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ANTIMICROBIAL ACTIVITY OF SYZYGIUM AROMACTICUM AGAINST POTENTIAL ENTERIC PATHOGENS

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ABSTRACT

Food borne diseases are a major cause worldwide. These diseases are caused by enteric pathogens such as Escherichia coli, Salmonella typhi and Staphylococcus aureus. These microorganisms have developed resistance against many antibiotics due to the increased use of drugs which reduces the effectiveness of conventional antibiotics. Spices and herbs are known to be natural sources consisting of antimicrobial activity. Clove is one spice which promises to possess antibacterial activity. The present study was carried out to determine the antimicrobial activity of cloves using methanol, ethanol and chloroform extracts against Escherichia coli, Salmonella typhi and Staphylococcus aureus strains. The extraction was carried out by the cold maceration technique. Antimicrobial activities of different solvent extracts were screened by disk diffusion. Minimum Inhibition Concentration (MIC) and Minimum Bactericidal Concentration (MBC) were determined by broth dilution method. Also, the study was done to investigate the phytochemical compounds in each extract. The results revealed that methanol and ethanol extract of clove showed a better antimicrobial activity against pathogens compared to chloroform extract of cloves. The results also demonstrated that the gram-positive bacteria were more sensitive to clove than gram negative bacteria. Phytoconstituents analysis demonstrated the presence of flavanoids, saponins, terpenoids, carbohydrate and tannins which are responsible for the antimicrobial activity.

Keywords: Enteric pathogens, Syzygium aromaticum, antimicrobial activity

INTRODUCTION

Food infection and intoxication are major causes of food borne diseases worldwide. These diseases occur when food borne pathogens come in contact through cross contamination, improper food handling and temperature misuse (Pandey and Singh, 2011). The most common food borne microorganisms that cause infection and intoxication are E. coli, S. aureus and Salmonella species (Braga et al., 2005). E. coli is a gram negative (consists of a thin cell wall with 1 or 2 layers of peptidoglycan) bacteria commonly found in the gastrointestinal tract and part of the normal bacteria flora. Most E. coli strains are harmless but certain serotypes cause food poisoning in their hosts (Blount, 2015). Salmonella species which are also gram-negative bacteria cause Salmonella food poisoning. S. aureus is a gram positive (consists of a thick cell wall with several layers of peptidoglycan) bacterium which can generate toxins that produce food poisoning in the human body (Zhou et al., 2014).

These microorganisms cause products to lose its quality which causes them unacceptable for consumption. As a result,

short shelf-life of food is a major problem in the food industry (Kable et al., 2018). Recent reports indicated that Salmonella Sp. and E. coli are responsible for economic losses amounting 5.9 billion dollars each year. Prevention of these pathogenic microorganisms in food is usually achieved by chemical preservatives. These chemicals can be responsible for many carcinogenic, teratogenic and residual toxicity (Shan et al., 2007). Therefore, investigations of naturally occurring antimicrobials for food preservation receive increasing attention.

Antibiotic resistance

Antibiotics are used to prevent and treat bacterial infections. Microorganisms have become resistance to many antibiotics due to increase use of drugs, which reduces the effectiveness of conventional medicines (Fait and Tor, 2014). Antibiotic resistance is a major rising cause throughout the world. Infections, such as pneumonia, tuberculosis, blood poisoning, gonorrhoea, and food borne diseases are becoming harder to treat as antibiotics become less effective (WHO, 2017). A case was reported in a hospital in Bangladesh indicating 75% of the bacterium species Salmonella typhi showed resistance against the antibiotic Ciprofloxacin (Kabir et al., 2017). Also, emergence of antibiotic-resistant strains of S. aureus such as methicillin-resistance S. aureus is a worldwide problem in clinical medicine (Solarte et al, 2017). Moreover, international committees of experts have indicated the need to search new alternatives for the treatment of infectious diseases (Atanasov et al., 2015). Therefore, new antimicrobial agents are discovered to counter the threat posed by pathogenic microorganisms. Hence, natural plants are a preferred choice where research has shown certain spices may be helpful as antibiotics in the future. There are several research carried out previously reporting the antimicrobial activity of cloves (Pandey and Signh, 2011; Kumar et al., 2014) Therefore clove is an appropriate choice of spice to carry out further studies.

