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Bacteriostatic and fungistatic activities of *Oreganum vulgare* extract and volatile oil and interaction studies in combination with antibiotics and antifungal agents against food poisoning pathogens

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Abstract

The use of food preservatives to prevent spoilage of the product during transportation and shelf life by food manufacturers is common. Artificial preservatives added may prevent the food but they may be carcinogenic and may harm the consumer's health. In food producing animals also, due to misuse of antimicrobials, antibiotic resistance have been developed which is affecting food industry. The present study was done to find out *Oreganum vulgare*, the most common food herb's antimicrobial potential against food poisoning organisms. The volatile oil was analysed by GC-MS and chloroform extract was fractionated into phenolic and non-phenolic part. The fractions and volatile oil were used alone and in combination with standard antimicrobials to evaluate the interaction effect. Volatile oil consisted of mainly Carvacrol (86.5%), Þ-cymene (7.2%) followed by bornyl acetate. The Minimum Inhibitory Concentration (MIC) was found to be lowest for volatile oil followed by phenolic fraction when used alone and both in combination against *Shigella flexneri*, *Aspergillus flavus* and *Salmonella enterica* ser. Typhi. Synergism was shown by volatile oil with a FICI of 0.265, 0.187, 0.280 when combined with ciprofloxacin and fluconazole respectively against *Shigella flexneri*, *Salmonella enterica* ser. Typhi and *Aspergillus flavus*. Obtained data suggest the potential use of volatile oil and phenolic fraction of chloroform extract as along with standard antimicrobials as more effective combination with lesser side effects. The demonstrated antimicrobial activity of phenolic fraction and volatile oil when used alone suggests their use in food industry as preservatives without any toxicity. The interaction studies data with standard antimicrobials indicates the use of these combinations to infected food producing animals and humans; hence may solve the problem of antibiotic resistance in future.

Introduction

Food-borne disease is defined as "A disease, usually either infectious or toxic in nature, caused by agents that enter the body through the ingestion of food". The main causes of food-borne intoxication are toxins produced by some bacteria like botulism toxin, fungal toxins like fusarium toxin, Aflatoxin, algal toxins or food borne chemical poisoning. Food poisoning is having symptoms like intestinal flu which are abdominal cramps, diarrhea which may or may not be bloody, vomiting, fever and dehydration generally. More than eighty one million people are affected by food-borne diseases every year. Bacterial toxins that produce intoxications are the exotoxin types of either enterotoxin (affecting the gut) as in staphylococcal intoxication or neurotoxin (affecting the nervous system) as in botulism. Shigellosis is caused by members of *Shigella flexneri* species which may lead to clinical symptoms like mild watery diarrhea in low infectious dose to dysentery in high infectious dose. High protein foods like eggs, poultry foods are often contaminated with Salmonella, the gram negative rods and causes Salmonellosis (Ramnathan, 2010).

Not only bacterial toxins but also mycotoxins are source of food spoilage. Aflatoxins are produced by almost 50 species of genus *Aspergillus* mainly *Aspergillus flavus* which also produces cyclopiazonic acid, the causative agent of necrosis in digestive tract and neurological disorders (Ramnathan, 2010). The development and spread of resistance to currently available antibiotics is a worldwide concern (Chanda and Rakholiya, 2011). Antibiotics that work today may not work tomorrow. So it is the major concern to develop newer drugs with lesser resistance (Sarkar *et al*., 2003). Antibiotics obtained from herbal origin

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are having fewer side effects than that of synthetic ones and are having great therapeutic potential to heal infections (Chanda *et al*., 2010; Habbal *et al*., 2011). Sometimes single antibiotic is not effective, hence a chemically compatible combination each having antibiotic effect may give the desired effect, may be by complex formation which is more effective in inhibition of micro-organism either by causing lyses of cell wall or by inhibiting its formation (Chanda and Rakholiya, 2011). So the present aim for this study is the comparison of effectiveness of newer herbal and synthetic antibiotic combination with that of synthetic antibiotic already in use clinically which will open doors for discovery of potential newer antibiotics with less resistance and side effects.

Materials and Methods

Plant material

The freeze dried leaves of *Oreganum vulgare* Linn. were procured from Aum Agreefresh Pvt. Ltd., Vadodara, Gujarat and were identified by the same company. The voucher specimen (Pcog1101) was deposited in Department of Pharmaceutical sciences, Guru Jambheshwar University of Science and Technology for future references.

