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Phyotchemical screening and antibacterial activity of fruit extract of *Syzygium aromaticum*

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ABSTRACT
India the land of spices has been employing these as not only an important component of our food but has also as potential Ayurvedic medicines for bioactive antimicrobial compounds. These have been the subject of attention owing to their cosmopolitan distribution, high degree of efficiency with no side effects. Among all Syzygium aromaticum (clove), a member of family Myrtaceae, has been used as a traditional medicine employed in the case of dental decay/ infections. It has been used to boost immune response, improvise digestion, enhances blood circulation and hepatic function. The associated phytochemicals tends to reduce tension, anxiety, and depression. In the present study phytochemicals and antibacterial activity of the fruit extracts of S. aromaticum in different solvents (Acetone, aqueous and chloroform) has been compared against different pathogenic bacteria. Phytochemical analysis clearly indicated that alkaloids and flavonoids were found to be absent in all the three extracts, while carbohydrates, glycosides, proteins, tannin and terpenoids were present in acetone, aqueous and chloroform extract. Saponins were limited to aqueous extract only. Antibacterial activities of the crude extracts were studied against two gram-positive and four gram-negative bacterial strains. All bacterial strains (E. coli, S. aureus, B. subtilis, P. aeruginosa, S. typhi and K. pneumonia) were procured from IMTech Chandigarh. The antibiogram of all fruit extract showed significant ($p < 0.01$) antibacterial activity. Maximum antibacterial activity was reported in the aqueous extract of fruit indicating its potential significance in the treatment of canine infections and diseases within the livestock population.
***Keyword:-** Syzygium aromaticum, fruit extract, phytochemical screening and antibacterial activity.*

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INTRODUCTION

Since ancient times, herbs and spices have been used to enhance the taste and fragrance of foods. In addition to this, they also act as preservatives and have therapeutic values (1). Natural herbal products have been used form prehistoric times and are also employed in traditional medicine system like Ayurveda, Chinese and Egyptian. In present scenario about 40% of total world's population utilized herbal medicines for maintaining their health status because they are considered to be safe for human consumption and also have effective results (2-4). India has fame for the utilization of herbal medicine since ancient times because in India approx 25000 species of medicinal plants are found, of which 150 species are used for extracting medicines or drug formulations. It was also estimated that about 25% of modern medicines are also obtained from plants (5).

Clove (*Syzygium aromaticum*) is a plant of high medicinal value. It is the native of Spice Islands, Indonesia, Pemba, Zanzibar, India and Ceylon belongs to family Myrtaceae (6). *Syzygium aromaticum* possesses several therapeutic values. It is widely used to prevent nausea, cough, vomiting, diarrhea, dyspepsia, flatulence, abdominal cramps and gastrointestinal disturbance. In addition it also provides relief in pain helps in uterine contractions and stimulate nerves (7, 8, 9, 10). Moreover, it can be used as an antiseptic (11), anti-mutagenic (12), anti-inflammatory (13), antioxidant (14), anti-ulcerogenic (15, 16), anti-thrombotic (17), antifungal (18, 19), antiviral (20), antibacterial (21) and antiparasitic (22). Eugenol, an important volatile compound present in the clove bud has the bactericidal and fungicidal effect hence used in the preparation of traditional medicines (23).

MATERIAL AND METHODS

Collection of and preparation of extracts of *Syzygium aromaticum*-

The clove (*Syzygium aromaticum*) fruit samples were collected from the local market of Mathura. Clove buds were washed with distilled water, sliced and dried. The dried material was crushed into fine powder and used for extraction.

Acetone, chloroform and aqueous extract were prepared by mixing 50 g of *Syzygium aromaticum* powder in 500 ml of each solvent. The extracts were kept as such for 24 hrs at 25˚C with continuous intermittent shaking. The crude extract in each case was filtered using Whatman filter paper and the filtrate collected was evaporated using rotary evaporator. Dry extracts obtained in each case were subjected to phytochemical screening and antimicrobial estimation (24).

