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IN-VITRO ANTIBACTERIAL EFFICACY OF COMMON SPICES AND THEIR EFFECT ON PERMEABILITY OF MEMBRANE

K. PAVITHRA VANI*[1] AND O. BHAGYA LAKSHMI[2]

[1]Department of Microbiology, Sarojini Naidu Vanita Maha Vidhyalaya, Hyderabad – 500 001.Telangana, India.*
[2]Department of Botany, Sarojini Naidu Vanita Maha Vidhyalaya, Hyderabad – 500 001. Telangana, India.

ABSTRACT

The aim of the present study is to investigate three different spices for antibacterial activity and also to study their effect on membrane permeability of bacteria. The spices selected were Black pepper (*Piper nigrum*), Cinnamon (*Cinnamomum zeylanicum*) and Cloves (*Syzygium aromaticum*) are routinely consumed in our diet. The spice extracts and essential oils were screened for antibacterial activity by Disc Diffusion Method, followed by determination of MBC by double dilution method. Minimum Bactericidal Concentration (MBC) was determined by the Double Dilution Method. Killing Kinetics were studied to know the time course of lethal action of these spices on *Escherichia coli* and *Staphylococcus aureus*. Effect of spices on membrane leakage of bacteria was studied by using a UV-spectrophotometer. The results indicate excellent inhibition on the growth of Gram-positive and Gram-negative bacteria. Among the spices tested cloves and cinnamon were found to be more efficient in killing bacteria followed by black pepper. Leakage of cellular constituents from bacterial cells into the extra cellular medium through membrane having absorption at 260nm (Nucleic acids) and 280nm (Proteins) gradually increased after treating the bacteria with the test compound and proved that this may be one of the modes of bactericidal action. From the experimental results it can be concluded that the above three spices are good antibacterial and prospective phytotherapeutic agents.

KEYWORDS: **Antibacterial, MBC, Killing kinetics, Spectrophotometer, Phytotherapeutic**

K. PAVITHRA VANI
Department of Microbiology*, Sarojini Naidu Vanita Maha Vidhyalaya, Hyderabad – 500 001,Telangana, India.

***Corresponding author**

INTRODUCTION

The rise in antibiotic resistant microorganisms in recent years has led to an increasing search for new antibiotics. Plant secondary metabolites have been used for centuries in traditional medicines and therefore represent a source of potentially active compounds[1]. Medicinal plant products could also prove useful in reducing/minimizing the adverse effects of various chemotherapeutic agents as well as in prolonging longevity and attaining positive health[2]. The plant derived foods contain a multitude of components that elicit several biological responses. Some of which are consistent with reduced risk of one or more diseases. The active constituents were aptly termed as phytochemicals and as phytonutrients, nutraceuticals, biotherapeutics etc., which form part of diet[3].Plants or plant products that form the part of food or as dietary components have been receiving considerable attention. Among the various plants that form part of diet, spices occupy a special place. Spices are known as appetizers and are considered essential in food preparations for their preservative values. Some of them have proved to possess antioxidant and antimicrobial properties[4]. Apart from enhancing taste, spices are believed to have medicinal value. They form an important part of the ayurvedic pharmacopeia (Indian System of Medicine). They are used in number of medicinal preparations for the treatment of several disorders of the body, particularly the digestive system[5] and have a profound effect on human health, since they affect many physiological disorders. For instance spices intensify salivary flow and the secretion of amylase which facilitates digestion[6]. Spices are considered as rich source of bioactive antimicrobial compounds[7]. Recently many researchers demonstrated the antibacterial activities of spices [8, 9, 10, 11].In the light of above information the present work on evaluation of antibacterial activity of three commonly used spices like Black pepper (*Piper nigrum*), Cinnamon (*Cinnamomum zeylanicum*) and Cloves (*Syzygium aeromaticum*) are carried out to test for their antibacterial activity and their effect on membrane permeability of bacteria.

MATERIALS AND METHODS

(i) Plant material

Black pepper, Cinnamon and Cloves were purchased from Spice Garden, Kerala, India.

(ii) Bacterial cultures

Gram-positive bacteria: *Bacillus cereus* (NCIM-2016), *Staphylococcus aureus* (NCIM-2079) and *Streptococcus pneumoniae* (NCIM-2080)

Gram-negative bacteria: *Escherichia coli* (NCIM-2089), *Klebsiella pneumoniae* (NCIM-2957), *Pseudomonas aeruginosa* (NCIM-2200) and *Salmonella typhi* (NCIM-2501) were obtained from NCIM, NCL, Pune, India.