Bacterial strains and antibiotics

The microorganisms used for antimicrobial studies of volatile oil and extract were procured from Microbial Type Culture Collection (MTCC), Institute of Microbial Technology, Chandigarh. The bacterial strains used were *Shigella flexneri* MTCC 1457, *Salmonella enterica* ser. Typhi MTCC 733 and fungal strain used was *Aspergillus flavus* MTCC 873. The media used for the growth and maintenance of microorganisms were nutrient agar (NA), for bacteria, potato dextrose agar (PDA) for fungi (Hi-media, Mumbai, India). The organic solvents used for extraction and fractionation of plant metabolites were of analytical grade.

Preparation of Plant Materials

500 g of drug was placed in a closed flask with chloroform and after 24 h, filtered and concentrated in rotary vacuum to yield 12.5 g of paste like extract. In order to separate the phenolic from non-phenolic fraction of the chloroform extract, a liquid-liquid extraction was done. In a separating funnel, 2 g of the extract was diluted in 40 ml of chloroform and washed three times with 120 ml of 0.1N sodium hydroxide. The chloroform phase was separated and was concentrated to obtain the crude non- phenolic fraction. To further purify this fraction, 0.3 g of it were diluted in ethanol and centrifuged at 3600×g at 10°C for 15 min. Ethanol was concentrated from the supernatant to obtain purified non-phenolic fraction. The basic aqueous phase was acidified with 6N HCl to pH 3.0 and 40 ml of chloroform was added to extract the phenolic fraction. The phenolic fraction was dissolved in chloroform and separated by preparative thin layer chromatography (TLC) on silica gel-G eluting with benzene-methanol 95:5 (Raul *et al.*, 2010).

Extraction of volatile oil

Volatile oil was extracted from freeze dried leaves (1000 g) by hydro-distillation method by using clevenger's apparatus. The yellowish oil (16.6 ml, yield=1.66% v/w) obtained was separated from aqueous phase and dried over anhydrous sodium sulphate and stored at 4°C until used.

GC-MS analysis of Volatile oil

The oil sample was diluted with hexane in ratio of 1:100 and used for the further analysis. The quantitative analysis was done with the help of chromatographer in gas phase (Agilent 7890A GC system) equipped with MS detector (5975C inert XL EI/CI MSD), HP-5MS capillary column (Agilent 19091S-433: 1548.52849 HP-5MS 5% Phenyl Methyl Silox) having dimensions 30 m x 250 µm x 0.25 µm. The column temperature was programmed from initial 80°C upto 300°C. The temperature of the injector was fixed to 270°C. The debit of gas (helium) vector was fixed to 1 ml/min and split injection with split ratio 50:1. The volume of injected sample was 2 µL of diluted oil in hexane (10%). The components were identified based on comparison of their relative retention time and mass spectra with those of standards, W9N08.L library data of the GC-MS system and literature data.

Minimum Inhibitory Concentration (MIC) determination and comparison of MIC determination by spectrophotometric and visual methods and growth curve

MIC was determined by modified method as described by Kaya and Ozbilge (2012). The concentration of stock solutions of phenolic and non-phenolic fractions were 10 mg/ml, and that of ciprofloxacin and fluconazole were 0.25 mg/ml in DMSO respectively for bacterial and fungal strains. 0.5 ml of phenolic and non-phenolic fractions and volatile oil were mixed with 0.5 ml of ciprofloxacin respectively. MIC of phenolic, non-phenolic fraction, volatile oil and ciprofloxacin was determined using two fold serial dilution method. For determination of interaction effect of phenolic, non-phenolic fractions and volatile oil, 0.5 ml of respective test sample were

mixed with 0.5 ml of ciprofloxacin stock solution and 0.5 ml of fluconazole for bacterial and fungal strains respectively. MIC was determined using two fold serial dilution method. Tubes containing only bacterial suspensions and nutrient broth were used as positive control and negative control were the tubes with only nutrient broth.

Optical Densities (ODs) were measured for at 35°C using Thermo Scientific 2000/2000 C nanodrop spectrophotometer at 405 nm. OD of each replicate at before incubation (T0) was subtracted from OD after incubation at 37°C (T24) for bacterial cultures and at room temperature for fungal strains respectively. The adjusted OD of each control tube was then assigned a value of 100% growth. The percent inhibition of growth was thus determined using the formula: Percent Inhibition = 1- (OD of tube containing test solution/OD of corresponding control tube) × 100.