Phytochemical screening of various extracts of *Syzygium aromaticum*-

Phytochemical screening of fruit extracts of *S. aromaticum* in different solvents was performed (25).

Test for Saponin:-

The extracted solution of clove (500 µl) was mixed with 7ml of distilled water. Froth formation indicates the presence of saponin.

Test for Flavonoids:-

To 1ml of NaOH add 100µl of sample. A serious yellow shading was delivered which winds up boring on expansion of a couple of drops of weaken corrosive demonstrates the nearness of flavonoids.

Test for Tannins:-

Prepare 5% solution of ferric chloride in distilled water. 0.5ml of this solution was added to 100 µl of sample. Dull green or dark blue shading showed that tannins are present.

Test for Carbohydrates:-

1ml of Fehling's A (few amount of copper sulphate in distilled water) and Fehling's B (potassium tartrate and sodium hydroxide mixed in distilled water) solution were shaken and heated for a minute. Same volume of extracted clove sample was added and heated on boiling water bath for 5-10mins. Appearance of yellow colour first and than brick red colour precipitates confirmed the presence of reducing sugars.

Test for Protein:-

500µl of 5% of sodium hydroxide and 1% copper sulphate were added in sample. Presence of purple colour confirms the presence of protein and free amino acids.

Test for Phlobatannins:-

500 µl plants extract of clove sample was boiled with 0.5ml 1% aqueous HCl. Deposition of red precipitate shows the availability of phlobatannins.

501 **Test for Terpenoids:-**

1ml of extracted clove sample was mixed in 500 µl of chloroform. Add 500 µl of conc. Sulphuric acid to form a layer. A raddish brown precipitate colour at the interface formed shows the presence of terpenoids.

Test for Alkaloid (Wagner's Test):-

Add 1ml of Wagner's reagent (iodine in potassium iodine) in 1ml of extract. Reddish brown colour precipitate shows the presence of alkaloids.

Test for Soluble Starch:-

500 µl of extracted sample was boiled with 1ml of 5% KOH, cooled and acidified with 200 µl sulphuric acid. Yellow colour was taken as the presence of soluble starch.

Test for Cardiac Glycosides:-

1ml of extract, 1ml of glacial acetic acid, on 100 µl 5% ferric chloride and conc. Sulphuric acid were added. Appearing reddish brown colour in between of 2 liquid layers indicates the presence of cardiac glycosides

Antibacterial activity of different extracts of *Syzygium aromaticum*-

Test microorganisms-

Escherichia coli (MTCC 294), *Staphylococcus aureus* (MTCC 3160), *Bacillus subtilis* (MTCC 2057), *Pseudomonas aeruginosa* (MTCC 2581), *Salmonella typhi* (MTCC 660) and *Klebsiella pneumonia* (MTCC 4030) bacterial cultures were used for the antimicrobial activity.

Preparation of different concentrations of extracts of *Syzygium aromaticum*-

Prepare different extracts (acetone, aqueous and chloroform) as per the method given in section 2.1. The dried extracts were diluted with appropriate solvent to make different concentrations (6.25 mg/ml, 12.5 mg/ml, 25 mg/ml, 50 mg/ml and 100 mg/ml).

Preparation of bacterial inoculum -

A loopful culture of each bacterium was inoculated into 4-5 ml peptone water and incubated at 37^0C for 24 hours. Now match this bacterial growth with that of 0.5 Mc Farland standard that is prepared by mixing 0.5 ml of 1.75 % (w/v) $BaCl_2.2H_2O$ with 99.5 ml of 1% (v/v) H_2SO_4 . If the bacterial growth is

dense then dilute it by adding more peptone water to match exactly with Mc Farland standard. This concentration is equivalent to 1-2 X 10^8 CFU/ml approximately (26).