(iii) Chemicals and Media

Purchased from Qualigens and Hi-Media laboratories India ltd.

(iv) Extraction of Spices

The spices were ground to a fine powder. The spice powder (50g) was then extracted with acetone and DCM (Dichloromethane) using soxhlet apparatus by continuous heat extraction for 24hrs. The extract obtained was concentrated to dryness by evaporating the solvent under reduced pressure[12]. The extracts were stored in the air tight vials in the refrigerator for further use. In the experiments the concentrate thus obtained was dissolved in dimethyl sulfoxide (DMSO) such that the final concentration of the extract would be 1g/ml of DMSO.

(v) Extraction of Spice Essential oils

Essential oils of spices were extracted by steam distillation. The essential oils obtained were transferred into separate air tight vials and preserved in the refrigerator at 4^0C until used.

(vi)Antibacterial Screening by Disc Diffusion Method

The *In-vitro* antibacterial activity of spice extracts were carried out by Disc Diffusion Method[13]. 0.5ml of 18hrs old actively growing

bacterial cultures were mixed in soft nutrient agar (Nutrient broth with 1% agar) and plated. The 1µl of spice extract (1mg/1µl) was loaded onto different sterile filter paper discs prepared from Whatman No 1 filter paper. Similarly 5µl essential oil was loaded on to the disc. The discs were then placed on the preseeded agar medium and incubated for 24hrs at 37^{o}C. The diameter of zone of growth inhibition was measured in mm diameter. The effects were compared with that of the standard antibiotic Ampicillin (20µg/disc) and DMSO alone served as control.

(vii) Determination of Minimum Bactericidal concentration

MBC was determined by Double Dilution Method[14]. Two fold serial dilutions of the spice extracts and essential oils were carried out in the nutrient broth to get the desired concentration. To each test tube an inoculum containing 10^5 cells/ml of actively growing bacterial culture was added. The culture tubes were incubated at 37^{o}C for 24hrs. After the incubation, a loop full of treated bacteria culture was streaked on nutrient agar media and incubated for 18hrs at 37^{0}C. The plates were checked for the growth of bacteria and MBC of the spice extracts and essential oils were determined.

(viii) Killing Kinetics

The method[15] was followed to study the killing time of spices on bacteria. In this assay *E. coli* and *S. aureus* was treated with spice extracts and essential oils at 1 MBC and at regular time intervals (30minutes and 15minutes respectively) aliquots of treated cultures was drawn, diluted appropriately and inoculated on nutrient agar plates. The inoculated plates were incubated at 37^{0}C for 24hrs for colony development. The number of colonies was counted and the cell viability was determined.

(ix) Effect of spice extracts and essential oils on membrane leakage of bacteria

The method[16] was followed to determine the leakage of 260nm and 280nm absorbing material from the bacterial cells. The cell suspension was prepared. *S. aureus* cells were grown overnight with continuous shaking in nutrient broth at 37^{0}C, harvested, washed with 10mM EDTA and then twice in distilled water by centrifugation each time at 6000rpm for 15 minutes at 4^{0}C and resuspended such that the absorbance of the final suspension was 2.0 at 450nm. After 30mins incubation at room temperature, the spice extracts and essential oils were added separately to suspension at 1 MBC. At regular intervals of time, aliquots of the samples were drawn, centrifuged and absorbance of supernatant at 260nm and 280nm was measured using UV-Spectrophotometer.

RESULTS

(i) Antibacterial Screening by Disc Diffusion Method

The antibacterial activity of all the three spice extracts and essential oils were measured in terms of zone of growth inhibition in mm diameter. The disc diffusion method results showed excellent growth inhibition against the tested bacteria (Table I)

(ii) Determination of Minimum Bactericidal concentration

The MBC of the three spice extracts and essential oils were expressed in µg/ml and µl/ml respectively. Compared to extracts, essential oils are found to be more efficient in bactericidal action. The MBCs were effectively compared with that of standard antibiotic ampicillin (Table II).

(iii) Killing Kinetics

Killing kinetics studies performed to know the time of lethal action i.e., 100% killing by the test compound (spice extracts and essential oils) are presented in minutes (Graph I and II).