The MIC is reported as the lowest concentration of test material which results in 100% inhibition of growth of the test organism. Visual MIC was determined by noting down the concentration of that first tube in which there is no appearance of turbidity after incubation of 24 h and it was compared with that of MIC determined by spectrophotometric method.

Fractional inhibitory concentration (FIC) index determination

The FIC index (FICI) was calculated by dividing the MIC of the combination of phenolic fraction, non-phenolic fraction, volatile oil and reference antibiotic respectively (Saad *et al*., 2010).

FIC of vol. oil = MIC of vol. oil in combination with antibiotic drug/ MIC of vol. oil

FIC of Phenolic Fraction = MIC of Phenolic Fraction in combination with antibiotic drug/ MIC of Phenolic Fraction

FIC of Non-Phenolic Fraction = MIC of Non-Phenolic Fraction in combination with antibiotic drug/ MIC of Non-Phenolic Fraction

FIC of antibiotic drug = MIC of antibiotic drug with particular fraction/ MIC of drug

FICI (Vol. Oil) = FIC of Vol. oil + FIC of antibiotic drug

FICI (Phenolic Fraction) = FIC of Phenolic Fraction + FIC of antibiotic drug

FICI (Non-Phenolic Fraction) = FIC of Non-Phenolic Fraction + FIC of antibiotic drug

Results and Discussion

GC-MS analysis of volatile oil indicates that total 35 compounds were characterized and quantified. The major component of volatile oil *Oreganum vulgare* is Carvacrol (86.5%), followed by Þ-cymene (7.2%), γ-Terpinene (0.642%), 3-Cyclohexen-1-ol (0.565%), δ-Cadinene (0.421%), β- Bisabolene (0.400%). Non-phenolic fraction shows MIC at 0.07800, 1.25 and 1.25 mg/ml against *Shigella flexneri*, *Salmonella enterica* ser. Typhi and *Aspergillus flavus* respectively as compared to MIC of standard drug ciprofloxacin at 0.03900 mg/ml and 0.01953 mg/ml respectively against *Shigella flexneri* and *Salmonella enterica* ser. Typhi and that of fluconazole at 0.156 mg/ml against *Aspergillus flavus*. MIC of phenolic fraction and volatile oil were found to be 0.03900, 0.00244 mg/ml respectively against *Shigella flexneri*, 0.312, 0.00970 mg/ml respectively against *Salmonella enterica* ser. Typhi, 0.312 and 0.01953 mg/ml respectively against *Aspergillus flavus* while MIC for the combination of non-phenolic fraction and standard (ciprofloxacin for bacterial strains and fluconazole for fungal strain) was observed at 0.07800, 0.312, 0.625 mg/ml against *Shigella flexneri*, *Salmonella enterica* ser. Typhi and *Aspergillus flavus* respectively and that of MIC exhibited by phenolic fraction and standard drug at concentration of 0.01953, 0.00488 and 0.07800 mg/ml respectively against *Shigella flexneri*, *Salmonella enterica* ser. Typhi and *Aspergillus flavus*. The interaction studies of volatile oil and standard were interesting as the new MIC lowered down to 0.00061, 0.00122 and 0.00488 mg/ml respectively against *Shigella flexneri*, *Salmonella enterica* ser. Typhi and *Aspergillus flavus*. The antimicrobial activity of volatile oil is due to terpenenes or terpenoids which are active against bacteria (Ahmed *et al*., 1993; Amaral *et al*., 1998) and fungi (Harrigan *et al.*, 1993; Ayafor *et al.*, 1994). The mechanism of action of terpenes is not fully understood but is speculated to involve membrane disruption by the lipophilic compounds (Coe, 1994). It has been hypothesized that phenolic compounds play a role of phytoanticipins in plants (Van *et al.*, 1994).

Fractional Inhibitory Concentration Index

FIC index was calculated to describe standard drugs ciprofloxacin and fluconazole interactions with volatile oil, phenolic and non-phenolic fractions respectively for bacterial and fungal strains. Synergy is defined as an FIC index of ≤ 0.5. Indifference was defined as an FIC index of ≥ 0.5 but of ≤ 4.0.