Clove

Clove which is scientifically named as Syzygium aromaticum is an aromatic flower bud which belongs to the Myrtacese family (Kamatou, Vermaak and Viljoen, 2012). Cloves play an important role in the human diet for improving flavour and aroma of food stuffs. Clove represents one of the major sources of phenolic compounds as flavonoids, hidroxibenzoic acids, hidroxicinamic acids and hidroxiphenyl propens. Eugenol is the main bioactive compound of clove's essential oils (EO). It is responsible for the characteristic, pungent odour of clove (Cortés-Rojas et al., 2014). The main feature of EOs is their complex chemical composition and the synergistic interaction of many of their active ingredients. The action of EOs is developed by various mechanisms that affect bacterial survival, which decrease the possibility of resistant strain selection (Nazzaro et al., 2013). Cloves are rich in secondary metabolites which include alkaloids, glycosides, flavonoids, steroids, tannins and saponins. The therapeutic value of clove lies in the secondary metabolites. Thus, they play an important role in developing of newer drugs because of their effectiveness, less side effects and relatively low cost when comparing with synthetic drugs (Raja et al., 2016).

Antimicrobial activity of cloves

The eugenol present in clove gives the spice a medicinal value. The antimicrobial activity of eugenol is due to the presence of a free hydroxyl group. Primarily, the eugenol molecule acts on the cytoplasmic membrane and increases the membrane nonspecific permeability and affects the transport of ions and ATP (Marchese et al.,

2017). Devi et al (2010) evaluated the mechanism of antibacterial activity against Salmonella typhi. He reported, eugenol molecule alters the membrane permeability resulting leakage of ions and cellular contents which ultimately results in cell death. Eugenol also modified the fatty acid profile of the cell membrane of different bacteria. Di Pasqua et al. (2007) measured changes in the principal fatty acid composition of Escherichia coli. Also, Das et al. (2016) explained the mechanism of action of eugenol against S. aureus. He demonstrated that eugenol was able to trigger cell cytotoxicity due to the production of intracellular reactive oxygen species (ROS) which induces the inhibition of the growth of cell, disruption of the cell membrane and DNA damage resulting in cell decomposition and death. In this study, clove powder was used for investigating antimicrobial potential. The aim of the present study work was to investigate the efficiency and compare different solvents extracts of Syzygium aromaticum against enteric pathogens and phytochemical compositions. Therefore, to determine the antibacterial activity disk diffusion along with the determination of the minimal inhibitory concentration (MIC) and minimal bactericidal concentration (MBC) was carried out.

Methodology Sample collection

The powdered form of Syzygium aromaticum (cloves) was obtained from the local market in the Colombo, Sri Lanka.

Extraction of cloves using the cold maceration technique

Powdered cloves (10g) were measured and were transferred to three falcon tubes each. 40ml of 80% methanol was added to each tube and was mixed. The tubes were sealed and left on the roller mixer overnight. After 24 hours the supernatants were separated from the residue by filtering through a muslin cloth and re-filtered by passing through Whatman no. 1 filter paper. The filtrate was collected into watch glasses and left in the fume hood overnight to air dry. After the filtrate was completely dried, the crude extract was scrapped and stored in a sterile glass bottle. The weight of the dry mass was determined and used to calculate the concentration of the extracts in mg/ml. Stock solutions were prepared by dissolving 1g of the dried extract in 10ml of the solvent to obtain a concentration of 100mg/ml and stored at 40C in sterile falcon tubes until further use. Likewise, clove extraction using ethanol and chloroform was carried out. The yield percentage was determined in all three extracts as follows,

Yield (%) = Final weight of extract X 100

Initial weight

Antimicrobial activity of clove extracts Bacterial strains

Test organisms including Escherichia coli (ATCC 25922), Staphylococcus aureus

(ATCC 25923) and Salmonella typhi were obtained from the BMS laboratory.

Preparation of subcultures

Subcultures of Escherichia coli, Salmonella typhi and Staphylococcus aureus were prepared. 10ml of nutrient broth was added to 5 test tubes each. Under sterile conditions, two test tubes were inoculated with E. E coli, two test tubes were inoculated with Salmonella typhi and one tube with Staphylococcus aureus. The subcultures were incubated at 370C overnight. After incubation, the cultures were streaked plated to prepare bacterial plates.

Disk Diffusion

The disk diffusion was carried out of the three extracts (methanol, ethanol and

chloroform) using the three bacterial strains.