(iv) Effect of Spice Extracts and Essential oils on Membrane Leakage of Bacteria

The membrane leakage of cellular constituents having absorption at 260nm and 280nm were observed over a period of 90 minutes in the presence of spice extracts and essential oils against *S. aureus*. The results showed that clove and cinnamon induced leakage rapidly than black pepper (Graph III and IV).

Table I

Antibacterial activity of spice extracts and essential oil determined by disc diffusion method

Bacterial Strain	Acetone Extract (1mg)			DCM extract (1mg)			Essential oil (5µl)			Ampicillin
	Black Pepper	Cinnamon	Cloves	Black Pepper	Cinnamon	Cloves	Black Pepper	Cinnamon	Cloves	
Bacillus subtilis	11	22	17	10	19	18	17	22	18	24
Staphylococcus aureus	16	23	22	16	22	20	18	24	23	32
Streptococcus pneumoniae	20	20	14	17	20	18	20	21	16	21
Escherichia coli	12	19	12	15	18	16	14	19	15	17
Klebsiella pneumoniae	14	20	14	11	17	16	16	19	15	18
Pseudomonas aeruginosa	11	19	18	10	18	15	13	20	17	17
Salmonella typhi	16	17	14	11	15	13	15	18	14	27

- ***The above values represent diameter of zone of growth inhibition in mm***
- ***Ampicillin disc at a concentration of 20µg are used for comparison.***
- ***The values are the average of three experiments.***
- ***Acetone, DCM and DMSO (Dimethyl Sulfoxide) are the solvent controls with no activity.***

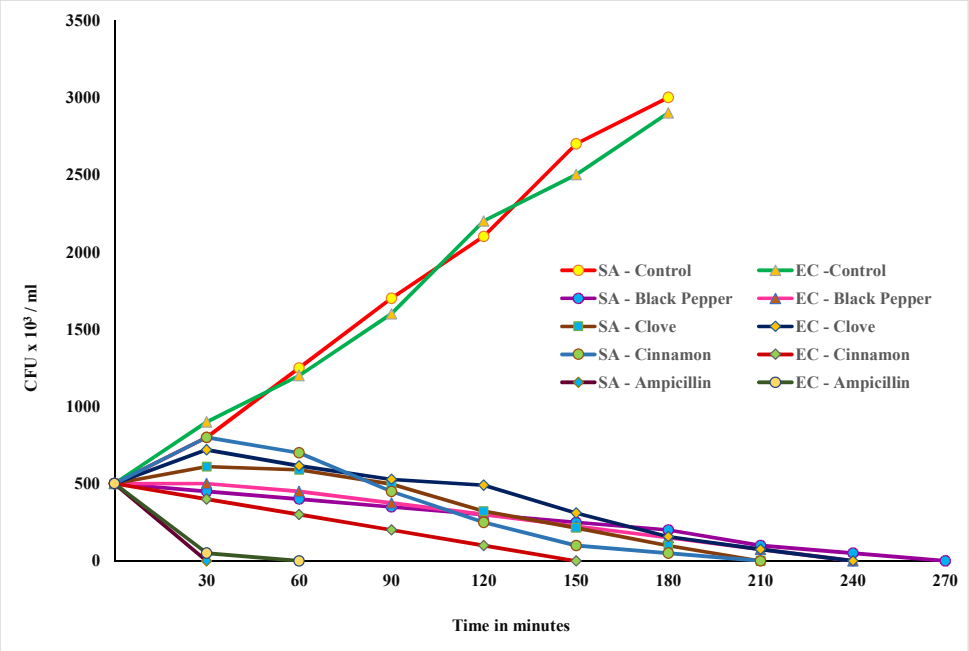
Table II

Determination of Minimum Bactericidal Concentration of spice extracts and essential oil

Bacterial Strain	Acetone Extract (1µg/ml)			DCM extract (1µg/ml)			Essential oil (5µl/ml)			Ampicillin
	Black Pepper	Cinnamon	Cloves	Black Pepper	Cinnamon	Cloves	Black Pepper	Cinnamon	Cloves	
Bacillus subtilis	250	31.30	31.30	62.50	31.30	31.30	15.62	15.62	15.62	125
Staphylococcus aureus	125	31.30	15.60	125	125	31.30	15.62	7.80	7.80	0.98
Streptococcus pneumoniae	500	31.30	31.30	125	125	31.30	15.62	7.80	15.62	125
Escherichia coli	125	62.50	31.30	250	125	31.30	3.90	3.90	7.80	1.95
Klebsiella pneumoniae	125	31.30	31.30	125	31.30	31.30	3.90	3.90	3.90	0.49
Pseudomonas aeruginosa	62.50	31.30	31.30	125	31.30	31.30	7.80	7.80	3.90	0.98
Salmonella typhi	250	125	31.30	250	125	31.30	15.62	15.62	15.62	15.60

