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Synergistic effect of some spices in combination with antibiotics against drug resistant pathogenic microorganisms

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Abstract

Aim: The present work was to evaluate the possible *in vitro* interaction of ethyl acetate and methanolic extracts of *Piper nigrum* (*P. nigrum*) (Seed), *Syzygium aromaticum* (*S. aromaticum*) (Bud), and *Cinnamomum zeylanicum* (*C. zeylanicum*) (Bark) with norfloxacin and ciprofloxacin, known antimicrobial drugs. **Methods:** This synergy study of traditional spices was carried out against Gram positive drug resistant strains of *Staphylococcus epidermidis* (*S. epidermidis*), *Streptococcus pneumoniae (S. pneumoniae)* and *Bacillus cereus* (*B.cereus*) using agar well diffusion method. **Result and conclusion:** The extraction yield of *S. aromaticum* (3.40±1.2%) was highest among the methanol extract while among ethyl acetate, the best extractive yield with 5±1.8% was calculated in *P.nigrum*. The concentration dependent activity of the *S. aromaticum* was recorded with zone diameter of inhibition 17.9-19.6 mm while inhibition zone of *P.nigrum* and *C.verum* ranged from 10.9-21.5 mm and 10.7-13.1 mm respectively against the test bacteria. During synergism testing, extracts of *S. aromaticum*, *P. nigrum*, and *C. verum* in combination with used antibiotics had growth inhibitory indices (GIIs), 0.47-1.35, 0.53-1.18 and 0.54-1.29 against *S. epidermidis*, *S. pneumoniae* and *B.cereus* strains, respectively. Ethyl acetate extract of *P. nigrum* found to be a synergistic enhancer though it does not have any antimicrobial properties alone, but when taken concurrently with ciprofloxacin and norfloxacin, it enhanced the effect of these drugs. These results may indicate the possibility of concurrent use of *P.nigrum* extract to provide an array of safe antimicrobial agents to control infections caused by *S. epidermidis*, *S. pneumoniae* and *B. cereus* drug-resistant bacteria.

Keywords
Ethyl acetate, growth inhibitory indices, Methanol, *Piper nigrum*, Synergism.

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INTRODUCTION

Many human pathogens have evolved to become resistant to various currently available drugs causing considerable mortality and morbidity all over the world. The discovery of antibiotics has inevitably fostered the development and widespread emergence of bacterial resistance to a large number of antimicrobial agents poses major health problems because of difficulties in treatment [1]. Prudent use of antibiotics in human's demands that physicians establish a bacterial infection responsible for the patient's symptoms before the prescription of antibiotics. The wide use of antibiotics for the treatment of bacterial infections lead to the emergence and spread of drug resistant strains. According to WHO [2], medicinal plants administration to man or animals exert a sort of pharmacological action on them. Herbs make up most of the plant sources for the production of useful drugs that are being utilized by people globally [3]. Most existing plants that have medicinal values used for scientific research to properly test and utilize these plants for therapeutic purposes. Hemaiswarya *et al*., 2008 [4] reported that the plants-derived antimicrobials are less potent and enhances the need to adopt a synergistic interaction between its bioactive compounds to fight against infections. Crude extracts or phytochemicals when combined with antimicrobial agents, efficacy of the antimicrobial agents increased and act as an alternative to fight with infections caused by multidrug resistant (MDR) microorganisms [5]. Indian intellect has classified the plant kingdom into convenient classes, and identified the medicinal use of the trees to the mankind. It was reported by various researchers that some of the herbs and spices which are used by humans to season foods, could yield useful medicinal compounds [6,7,8]. The use of plant-derived natural products in medical treatments is attracting more attention due to its potential efficacy and no side effects [6]. Traditional medical practitioners (TMPs) play a pivotal role in providing healthcare need in underdeveloped countries and rural population all over the world. This study was aimed to ascertain whether spices available in local markets that are prescribed in indigenous system of medicine have any antimicrobial activity against potential human bacterial pathogens that have developed resistance to conventional antibiotics.

MATERIAL AND METHODS

Processing of plant material: All collected spices were purchased from local market of Dehradun, Uttarakhand. These spices include *P. nigrum*, *C. zeylanicum* and *S. aromaticum*. The powder of each spice given in **table 1** was kept in an air tight container for further use.