Preparation of inoculums

A single colony of bacterial strain was picked from a sterile inoculation loop and dissolved in 10ml of NaCl solution. The turbidity was checked with the McFarland turbidity. Sample concentrations: 20mg/ml, 40mg/ml and 60mg/ml. The Muller Hinton agar (MHA) plates were labelled including date, sample and the microorganism used. From the NaCl solution containing the bacterium, 250µl was added onto the agar and was spread uniformly using a glass spreader and kept for 5 minutes. The sterile paper discs (6mm) were dipped in the sample (20mg/ml, 40mg/ml and 60mg/ml) and were placed on the plate as triplicates. Gentamycin disc was used at the positive control, 20% of the solvent was used at the negative control. The plates were incubated at 370C for 24 hours. Likewise, disk diffusion was carried out for all three extracts for the three bacterial strains.

The inhibition zones were measured using a ruler by placing it across the zone of inhibition, measuring from one edge of the zone to the other edge.

Determination of the minimal inhibitory concentration (MIC) by broth dilution method

Preparation of the bacteria inoculums

A volume of 5ml Muller Hinton (MH) broth was added to a test tube and inoculated with the bacteria until it reached the MacFarland standard. The required amount of cfur MIC was 1x106cfu/ml. Therefore a 1:150 dilution of the bacterial inoculums was done by adding 100µl of the prepared inoculums to 14.9ml of MH broth.

Dilution series preparation

The clove extract was dissolved in NaCl solution and 1:2 dilution series was prepared (final volume 1ml). Then each tube was inoculated with 1ml of the prepared diluted microbial inoculums, therefore the final concentrations of the samples are 2mg/ml, 4mg/ml, 8mg/ml, 16mg/ml, 32mg/ml and 64mg/ml. After mixing, the inoculated tubes were incubated at 370C overnight. 2ml of MH broth was used as the negative control and as the positive control 1ml of the MH broth was added to 1ml of the bacterial inoculums.

Determination of minimum bactericidal concentrations (MBC) of the clove extracts

The dilution represented the MIC and two of the more concentrated test product dilutions were plated in nutrient agar plates. The plates were incubated at 370C for 24 hours. The bacterial growth was observed. MBC was taken as the concentration of clove extract that did not exhibit any bacterial growth on the agar plates.

Phytochemical screening of clove

Phytochemical screening was carried out in the three extracts of cloves. The following tests were performed to detect phytochemical constituents present in them.

Test for tannins

2ml of the extract was added to a test tube. Then few drops of 1% FeCl3 were added.

Test for flavonoids

1ml of the extract was added to a test tube. Then few drops of 10% NaOH were added.

Test for carbohydrates

2ml of extract was added to a test tube. Then 2ml of the Molisch reagent was added. Few drops of conc. H2SO4 were added along the test tube wall.

Test for terpenoids

2ml of extract was added to a test tube. Then 1ml of chloroform was added. Then 1.5ml of Conc. H_2SO_4 was added.

Test for saponins

2ml of distilled water was added to 2ml of the extract and was shaken vigorously.

DATA ANALYSIS

Extract yield of different solvents

The extract of 30g of the powered cloves with ethanol, methanol and chloroform yielded residues ranged from 1g-2g. The highest yield was obtained from the methanol extract 2g, while the chloroform extract gave the lowest yield.

Table 1: The extraction yield of cloves of different solvents

Extract of clove	Yield (% w/w)
Methanol	6.6
Ethanol	5
Chloroform	3.3

Antimicrobial screening

Disk diffusion

Figure 1: Inhibition zones of ethanol extract against S. Aureus. 1) 20mg/ml concentration of the extract- S1, S2 and S3, 2) 40mg/ml concentration of the extract- S4, S5 and S6, 3) 60mg/ml concentration of the extract- S7, S8 and S9, 4) Positive control- Gentamycin, 5) Negative control- 20% ethanol

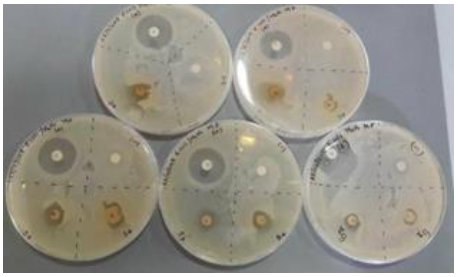

Figure 2: Inhibition zones of methanol extract against E. Coli,1) 20mg/ml concentration of the extract- S1, S2 and S3, 2) 40mg/ml concentration of the extract- S4, S5 and S6, 3) 60mg/ml concentration of the extract- S7, S8 and S9, 4) Positive control- Gentamycin, 5) Negative control- 20% methanol