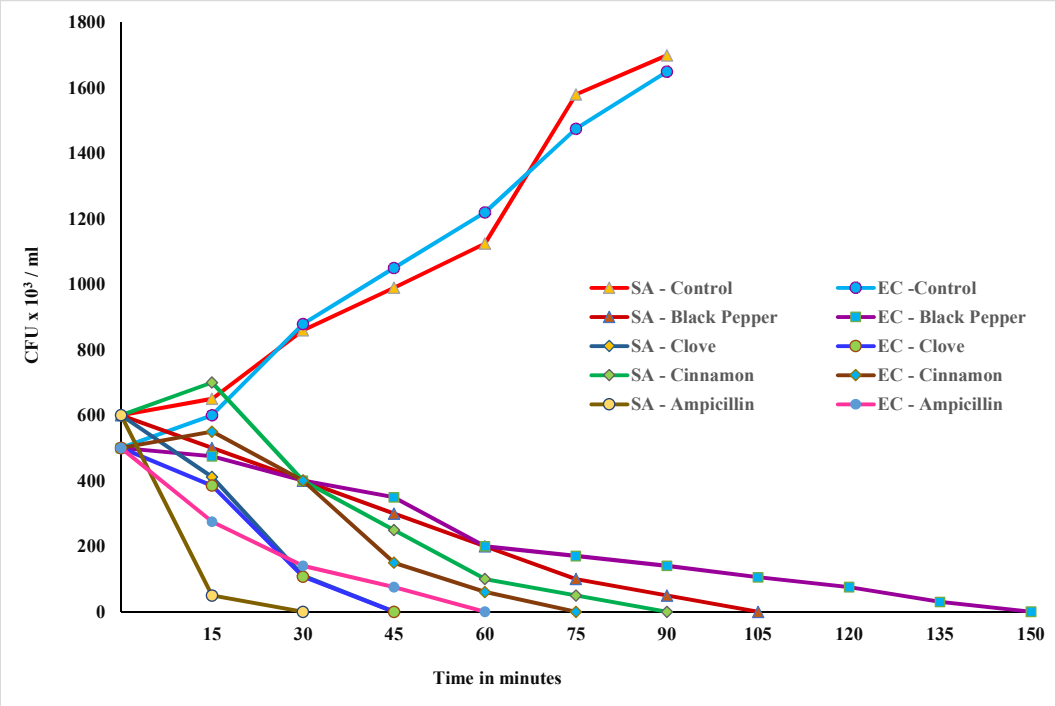
Graph I

Killing kinetic studies of spice extracts against S. aureus and E. Coli



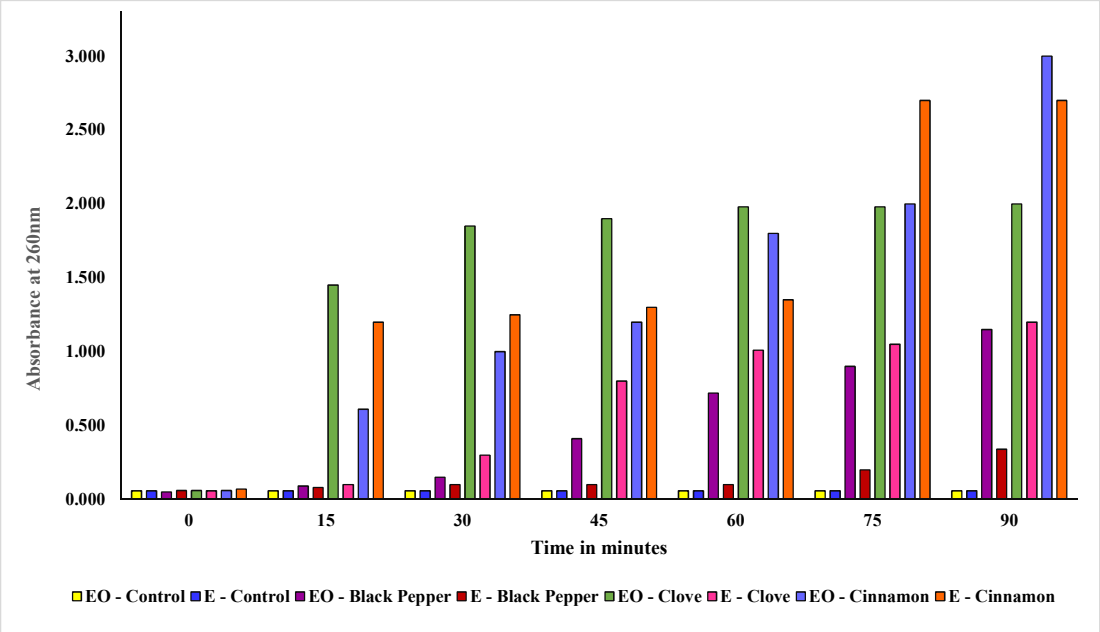
Graph II

Killing kinetic studies of spice essential oils against S. aureus and E. coli