Screening of antibacterial activity-

Antibacterial activity was carried out by using disc diffusion method (24, 27). Sterile plain discs (Hi Media) were used for this purpose. Melted Mueller-Hinton agar (MHA) medium was poured into pre-sterilized petriplates and when it becomes solidified 0.1 ml of bacterial inoculum (size $1X10^8$) was spread over the surface of Mueller-Hinton agar. Now, plates were incubated at 37^0C for one hour. Sterile plain discs (Hi Media) were impregnated with different concentration of extracts (6.25 mg/ml, 12.5 mg/ml, 25 mg/ml, 50 mg/ml and 100 mg/ml) were placed over the Mueller-Hinton agar surface at specific distance. Now plates were incubated at 37^0C for 24 hours for the development of zone of inhibition (28). Results were compared with the standard Hi Media antibiotics discs i.e. Gentamycin (G) and Tetracyclin (T) which serves as control (29).

Each experiment was repeated three times and average zone of inhibition were recorded for all the three extracts and compared it with standard antibiotics i.e. control (24).

Statistical analysis-

Statistical analysis of the results (zone of inhibition) obtained was performed by one way analysis (ANOVA) using SPSS ver. 20.0 software and Duncan's multiple test (DMRT) at $p < 0.05$ and $p < 0.01$ to determine the significant difference in mean values among the treated and control. All values were expressed as mean ± S.E.M. (standard error of mean).

RESULT AND DISCUSSION

Phytochemical screening

Phytochemical analysis of acetone, aqueous (water) and chloroform extract of fruit of *S. aromaticum* displayed the presence of carbohydrates, glycosides, proteins, tannin and terpenoids ("+" for the presence) while alkaloids and flavonoides were found absent ("-'' for the absence) among all the extracts. The presence of saponin was only limited to the aqueous extract of fruit. Similar findings have also been reported by other research workers (5, 30, 31, 32). The presence/ absence of various phytochemicals in different extracts are enlisted in table-1. The phytochemicals classes reported in the aqueous extracts of *S. aromaticum* have been found to be associated with the antidiarrheal, anti inflammatory, antimicrobial, insecticidal and antioxidant activity (33). The presence of glycosides has been reported to have potential therapeutic actions such as regulating blood pressure, treating congenital myocardial infractions and cardiac arrhythmia (34). The occurrence of terpenoids and tannins as indicated though the study of independent researchers (35) has highlighted its use in the treatment of asthma, cough and hay fever in addition to their antimicrobial and antioxidant activity.

Antibacterial activity-

Antibacterial activities of fruit extract in different solvents (acetone, aqueous and chloroform) were tested against selected pathogenic bacteria and their zones of inhibition were recorded (table-2). All the fruit extracts of *S. aromaticum* showed significant ($p<0.01$) bactericidal potential against both gram-positive (*Bacillus subtilis* (MTCC 2057), *Staphylococcus aureus* (MTCC 3160)) and gram-negative (*Escherichia coli* (MTCC 294), *Klebsiella pneumonia* (MTCC 4030), *Pseudomonas aeruginosa* (MTCC 2581), *Salmonella typhi* (MTCC 660)) (Fig-2). A dose dependent bactericidal activity was noticed in all the fruit extracts. From the results obtained, aqueous (water) extract of fruit displayed utmost antibacterial activity among which maximum was recorded against *S. aureus* (24 mm) followed by that of *B. subtilis*, *E. coli, K. pneumonae, P. aeruginosa* and *S. typhi* (20 mm each) at 100 mg/ml concentration (table 2) (fig. 2).

The chloroform extract showed enhanced concentration dependent inhibition against *S. aureus* and *S. typhi* (20 mm each) followed by *B. subtilis*, *E. coli*, and *P. aeruginosa* (18 mm each) while minimum for *K. pneumonae* (15 mm) at 100 mg/ml (table 2) (fig. 2). *E. coli*, *S. typhi* and *K. pneumonae* showed antimicrobial resistance at 6.25 mg/ml conc. of chloroform extract while *B. subtilis* displayed antimicrobial resistance against acetone extract at 6.25 mg/ml conc. Acetone extract displayed maximum zone of inhibition against *E. coli* and *K. pneumonae* (18 mm each) followed by *P. aeruginosa* (17 mm), *B.subtilis*, *S. aureus* and *S. typhi* (15 mm each). Similar findings were obtained by other workers (5, 30, 31, 32). The antibacterial activities accessed were compared with that of standard broad spectrum antibiotics Tetracycline and Gentamycin at 30 mcg and 120 mcg respectively (table 3) (fig 3.).