Table 1: Spices used for the study

S.No.	Botanical name	Common name	Family	Part used
1.	*Piper nigrum* L.	Black pepper	Piperaceae	Seeds
2.	*Cinnamomum zeylanicum* Nees	Cinnamon	Lauraceae	Bark
3.	*Syzygium aromaticum* L.	Clove	Myrtaceae	Flower Bud

Preparation of organic solvent extracts:
Shade-dried powder (10 g) was thoroughly mixed with 100 ml organic solvents (ethyl acetate and 80% methanol) in a conical flask. The mixture was placed at room temperature on shaker with 200 rpm and after 48 hrs, mixture was filtered through a sterilized Whatman No. 1 filter paper. The plant residue was re-extracted with the addition of solvents, and after 24 hrs, it was filtered again to get final concentrated slurry. The combined filtrate thus obtained was concentrated by complete evaporation of solvent at 40 °C on a water bath to yield the crude extract [9]. The dried extract, thus obtained was sterilized by UV-irradiation, checked for sterility on nutrient agar plates and stored at 4 °C in a refrigerator for further use [10]. The dry weight of extracts obtained by the solvent evaporation was used to determine the concentration in mg ml^{-1} [11].

Stock solutions of crude extracts for organic solvent were prepared by mixing well the appropriate amount of dried extracts with 5% Dimethyl sulfoxide (DMSO) solvent to obtain a final concentration of 100 mg ml^{-1}.

Determination of the extraction yield
The extraction yield is a measure of the solvent efficiency to extract specific phytocompounds from the plant material. The extraction yield was calculated according to the method of Zhang *et al*. [12]. Briefly, the dried extracts were weighed, and then the extraction yield was calculated as the percentage of the weight of the crude extract to the powder of raw material.

$$\textbf{Extraction yield (\%)} = \frac{\textbf{Weight of the dried extract x 100}}{\textbf{Weight of the plant sample}}$$

Antimicrobial agents: Fluoroquinolones including Norfloxacin (SRL) and Ciprofloxacin (Himedia) were used after diluting it to a final concentration of 10 µg/ml and 5 µg/ml respectively.

Microorganisms: MTCC strains of *S. epidermidis, S. pneumonia* and *B. cereus* were used as test organisms. All the strains were maintained in microbiology laboratory of UCST college, Dehradun. Their morphological, cultural and biochemical characteristics were confirmed by the standard methods [13].

Antibacterial activity of plant extract

The antibacterial activities in methanolic and ethyl acetate extract of *S. aromaticum*, *P. nigrum* and *C. verum* were determined by agar well diffusion method. In this process, sterile Mueller Hinton agar (MHA) plates were swabbed with young broth culture of the test bacteria and dried. Plates were punched for four wells each of 6 mm diameter using sterile aluminium borer. Each extract of spice used at 25, 50, and 100 mg/ml concentration. ZDI were recorded and interpreted according to the CLSI criteria [14] for resistance and sensitivity of the pathogens.

Combined antibacterial activity of plant extract and Antibiotic: MHA plates were swabbed with a 24 hr old bacterial culture for even growth and inoculums was adjusted to an optical density of 0.8 at 595 nm wavelength. The plates were kept at room temperature for 15 minutes to allow any surface moisture to be absorbed before the action of antimicrobial agents. Four wells (6 millimeter diameter) were punched at 24 millimeters apart in the agar plates. The wells were filled with 100 µl of spice extract and antibiotic alone as well as mixture of 100 µl of spice extract and antibiotics for evaluation of synergistic activity. Dimethyl sulfoxide (DMSO) was used as the negative control in the remaining well. After incubation at 37^{0}C for 24 hrs, the plates were observed for the synergistic activity, and ZDI for each extract was measured in terms of a millimeter (mm). The synergistic effect of the spice extract was considered when combination exhibited with enlargement of combined inhibition zone size by at least 4 mm.

Growth inhibitory index

The growth inhibitory indices (GIIs) were calculated following the formula:

$$\textbf{GIIs} = \frac{\textbf{Zone of inhibition in combination (antibiotic + spice extract)}}{\textbf{Total of zone of inhibition alone}}$$

in order to corroborate the synergistic activity of the antibiotics in combination with the spice extract. The synergistic, additive or antagonistic activities, if any, in between the extract and antibiotics were defined with GIIs >0.5, 0.5 and <0.5, respectively [15]

RESULTS

Extraction yield: The extraction yield of *S. aromaticum* (3.40±1.2%) was highest among the methanol extract while among ethyl acetate, *P.nigrum* showed the best extractive yield with 5±1.8% (**Table2**).