Table 1. MIC of volatile oil, phenolic, non-phenolic fractions of chlorofosrm extract alone and in combination with ciprofloxacin against *Shigella flexneri* and *Salmonella enterica* ser. Typhi by microdilution method

	S. flexneri							*Salmonella enterica ser.* Typhi						
Conc. mg/ml	NP % in	NP+S % in	P % in	P+S % in	O % in	O+S % in	S % in	NP % in	NP+S % in	P % in	P+S % in	O % in	O+S % in	S % in
0.00015	15.2	19.9	24.3	34.8	66.5	87.1	23.7	5.1	16.3	9.6	58.1	44.6	77.4	35.6
0.00030	20.5	29	31.9	44.8	75.9	94.4	31.1	12.2	22.8	18.6	61.4	58.5	88.4	40.5
0.00061	29.6	36.3	43.6	54.2	85.6	100	43.6	15.1	38.9	20.9	72.9	67.8	91.3	51.7
0.00122	33.4	42.5	52.7	65.1	93.8	>100	50.1	22.1	43.08	32.4	83.2	74.2	100	63.0
0.00244	41.6	52.1	63.3	74.4	100	>100	63.3	35.6	46.9	46.3	90.3	86.4	>100	71.1
0.00488	50.1	63.9	73	83.5	>100	>100	73.6	47.9	50.8	56.5	100	91.9	>100	78.1
0.00970	61.2	71.5	81.8	92.3	>100	>100	82.6	53.3	56.5	63	>100	100	>100	90.3
0.01953	70.6	85.3	91.4	100	>100	>100	93.8	59.1	63.3	74.9	>100	>100	>100	100
0.03900	90.9	92.1	100	>100	>100	>100	100	64.6	74.9	78.4	>100	>100	>100	>100
0.07800	100	100	>100	>100	>100	>100	>100	71.3	84.8	88.4	>100	>100	>100	>100
0.15600	>100	>100	>100	>100	>100	>100	>100	79.4	90.6	91.3	>100	>100	>100	>100
0.31200	>100	>100	>100	>100	>100	>100	>100	84.2	100	100	>100	>100	>100	>100
0.62500	>100	>100	>100	>100	>100	>100	-	90.6	>100	>100	>100	>100	>100	>100
1.25000	>100	>100	>100	>100	-	-	-	100	>100	>100	>100	-	-	-
2.50000	>100	>100	>100	>100	-	-	-	>100	>100	>100	>100	-	-	-
5.00000	>100	>100	>100	>100	-	-	-	>100	>100	>100	>100	-	-	-
10.0000	>100	-	>100	-	-	-	-	>100	-	>100	-	-	-	-

1. NP= non-phenolic fraction, 2. P= phenolic fraction, 3. O= volatile oil, 4. S= standard drug ciprofloxacin, 5. % in= inhibition

Table 2. MIC of volatile oil, phenolic, non-phenolic fractions of chloroform extract alone and in combination with fluconazole against *Aspergillus flavus* by microdilution method

	Aspergillus flavus						
Conc. (mg/ml)	NP % in	NP+S % in	P % in	P+S % in	O % in	O+S % in	S % in
0.00015	7.2	23.1	15.9	17.3	33.3	54.4	11.5
0.00030	10.1	34.7	21.7	25.1	47.2	69.5	22.6
0.00061	13.0	43.4	30.4	36.2	56.5	78.2	32.7
0.00122	20.2	52.1	42	44.9	66.1	84.6	44.3
0.00244	31.8	53.6	55	57.9	75.9	98.5	57.3
0.00488	40.5	65.2	66.6	68.1	81.7	100	60.8
0.00970	50.7	67.5	72.4	79.7	97.1	>100	77.6
0.01953	62.3	73.3	76.8	86.9	100	>100	88.4
0.03900	71.0	75.3	85.5	95.6	>100	>100	89.8
0.07800	73.9	82.6	87.8	100	>100	>100	97.9
0.15600	81.1	86.9	92.7	>100	>100	>100	100
0.31200	84.1	94.2	100	>100	>100	>100	>100
0.62500	91.3	100	>100	>100	>100	>100	-
1.25000	100	>100	>100	>100	-	-	-
2.50000	>100	>100	>100	>100	-	-	-
5.00000	>100	>100	>100	>100	-	-	-
10.0000	>100	-	>100	-	-	-	-