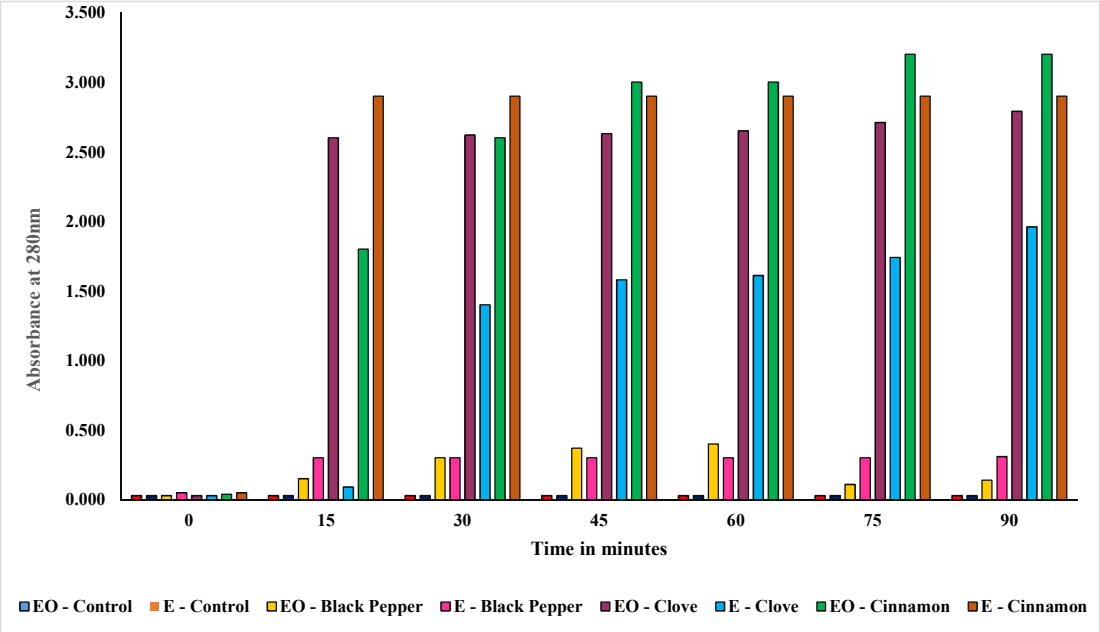
Graph III

Effect of spice extracts and essential oils on membrane leakage of 260nm absorbing material from S. aureus



Graph IV

Effect of spice extracts and essential oils on membrane leakage of 280nm absorbing material from S. aureus