Table 2: Extraction yield (%) of methanolic and ethyl acetate extracts of different spices (n=3)

S.No.	Botanical name	Extraction yield (%)	
		Methanol	Ethyl acetate
1.	*Piper nigrum*	2.33±0.8%	5±1.8%
2.	*Syzygium aromaticum*	3.40±1.2%	2.36 ±0.9%
3.	*Cinnamomum verum*	3.33±1.9%	3.43±1.2%

***Data are given as mean ± S.D. from three replicates in each extract**

The antibacterial activity of the plant extracts is shown in **Table 3**. Both extracts (Methanol and ethyl acetate) of used spices were tested at different concentrations (25, 50, and 100 mg/ml), against *B. cereus*, *S. epidermidis* and *S. pneumonia*. The *S.aromaticum* displayed ZDIs of 17.9-18.6, 18.4-18.8, and 19.4-19.6 mm against *B. cereus*, *S. epidermidis* and *S. pneumonia* respectively. *P. nigrum* showed antibacterial activity with ZDIs 10.9-20.7, 11.2-21.2, and 11.2-21.5 mm. *C. verum* exhibited ZDIs 12.5 mm for *B. cereus*, 11.2-11.3 mm for *S. epidermidis* and 10.7-12.8 mm for *S. pneumoniae* strain.

Table 3. Antibacterial activity of spice extract against Gram positive pathogenic bacteria

Pathogens	Type of Extract	Zone Diameter of inhibition (mm)								
		S.aromaticum			*P.nigrum*			*C.verum*		
		25mg	50mg	100mg	25mg	50mg	100mg	25mg	50mg	100mg
B.cereus	M	18.6	18.8	19.4	20.7	21.2	21.5	12.5	12.8	13.1
	EA	-	-	-	-	-	-	-	-	-
S.epidermidis	M	-	-	-	10.9	11.1	11.2	-	-	-
	EA	-	-	-	-	-	-	11.2	11.3	11.3
S.pneumoniae	M	17.9	18.4	19.6	12.3	12.9	13.1	10.7	11.1	11.2
	EA	-	-	-	-	-	-	12.6	12.7	12.8

M= Methanol; EA= Ethyl acetate; (-) = No zone of inhibition

Evaluation of synergistic activity of spices in combination with Ciprofloxacin

Methanolic and ethyl acetate extracts of spices when tested individually for their antibacterial activity showed various degree of activity. When used in combination with used spices, it rendered sensitivity to the bacterial strains and growth inhibitory indices (GIIs) of combined action are represented in **Table 4**.

Table 4. Synergistic effect between spice extracts (Methanolic and Ethyl acetate) with Ciprofloxacin (Average ± S D) by well diffusion method.

Spice (part used	Type of extract	*S. epidermidis*				*S. pneumoniae*				*B. cereus*			
		P	*A*	P+A	GIIs	*P*	*A*	*P+A*	GIIs	*P*	A	P+A	GIIs
S. aromaticum	**M**	-	12.5 ±0.8	12.9 ±0.9	**1.03**	11.1 ±0.3	17.3 ±0.5	18.9 ±0.8	**0.67**	19.5 ±0.2	16.3 ±0.4	17.4 ±0.4	**0.49**
	EA	-	16.5 ±0.3	22.3 ±0.6	**1.35**	13.1 ±0.3	20.5 ±0.2	18.7 ±0.5	**0.56**	22.1 ±0.4	18.3± 0.1	18.8 ±0.5	**0.47**
P.nigrum	**M**	11.2 ±0.2	17.8 ±0.5	18.4 ±0.2	**0.63**	-	21.6 ±0.3	21.7 ±0.6	**1.00**	-	19.2± 0.3	21.3 ±0.6	**1.10**
	EA	-	18.2 ±0.6	19.4 ±0.3	**1.07**	-	20.4 ±0.4	21.9 ±0.3	**1.08**	-	21.3± 0.2	23.1 ±0.5	**1.08**
C.verum	**M**	-	19.3 ±0.3	20.5 ±0.5	**1.06**	12.5 ±0.3	21.7 ±0.3	23.6 ±0.4	**0.70**	13.1 ±0.3	21.6± 0.5	23.9 ±0.8	**0.69**
	EA	11.3 ±0.3	21.6 ±0.7	22.8 ±0.9	**0.69**	24.6 ±0.1	25.8 ±0.4	27.6 ±0.7	**0.54**	-	14.7± 0.3	18.9 ±0.5	**1.29**