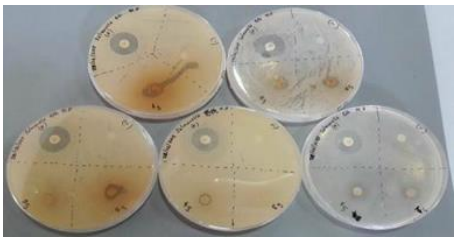

Figure 3: Inhibition zones of ethanol extract against Salmonella, 1) 20mg/ml concentration of the extract- S1, S2 and S3, 2) 40mg/ml concentration of the extract- S4, S5 and S6, 3) 60mg/ml concentration of the extract- S7, S8 and S9, 4) Positive control- Gentamycin, 5) Negative control- 20% ethanol

Statistical analysis of antibacterial activity of different extracts between S.Aureus, E.Coli and Salmonella

According to the figure 1, 2 and 3 the inhibition zone has been increased as the concentrations increased. 60mg/ml concentration gave the highest diameter of inhibition zone against all three microorganisms. However, since the p value is less than 0.05, there is a statistically significant difference in the

mean diameter of inhibition zones given by all three different concentrations of the extracts against all three microorganisms (Table 2-B, D, F). Therefore, at the maximum concentration the antimicrobial activity of clove is high.

According to the table 2-A, C, E there is no significance interaction between the extractions and concentration to the antimicrobial activity in all three microorganisms. However, there is significance different between the extractions on the antimicrobial activity since the p value is than 0.05. Hence, different extractions have an impact on the antimicrobial property of the microorganism.

(A) ANOVA for S. Aureus, Inhibition zone

Source	df	F	Sig.
Corrected Model	5	3.952	.024
Intercept	1	135.561	.000
Extraction	1	12.771	.004
Concentration	2	3.243	.075
Extraction* Concentration	2	.252	.781
Error	12		
Total	18		
Corrected Total	17		

Table 2: The statistical data for antibacterial activity

(B)ANOVA,Multiple Comparisons

(I) Concentration	(J) Concentration	Mean Difference (I-J)	Std. Error	Sig.
	40	-2.6667	2.36095	.000
20				
	60	-6.0000	2.36095	.002
	20	2.6667	2.36095	.001
40				
	60	-3.3333	2.36095	.000
	20	6.0000	2.36095	.001
60				
	40	3.3333	2.36095	.001

The mean difference is significant at the 0.05 level

(C)ANOVA for E. Coli, Inhibition zone

Source	df	F	Sig.
Corrected Model	5	29.486	.000
Intercept	1	2100.000	.000
Extraction	1	32.190	.000
Concentration	2	57.571	.000
Extraction * Concentration	2	.048	.954
Error	12		
Total	18		
Corrected Total	17		

(D) ANOVA, Multiple Comparisons

(I) Concentration	(J) Concentration	Mean Difference (I-J)	Std. Error	Sig.
40		-2.8333*	.62361	.002
20		-6.6667* 2.8333*	.62361	.000
60		-3.8333* 6.6667*	.62361	.002
20		3.8333*	.62361	.000
40			.62361	.000
60			.62361	.000
20				
60				
40				

The error term is Mean Square (Error) = 1.167. The mean difference is significant at the 0.05 level

E) ANOVA for Salmonella Inhibition zone

Source	df	F	Sig.
Corrected Model	5	8.257	.001
Intercept	1	171.920	.000
Extraction	1	.102	.755
Concentration	2	20.591	.000
Extraction* Concentration	2	.000	1.000
Error	12		
Total	18		
Corrected Total	17		

(F) ANOVA, Multiple Comparisons

(I) Concentration	(J) Concentration	Mean Difference (I-J)	Std. Error	Sig.
	40	-6.3333*	1.27657	.001
20				
	60	-7.6667*	1.27657	.000
	20	6.3333*	1.27657	.001
40				
	60	-1.3333	1.27657	.564
	20	7.6667*	1.27657	.000
60				
	40	1.3333	1.27657	.564

Comparison of antibacterial activity of different extracts between S.Aureus, E.Coli and Salmonella