The values in terms of zone of inhibition represent the average of standard error of mean (SEM) for experiments performed in triplets. Statistical analysis was performed using one way ANOVA followed by DMRT revealed the result to be significant ($p<0.01$).

Table-1: Phytochemical screening of different fruit extract of *S. aromaticum.*

Extracts	Phytochemical tests							
	Alkaloides	Carbohydrate	Flavonoides	Glycosides	Proteins	Saponin	Tanins	Terpenoids
Acetone	-	+	-	+	+	-	+	+
Aqueous	-	+	-	+	+	+	+	+
Chloroform	-	+	-	+	+	-	+	+

Table-2: Antibacterial activity of different fruit extracts of *S. aromaticum* against pathogens.

Extract (ml/disc)	Solvent	Type	Bacteria	Zone of inhibition* (mm) at diverse fruit extract conc.				
				6.25 mg/ml	12.5 mg/ml	25 mg/ml	50 mg/ml	100 mg/ml
(10 µl/ disc)	Acetone	Gram '+'	*Bacillus subtilis*	Nil	5 ± 0.2	7 ± 0.2	10 ± 0.1	15 ± 0.15
			Staphylococcus aureus	7 ± 0.2	8 ± 0.1	1 ± 0.1	1.3 ± 0.1	15 ± 0.2
		Gram '-'	*E. coli*	6 ± 0.2	10 ± 0.2	13 ± 0.2	15 ± 0.2	18 ± 0.25
			Klebsiella pneumonae	5 ± 0.1	6 ± 0.1	10 ± 0.1	13 ± 0.2	18 ± 0.25
			Pseudomonas aeruginosa	5 ± 0.1	6 ± 0.2	7 ± 0.1	12 ± 0.1	17 ± 0.15
			Salmonella typhi	5 ± 0.1	6 ± 0.2	8 ± 0.1	12 ± 0.1	15 ± 0.1
	Aqueous	Gram '+'	*Bacillus subtilis*	6 ± 0.2	10 ± 0.25	13 ± 0.1	15 ± 0.2	20 ± 0.2
			Staphylococcus aureus	7 ± 0.1	10 ± 0.1	14 ± 0.2	15 ± 0.1	24 ± 0.25
		Gram '-'	*E. coli*	6 ± 0.2	10 ± 0.2	15 ± 0.1	18 ± 0.2	20 ± 0.25
			Klebsiella pneumonae	8 ± 0.1	10 ± 0.15	14 ± 0.2	17 ± 0.1	20 ± 0.15
			Pseudomonas aeruginosa	5 ± 0.2	10 ± 0.2	12 ± 0.1	15 ± 0.15	20 ± 0.2
			Salmonella typhi	7 ± 0.1	10 ± 0.1	15 ± 0.1	18 ± 0.25	20 ± 0.25
	Chloroform	Gram '+'	*Bacillus subtils*	5 ± 0.1	6 ± 0.25	10 ± 0.2	14 ± 0.15	18 ± 0.15
			Staphylococcus aureus	5 ± 0.2	6 ± 0.1	10 ± 0.2	15 ± 0.2	20 ± 0.25
		Gram '-'	*E. coli*	Nil	6 ± 0.15	10 ± 0.1	14 ± 0.2	18 ± 0.2
			Klebsiella pneumonae	Nil	7 ± 0.2	10 ± 0.2	13 ± 0.2	15 ± 0.15
			Pseudomonas aeruginosa	5 ± 0.1	6 ± 0.1	8 ± 0.25	10 ± 0.1	18 ± 0.25
			Salmonella typhi	Nil	5 ± 0.1	6 ± 0.2	10 ± 0.1	20 ± 0.25

*Statistical analysis using one way ANOVA/ DMRT revealed results to be significant ($p < 0.01$).