A=ciprofloxacin; (-) = No activity; P= Spice extract; P+A=Spice extract + ciprofloxacin; DMSO= Dimethyl sulfoxide; GIIs = Growth inhibitory indices

Evaluation of synergistic activity of spices in combination with Norfloxacin

Methanolic and ethyl acetate extracts of spices when tested individually for their antibacterial activity showed various degree of activity. When used in combination with used spices, it rendered sensitivity to the bacterial strains and growth inhibitory indices (GIIs) of combined action are represented in **Table-5**. GIIs of 1.04 value was maximum in *C.verum* against *S. epidermidis* while minimum GIIs was calculated in *S. aromaticum* against *S. pneumoniae* and *B. cereus*

Table 5. Synergistic effect between plants extracts (Methanolic and Ethyl acetate) with Norfloxacin (Average ± S D) by well diffusion method.

Spice (part used)	Type of extract	*S. epidermidis*				*S. pneumoniae*				*B. cereus*			
		P	*A*	P+A	GIIs	*P*	*A*	*P+A*	GIIs	*P*	A	P+A	GIIs
S. aromaticum	**M**	-	24.3± 0.5	22.1± 0.9	**0.91**	19.6± 0.2	19.3± 0.5	19.8± 0.7	**0.51**	19.4± 0.4	19.2± 0.8	19.5± 0.7	**0.51**
	EA	-	23.2± 0.5	24.8± 0.8	**1.07**	-	20.3± 0.4	24.1± 0.7	**1.19**	22.1± 0.4	21.6± 0.5	19.5± 0.4	**0.45**

P.nigrum	**M**	11.2±0.2	24.5±0.6	26.2±0.3	**0.73**	13.1±0.3	22.6±0.3	21.9±0.8	**0.61**	21.5±0.3	23.2±0.5	24.1±0.9	**0.53**
	EA	-	25.1±0.4	25.8±0.8	**1.02**	-	20.3±0.7	23.9±0.6	**1.18**	-	21.5±0.4	22.7±0.9	**1.06**
C.verum	**M**	-	23.7±0.3	24.8±0.6	**1.04**	11.2±0.6	19.5±0.4	20.6±0.7	**0.68**	13.1±0.3	20.1±0.8	21.7±0.4	**0.65**
	EA	11.3±0.3	22.6±0.5	23.8±0.6	**0.70**	12.8±0.4	21.9±0.3	22.8±0.9	**0.66**	-	22.3±0.3	24.4±0.7	**1.10**

A=Norfloxacin; (-) = No activity; P= Spice extract; P+A=Spice extract+Norfloxacin; DMSO= Dimethyl sulfoxide; GIIs = Growth inhibitory indices

DISCUSSION

Epidemiological evidence supports the concern regarding the emergence of Gram-negative bacteria and their role in serious healthcare-associated infections due to antibiotic resistance. Spices extracts are generally a mixture of active and non-active compounds. In the present study, the amount of phytochemical extracted was higher with ethyl acetate followed by methanol in two of the cases. The extractive yield in ethyl acetate extracts varied from 2.36±0.9 to 5±1.8%, while in methanolic extracts the range varies from 2.33±0.8 to 3.40±1.2%. In the present investigation, extractive yield of *Syzygium aromaticum* (3.40±1.2%) was highest among the methanol extract while among ethyl acetate extracts, extractive yield was best in *P. nigrum* (5±1.8%) (**Table 2**). The yield of the extract depends on the characteristics of the solvent and spice material used [16]. Generally, the variation in extraction yield among the different spices might be due to the different availability of extractable components, resulting from the various chemical compositions of spices [9].