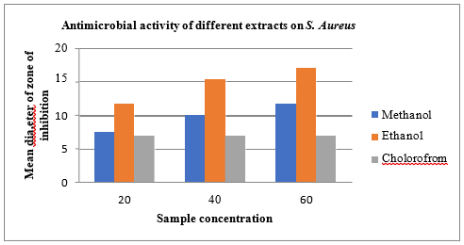

Figure 4: Antimicrobial activity of different extracts on S. Aureus

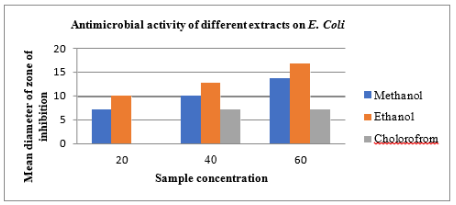

Figure 5: Antimicrobial activity of different extracts on E. Coli

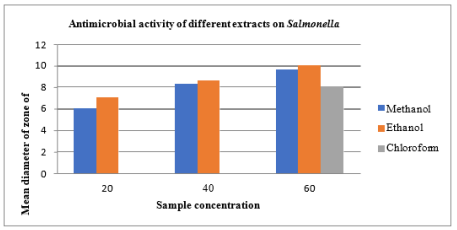

Figure 6: Antimicrobial activity of different extracts on Salmonella

The above grafts indicate that ethanol extraction has the highest antimicrobial activity.

Minimal inhibitory concentration

According to the disc diffusion results significant zones of ethanol and methanol extracts of clove against E. coli and S. aureus were observed, therefore the MIC was carried out.

Microorganism	Clove extract	MIC value (mg/ml)
S. aureus	Methanol	8
	Ethanol	8
E. coli	Methanol	16

Table 3: MIC results of methanol and ethanol extracts against S. aureus and E. coli

According to table 3, a visible solution was observed in 8mg/ml in both methanol and ethanol extracts for S. aureus. On E. coli a visible solution was observed in 16 mg/ml in the methanol extract and 8mg/ml in the ethanol extract of cloves.

Minimal bactericidal concentration

The MIC concentrations and two other concentrations were plated to determine the MBC value.

Table 4: Spread plate results for methanol and ethanol to determine the MBC value (Author developed)

Microorganism	Clove extract	Sample concentration that showed no visible growth (mg/ml)
S. aureus	Methanol	8 16
	Ethanol	8 16
E. coli	Methanol	16 32
	Ethanol	8 16

As mentioned in the table, both the extracts 8mg/ml and 16mg/ml did not show visible growth for S. aureus and E. coli. Therefore, the MBC value should be present (8 > x > 4) mg/ml concentration.

Phytochemical screening results

Table 5: Results of phytochemical analysis of cloves

Test	Methanol extract	Ethanol extract	Chloroform extract
Tannins	+	+	-
Flavonoids	+	+	-
Carbohydrate	+	+	-
Terpenoids	+	+	+
Saponins	+	-	-

DISCUSSION

In the past few decades, the incident of spread of drug resistant pathogens was found to be a crucial threat towards the treatment of various microbial diseases (Hemalatha et al., 2016). Due to this major crisis, there is a need of discovering new sources that have antimicrobial properties. Therefore, several studies have provided evidence that clove possess such antimicrobial activity and these natural plant derived products and EO have been studied as diseases control agents (Shaheen et al., 2015). In the present study, medicinally important Syzygium aromaticum EO was screened to determine the antimicrobial potential using different solvents and to determine the phytochemical components present. Three solvents (ethanol, methanol and chloroform) were used for extraction of phytochemicals and their extraction values were determined. Extraction of methanol yield was 2g whereas ethanol and chloroform yielded 1.5 g and 0.8 g respectively (Table 1). Methanol extraction showed better efficiency than the other solvents. This result was found to be similar with the existing literature (Nazrul et al. 2010; Edziri et al. 2011). In this study, gram positive Staphylococcus aureus and two gram negative bacterial pathogens E. coli and Salmonella typhi were selected to determine the antimicrobial activity of different solvent extracts of clove. Methanol and ethanol extract showed inhibition zones after disk diffusion against E.Coli, Salmonella and S.Aureus (Figure 1, 2, and 3). Whereas no significant inhibition zones were obtained for chloroform extract against E.Coli, Salmonella and S. Aureus. The antimicrobial activity of cloves against the tested pathogens revealed that methanol and ethanol extract of clove showed a better antimicrobial activity against pathogens (Fig 4,5 and 6). This may be due to the better solubility of active ingredients of cloves in methanol and ethanol solvents than chloroform (Rohini and Padmini, 2016).

