eukaryotic in nature as such may require high concentration of the extract than bacteria for them to be inhibited. According to Gulshan and Moye-Rowley [33] most of the multidrug resistance associated with fungi was traced to the presence of gene loci designated pleiotropic drug resistance loci. Finose and Gopalakrishnan, [34] had reported that ginger oil showed significant activities against the human pathogenic fungi, *Candida glabrata*, *Candida albicans,* and *A. niger*.

The result obtained in this study justify the use of ginger (*Z. officinale)* as an ethnomedicine during the ancient times and proves its potential

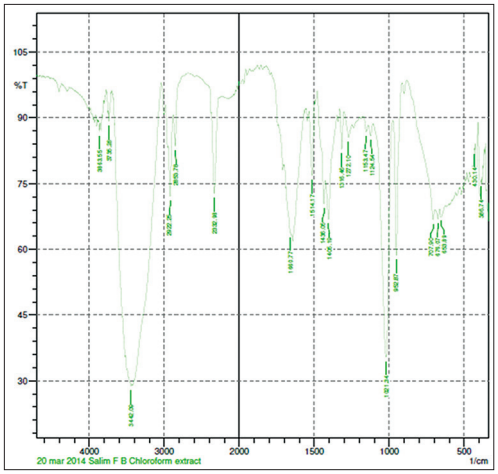

Fig. 1: Fourier transform infrared spectrum of chloroform extract of ginger

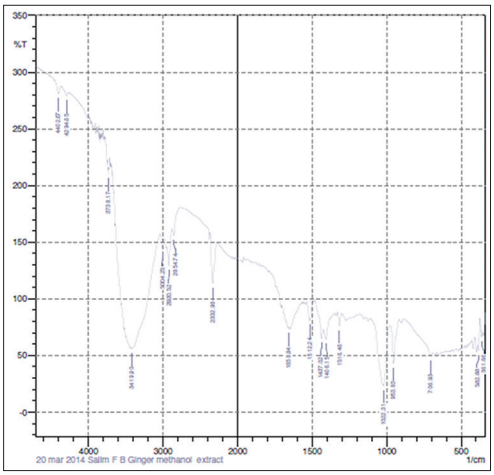

Fig. 2: Fourier transform infrared spectrum of methanolic extract of ginger

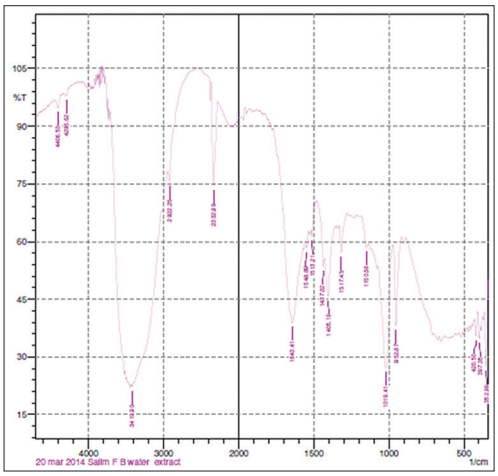

Fig. 3: Fourier transform infrared spectrum of hot water extract of ginger

to be used in modern drug development. The range of bacterial and fungal isolates sensitive to these extracts suggest the use of this plant in controlling microbial proliferation in various environmental settings such as medically as well as in food preparations. The result obtained

also showed that heat has effect on the bioactivity of the plant extract as it showed that upon exposure to hot water, the extract loses its ability to be active on the bacterial and fungal isolates with the exception to *A. niger.*

From the work carried out, it can therefore be recommended that further research should be carried out to find the optimum conditions at which these extracts are most effective, there is a need to extend the range of the test isolates to find whether or not they are sensitive to the extracts in addition to the ones used in this study. There is a need to isolate and characterize the bioactive components of the ginger extracts and test their individual effects on various test organisms as well as in combination.

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