In this study, three commonly available spices used by traditionally were tested against three different bacteria (*S.epidermidis, S.pneumoniae, B.cereus*). The result of synergistic activity testing showed that are susceptible to ciprofloxacin with diameter zone of inhibition from 12.5±0.8 mm to 25.8±0.4 mm against test bacterial strains. In combination with ethyl acetate extract of *S.aromaticum,* highest GII value 1.35 was calculated while methanolic extract of *P.nigrum* showed lowest GII value 0.63 with zone of inhibition of 18.4±0.3 mm against *S. epidermidis.* Ethyl acetate extract of *P. nigrum* and *C.verum* showed zone of inhibition of 21.9±0.3mm and 27.6±0.7 mm having GII value 1.08 and 0.54 respectively against *S. pneumoniae* strain.

The result of synergistic activity testing showed that are susceptible to Norfloxacin with diameter zone of inhibition from 19.2±0.8 mm to 25.1±0.4 mm against test bacterial strains. In combination with ethyl acetate of *S.aromaticum*, norfloxacin showed highest synergistic activity with zone of inhibition of 24.1±0.7 mm against *S. pneumoniae* while lowest synergism was found against *B. cereus* with 19.5±0.4 mm of inhibition zone. This implies that these spice extracts increased the antibacterial activity of the antibiotics against the test strains and showed interaction.

Medicinal plants are important elements of indigenous medical systems in India where different parts of various medicinal plants are used to cure specific ailment and interest in medicinal plants has revived as a consequence of current problems associated with the use of antibiotics [17, 18]. Bacterial resistance can be restored by synergistic action of antibiotics and bioactive plant extracts which is a novel concept and could be beneficial [19]. In the present study, Methanolic and ethyl acetate extracts of 3 species were screened for antibacterial and synergistic activity with two fluoroquinolones (Ciprofloxacin and Norfloxacin) against *B. cereus*, *S. epidermidis* and *S. pneumoniae*. Among the three extracts, Ethyl acetate extract of *P.nigrum* was found effective against all bacterial strains while Zarai *et al*., [20] found that the ethanol extract was the most effective in his study. All spice extracts exhibited synergistic activity but the results obtained were the variable in all the three test strains and the drug.

In this study ethyl acetate extract of *P. nigrum* have been found to be synergistic enhancer though it does not have any antimicrobial properties alone, but when taken concurrently with standard fluoroquinolones it enhances the effect of that drug. *S. aromaticum* showed antagonism against bacterial strain *B. cereus* while all other spice extracts showed synergism with the fluoroquinolones (Ciprofloxcin and Norfloxacin) against all bacterial strains. Various other authors have also reported the antimicrobial and synergistic activity of most of the plant (*Allium sativum, Baccharis trimera, Cymbopogon citrates, Zingiber officinale, Laurus nobilis, Majorana syriaca, Mentha piperita, Mikania glomerata, Ocimum basilicum, Psidium guajava, Rosa damascene, Rosamarinus officinalis, Rubia cordifolia, Salvia fruticosa and Syzygium aromaticum)* extracts against bacterial strains [21, 22, 23, 24, 25, 26].

Ali *et al*., 2011 [27] tested that aqueous extract of *S. aromaticum* showed antibacterial activities for *S. aureus*, *S. epidermidis*, *S. pyogenes*, *S. Typhi*, *A. calcoaceticus*, and *P. aeruginosa*. *C. verum* oil possessed stronger antimicrobial activities than used extracts [28]. The spices used in the present study might be potential source of non-antibiotic drugs that might potentially improve the performance of fluoroquinolones against infections by inhibiting the efflux. The bioactive compounds can be isolated from synergistic enhancer for use in future as adjuvants with antibiotics.

CONCLUSION:

This study has shown that ethyl acetate extract of *P.nigrum* exhibits potentials of synergy in combination with selected antibiotics against used strains, often presenting with problems of drug resistance. Moreover, this study established a good base for developing future efflux pump inhibitors from *P.nigrum* as adjuvant of antibiotics.

From this study, we have concluded that with the passage of time the antimicrobial therapy is drifting towards drug treatments due to microbial resistance developed because of already existing antibiotics. Therefore, there is a great need for the development of more innovative alternatives to deal with these stubborn microbes. Using combinations of traditional spices with the existing drugs can serve as a powerful tool in the modern world's drug resistance. This can be useful if this combination has synergestic effects on the drug resistant microbes. Some of these commonly used antibiotics which don't render any sensitivity to the pathogenic strains were found be highly active when combined with used traditional spices. Therefore, our study is a little step in this regard in search of an alternative.

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