Table-3: Antibacterial activity of extract against standard antibiotics.

S. No.	Type	Bacteria	Zone of inhibition (in mm)	
			Gentamycin (G) (120 mcg)	Tetracyclin (TE) (30 mcg)
1.	Gram '+'	*Bacillus subtilis*	25 ± 0.15	Nil
2.		*Staphylococcus aureus*	40 ± 0.2	Nil
3.	Gram '-'	*E. coli*	Nil	25 ± 0.15
4.		*Klebsiella pneumonae*	30 ± 0.25	Nil
5.		*Pseudomonas aeruginosa*	Nil	35 ± 0.25
6.		*Salmonella typhi*	Nil	26 ± 0.1

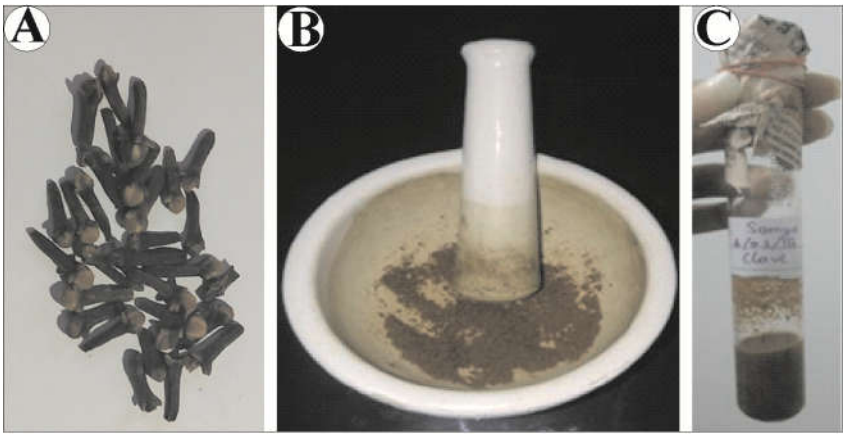

Fig. 1. Preparation of fruit extract of *S. aromaticum*. **(A)** Fruiting body **(B)** Crushed fruit powder **(C)** Aqueous extract obtained after filtration.

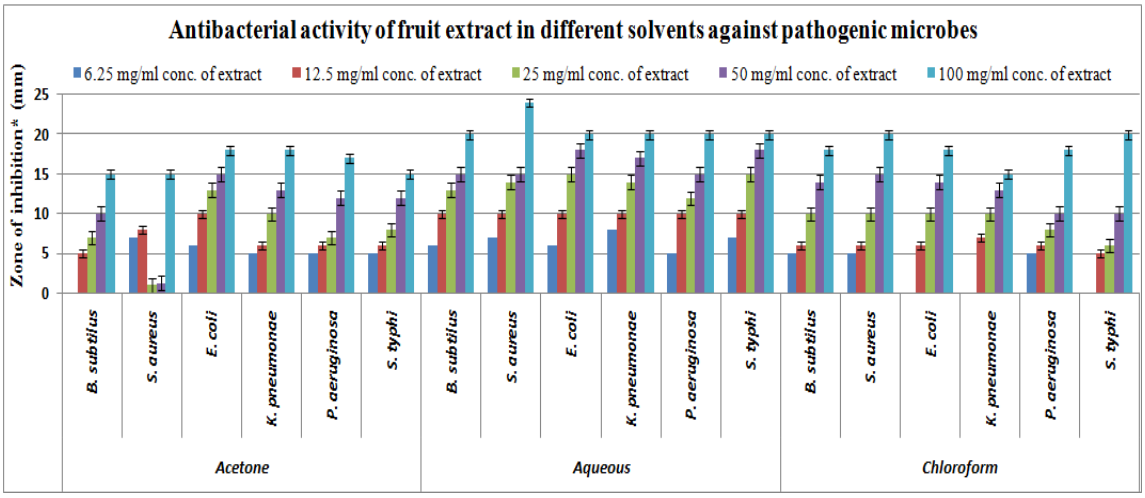

Fig. 2. Comparative antibacterial activity of different fruit extracts of *S. aromaticum* in different solvents. Considerable high antibacterial activity was found to be associated with aqueous extract followed by chloroform and acetone. The antibacterial activity was also compared with that of control.