DISCUSSION

Spices are used as active ingredients in ayurvedic medicines and reported to possess a number of pharmacological effects to treat different human ailments[17]. Preliminary antibacterial screening of black pepper, cinnamon and clove extracts and essential oils were done by disc diffusion method. It is a practical approach for screening the antibacterial potential of various compounds. The results revealed that the cinnamon and cloves showed greatest activity followed by black pepper. The essential oil of spices showed antibacterial activity with very high diameter of zone of growth inhibition against all the tested bacteria with more or less same as ampicillin. Gram positive bacteria are found to be more sensitive than Gram negative bacteria. The differences in the zones of growth inhibition which may depend on the solubility, evaporation, rate of diffusion of the test compounds into the agar medium.In an investigation[18], clove essential oil showed highest antibacterial activity against the tested bacteria. In their study, the recorded inhibition zones exhibited by clove essential oil against *E.coli* 11.87±3.22 mm, *K. pneumoniae* 18.86±1.46 mm and *S. typhi* 18.00±3.08 mm. The values are approximately same as in our study. Hence, their study supports the present investigation. In another study[9] ethanolic and methanolic extracts of clove and cinnamon were demonstrated for the antibacterial activity. The results showed 16mm, 20mm, and 18mm diameter of zone of growth inhibition for ethanolic extract of clove against *S. aureus*, *P. aeruginosa* and *E. coli* respectively. Methanolic extracts showed 24mm, 19mm, and 20mm against the above tested organisms.The MBC results revealed that acetone and DCM extract of clove had the highest antibacterial activity against the bacteria tested. Acetone extract of cinnamon is more effective than DCM extract. Black pepper is also showing good MBC results, but compared to clove and cinnamon the antibacterial activity is less. All the three spice essential oils tested showed excellent bactericidal action even at low concentrations as same as the standard antibiotic (ampicillin) used. In a study[10] the MIC of clove against Methicillin Resistant *Staphylococcus aureus* (MRSA) was observed in the range of 64–512 µg/ml and cinnamon as 64–256 µg/ml. It supports the present study and proves that these spice extracts are effective even against drug resistant bacteria. In an investigation[19],

the antimicrobial effectiveness of clove and cinnamon against five spoilage organisms from meat were studied. The MIC of the clove and cinnamon powder were from 1.0 to 1.5% (w/v) against *E. coli*, *S. aureus*, *Brochothrix thermosphacta* and *Lactobacillus rhamnosus* and the same against *P. aeruginosa* were 2.0 to 2.5% respectively. Generally, the composition, structure and functional groups of essential oils play an important role in determining their antimicrobial capacity. Compounds containing phenolic groups are responsible for these antimicrobial properties, although other compounds also present the same properties[20]. Killing kinetics showed that cinnamon and clove extracts took less time for complete killing of *E. coli* and *S. aureus* than black pepper. Spice essential oils are much more efficient in killing in shorter time than extracts. Clove essential oil is as effective as ampicillin and even more effective against *S. aureus* and *E. coli*. Eugenol and cinnamaldehyde was reported to have bactericidal action, the reduction in the number of CFU/ml in 1 hour to *Listeria monocytogenes* and *Lactobacillus sakei* was observed[21]. Membrane leakage studies showed that the cinnamon, clove extracts and essential oils were the most effective compounds in terms of inducing leakage of cellular constituents which has absorption at 260nm and 280nm followed by black pepper. Membrane leakage observed was high in *S. aureus*. Both cinnamon extract and essential oil induced gradual leakage of 260nm absorbing material followed by clove essential oil and reached maximum at 90minutes. Bacterial cells when treated with cinnamon extract and essential oil the leakage of 280nm absorbing material was sudden and very high at the initial stages only. While, clove essential oil also caused sudden leakage but its extract was slow. Black pepper has less effect on membrane leakage compared to cinnamon and clove (Graph III and IV). Membrane permeabilization is thought to provide access to the cytoplasm in addition to contributing to killing[22]. The possible action modes of the constituents of spices have been proposed[23, 24], although no specific mechanism has been confirmed. A researcher[25] claimed that effect of phenolic compounds is dose dependent. According to a study[26] synergistic activity suggests different modes of actions of the combining compounds. An investigators[27] reported in their study that the clove essential oil as well as the major components were capable of inducing cell lysis and electronic microscope observations revealed that both cell wall and cell membrane of the treated bacteria were significantly damaged which supports the present study. Results of the present study shows that these spices extract and essential oils affect the membrane integrity of bacteria resulting in the leakage of intracellular constituents which lead to cell death although the presence of additional mechanisms or targets cannot be ruled out.

CONCLUSION

The present study and earlier reports reveal that black pepper, cloves and cinnamon are effective antimicrobial agents. Thus, it supports the use of these spices in traditional medicine for the treatment of various bacterial infections. The phytochemicals acting as antimicrobials represent a vast untapped source of medicines and hence have enormous therapeutic potential. They could also help curb the problem of the multi-drug resistant bacteria. The investigation can be further extended for the development of commercial drug formulation.

CONFLICT OF INTEREST

Conflict of interest declared none.

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