1. NP= non-phenolic fraction, 2. P= phenolic fraction, 3. O= volatile oil, 4. S= standard drug ciprofloxacin, 5. % in= inhibition

Antagonism was defined as an FIC index of >4.0 (Agrawal *et al*., 2007). Synergism was shown by volatile oil with a FICI of 0.265, 0.187, 0.280 when combined with ciprofloxacin and fluconazole respectively against *Shigella flexneri*, *Salmonella enterica* ser. Typhi and *Aspergillus flavus* and by the phenolic fraction with FICI of 0.264 in combination with ciprofloxacin against *Salmonella enterica* ser. Typhi. Indifference was exhibited by phenolic fraction with FICI of 1.000 and 0.750 respectively against *Shigella flexneri* and *Aspergillus flavus* while antagonism was shown by non-phenolic fraction with FICI of 16.224 and 4.506 respectively against *Salmonella enterica* ser. Typhi and *Aspergillus flavus* upon addition of ciprofloxacin and fluconazole respectively. The antifungal activity of polyphenolic compounds might be due to formation of multinucleate stage by breakage of intersepta in mycelium and cell surface damage by pilferage (Bais *et al*., 2002).

This study has demonstrated for the first time the interaction study of the volatile oil, phenolic and non-phenolic fraction of chloroform extract of *Oreganum vulgare* and standard drugs to inhibit food poisoning bacteria and fungal strains effectively. The results show that volatile oil alone and in combination with standard drugs is very effective against bacteria

Table 3. FIC determination of volatile oil, phenolic, non-phenolic fractions of chloroform extract and standard antibiotic/antifungal drug and FICI determination

Strain	FIC		FIC		FIC		FICI		
	O	S	P	S	NP	S	O	P	NP
Shigella flexneri	0.250	0.015	0.500	0.500	1.000	2.000	0.265	1.000	3.000
Salmonella enterica ser. typhi	0.125	0.062	0.015	0.249	0.249	15.975	0.187	0.264	16.224
Aspergillus flavus	0.249	0.031	0.250	0.500	0.500	4.006	0.280	0.750	4.506

1. O= volatile oil, 2. P= phenolic fraction, 3. NP= non-phenolic fraction, 4. S= standard (ciprofloxacin for bacterial strain and fluconazole for fungal strain)

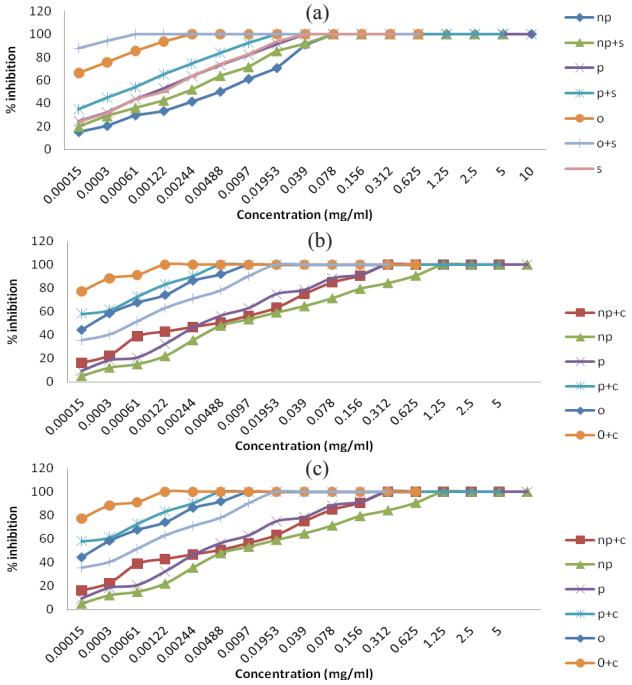

Figure 1. Growth curve (% inhibition against concentration in mg/ml) of (a) *Shigella flexneri* (b) *Salmonella enterica* ser. Typhi (c) *Aspergillus flavus* in presence of phenolic, non-phenolic fractions and volatile oil alone and in combination with ciprofloxacin and fluconazole

as well as fungus. The phenolic fraction was also found to be effective alone and in combination but the concentration is higher than volatile oil at which it inhibits microbes. Hence, both phenolic fraction and volatile oil may act as promising antimicrobial agent used by food industry with lesser side effects as compared to synthetic antimicrobial agents.

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