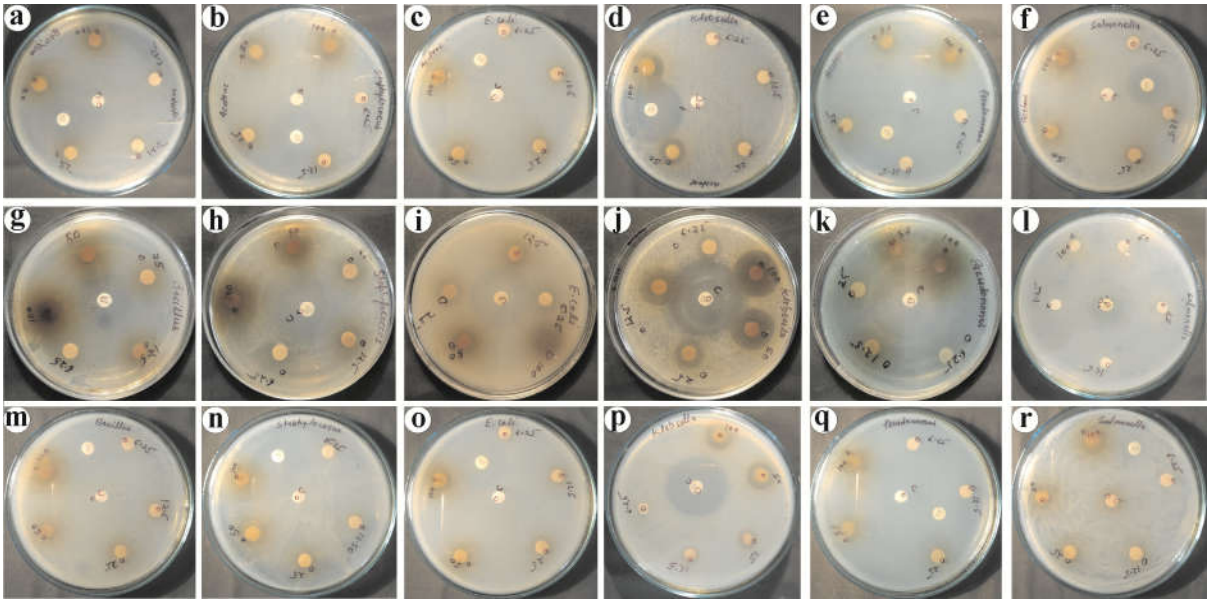

Fig. 3. Antibacterial activity of fruit extract of *S. aromaticum* in Acetone **(a-f)**, Aqueous **(g-l)** and Chloroform **(m-r)** against *B. subtilis* (MTCC 2057), *S. aureus* (MTCC 3160), *E. coli* (MTCC 294), *K. pneumonae* (MTCC 4030), *P. aeruginosa* (MTCC 2581) and *S. typhi* (MTCC 660).

CONCLUSION

From this study it becomes clear that active phytochemicals were present in the various extracts of *S. aromaticum* and due to the presence of these phytochemicals, all three extracts showed significant ($p<0.01$) antibacterial activity. Among them aqueous extract revealed maximum zone of inhibition at concentration 100 mg/ml followed by chloroform and acetone extract. Due to its potential antibacterial activity, *S. aromaticum* is used in mouth fresheners and toothpastes for inhibiting tooth gums and dental cavities and also provide relief in pain.

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