Solvents (methanol, ethanol and distilled water) were used as negative controls. The negative control did not show any zone thereof no contamination was observed. The antimicrobial activity of clove was compared with a standard antibiotic, Gentamycin. According to the studies carried out by Soni and Dhahiya, 2014; Upadhyaya et al., 2017; Kumar et al., 2014 the concentration used were much lower compared to the concentrations that were used in this investigation. Furthermore, a study showed high antimicrobial inhibition zones (17mmm -22mm) at the concentrations used in this study against these pathogens. In the present study inhibition zones obtained were relatively small (7mm-17mm). This could be due to thick depth of the agar or due to the use of heavy inoculates (King and brown, 2015). The results also demonstrated from the mean diameters of inhibition zones that the antibacterial activity was higher against S.aureus compared to that of E.coli and Salmonella (Fig 5, 6). The natural substance of clove as flavonoids can disrupt the cell wall and membrane of bacteria and inhibit their protein synthesis and eventually destroy the bacteria. Gram negative bacteria contains an outer membrane along with thin a cell wall, therefore it is more resistance against antibiotics. This may be the same reason that a higher antibacterial activity was demonstrated against S. aureus then E. coli

and Salmonella. Since significant zones were obtained for methanol and ethanol extracts of clove against E. coli and S. aureus the MIC was carried out. The MIC is the lowest concentration of a chemical which prevents visible growth of a bacterium. The MIC values of methanol and ethanol extracts ranged from 8mg/ml for S. aureus. For E. coli methanol extract the MIC was 16mg/ml, for ethanol extract 8mg/ml (Table 3).

The MBC is the concentration resulting in microbial death as defined by the inability to re-culture bacteria. As mentioned in table 4, both the extracts 8mg/ml and 16mg/ml did not show visible growth for S. aureus. For E..coli. Methanol extract 16mg/ml and 32mg/ml did not show visible growth, whereas for the ethanol extraction 8mg/ml and 16mg/ml did not show growth. Therefore, the MBC value should be present ($8 > x > 4$) mg/ml concentration. The antibacterial properties of cloves are mainly due to presence of different chemical agents which were classified as bioactive antimicrobial compounds ((Nikousaleh and Prakash, 2015). Phytochemical constituents such as alkaloids, glycosides, flavonoids, tannins, steroids, terpenoids are secondary metabolites of plants that serve as a defence mechanism against many microorganisms (Jyothiprabha and Venkatachalam). The present study also revealed the presence of medicinally active compounds like flavonoids, terpenoid, saponins, carbohydrates and tannins in cloves which could be responsible for the observed antibacterial property. According to table 5, tannins, flavonoids, carbohydrates, terpenoids and saponins were present in methanol extract of clove, whereas saponins and terpenoids were present in chloroform extract of clove; whereas tannins, flavonoids, carbohydrates and terpenoids were present in the ethanolic extract of clove. Phytochemicals detected in cloves, are used as various antibiotics in treating common pathogenic strains (Kubmarawa, 2007 and Mensah, 2008). It has been reported that tannins bind to protein and interfere with protein synthesis. Flavonoids are hydroxylated phenolic which have the ability to complex with extracellular and soluble proteins and to complex with bacterial cell wall (Ayoola et al., 2010). Therefore, these phytochemicals show activities including antimicrobial, antioxidant and anti-inflammatory activities. Terpenoids have been reported to possess anti-carcinogenic, anti-ulcer and antimicrobial activities. Saponins have been reported as antioxidant and antimicrobial in activities (Kumaravel and Alagusundaram). The present study results confirmed that the methanol and ethanol extraction contain a significant amount of phytochemicals. Thus suggest these compounds may be the bioactive constituents, which is a valuable reservoir of bioactive compounds of significant medical merit.

CONCLUSION

According to the present study cloves may possess effective anti-bacterial activity against micro-organisms and can be used for prevention of drug resistant microbial diseases and further evaluation is necessary. The phytochemicals (flavonoids, saponins, terpenoids, tannins and carbohydrates) were present mainly in methanol and ethanol extracts. This study opens up the possibility for the search of new antimicrobials as an alternative to the antibiotics by using the methanol and ethanol extracts of cloves.

This investigation focused on antibacterial and phytochemical components however cloves possess other beneficial properties as well. Eugenol present in clove is believed to have anticancer properties and anti-inflammatory properties too (Han and Parker, 2017). Therefore, further studies

can be carried out on these properties of cloves as well.

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