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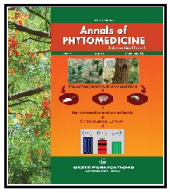

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Evaluation of the *in vitro* antibacterial activity and minimum inhibitory concentration of *Curcuma longa* L., *Ocimum sanctum* L. and *Piper nigrum* L. ethanolic and aqueous extracts

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Abstract

The study was conducted to evaluate the *in vitro* antibacterial activity and minimum inhibitory concentration (MIC) of *Curcuma longa* L., *Ocimum sanctum* L. and *Piper nigrum* L. ethanolic and aqueous extracts against *Staphylococcus aureus*, *Streptococcus agalactiae*, *Bacillus cereus*, *Listeria monocytogenes*, *Escherichia coli* and *Pseudomonas aeruginosa* was carried out. ABST was performed using the disc diffusion method while MIC of all six extracts were determined by micro-broth dilution technique against all six bacteria. The extracts were suspended in a solution containing 10% dimethyl sulfoxide and 0.5% tween 80. Under aseptic condition, empty sterilized discs were impregnated with 50 µl of different (50%, 20%, 10% and 5%) of all six extracts and placed on the agar plate surface. Sterile disc moistened with vehicle (DMSO plus tween 80) was placed on the seeded petri plate as a vehicle control. Standard disc containing antibacterial drugs (gentamicin, tetracycline, cefpirome and ampicillin) were used as reference control. The petri plates were incubated at 37°C for 18 h. After the incubation period, the zone of inhibition was measured. Antibacterial sensitivity test revealed that ethanolic extracts of *C. longa*, *O. sanctum* and *P. nigrum* showed antibacterial activities against *Staphylococcus aureus*, *Bacillus cereus* and *Listeria monocytogenes* whereas *O. sanctum* ethanolic extracts also showed inhibitory effect against *Streptococcus agalactiae*. *O. sanctum* aqueous extract exhibited antibacterial activity against *Staphylococcus aureus*, *Bacillus cereus*, *Listeria monocytogenes* and *Escherichia coli*, while *C. longa* aqueous extract showed antibacterial activity against *Listeria monocytogenes*. Standard antibiotics (gentamicin, tetracycline, cefpirome and ampicillin) were also found active against tested bacteria. The results revealed that ethanolic extract of *O. sanctum* showed lower MIC against *Staphylococcus aureus*, *Streptococcus agalactiae*, *Bacillus cereus*, *Listeria monocytogenes* and *Escherichia coli* than *C. longa* and *P. nigrum* ethanolic extracts. Aqueous extract of *C. longa* showed lower MIC against *Staphylococcus aureus*, *Bacillus cereus*, *Listeria monocytogenes* and *Escherichia coli* than *O. sanctum* and *P. nigrum* aqueous extract. *P. nigrum* showed lower MIC against *Streptococcus agalactiae* than *C. longa* and *O. sanctum* aqueous extract. All six ethanolic and aqueous extracts were showed no antibacterial activity against *Pseudomonas aeruginosa*.

1. Introduction

Phytobiotics are natural bioactive compounds that are derived from plants. Herbs and spices are well identified to exert potent antimicrobial properties *in vitro* against various pathogens (Burt, 2004; Lee *et al*., 2013). The mechanism of action of phytobiotics is not clearly understood but may depend upon the composition of the active ingredients in the product being used. Some mechanisms suggested to be responsible for their beneficial properties include: (1) disruption of the cellular membrane of pathogens; (2) modification of the surface of the cells affecting to the hydrophobicity and, therefore, their virulence capacity; (3) stimulating the immune system, specifically activation of lymphocytes, macrophages, and natural killer cells; (4) protecting intestinal mucosa from bacterial pathogens colonization; and (5) promoting the growth of beneficial bacteria such as *Lactobacilli* and *Bifidobacteria* (Vidanarachchi *et al*., 2005). The main bioactive compounds of the phytobiotics are polyphenols, and their composition and concentration vary according to the plant, parts of the plant, geographical origin, harvesting season, environmental factors, storage conditions, and processing techniques. Researches on phytogenic herbs have revealed several various characteristics such as antioxidative and antimicrobial effects (Windisch *et al*., 2008). Phytobiotics also exert their action through immunomodulatory effects such as increased proliferation of immune cells, modulation of cytokines and increased antibody titers (Lee *et al*., 2010).

Curcuma longa L. is a perennial herb and member of the Zingiberaceae family and is cultivated extensively in Asia mostly in

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India and China. The rhizome portion of the *C. longa* plant used medicinally. It has many names such as Curcum in the Arab region, Indian saffron, Haridra (Sanskrit, Ayurvedic), Jianghuang (yellow ginger in Chinese), Kyoo or Ukon (Japanese) (Goel *et al*., 2008). The active constituents of turmeric are the flavonoid curcuminoids which is a mixture of curcumin (diferuloylmethane), monodexmethoxy curcumin, and bisdesmethoxy curcumin. Curcumin makes up approximately 90% of the curcuminoid content in turmeric (Wei and Shibamoto, 2007).

The genus Ocimum belongs to the family Lamiaceae, comprises about 68 species including indigenous to tropical regions of Asia, Africa and central and South America. *Ocimum sanctum* L. is a short-lived perennial shrub of 30-60 cm height with hairy stems and sparsely hairy leaves, which distributed in the Himalayas up to an altitude of 6000 feet (Watt, 1893). There are many chemical constituents present in *O. sanctum* such as oleanolic acid, rosmarinic acid, ursolic acid eugenol, linalool, carvacrol, b elemene, b caryophyllene and germacrene (Falagas and Bliziotis, 2007). It has various pharmacological actions like antimicrobial, antifungal, anticancer, antiarthritis, antifertility, hepatoprotective, antispasmodic, analgesic, antiemetic, and cardioprotective (Rao *et al*., 2013).

Piper nigrum L. (Black pepper) belongs to the family Piperaceae, cultivated for its fruit (berries) that are usually dried and used as a spice. Black pepper is native to Southern India and is extensively cultivated in this tropical region. Black pepper is referred to as "King of Spices" and represents one of India's major commodities (Srinivasan, 2000). It is also known that black pepper was once used as a food preservative. It contains major pungent alkaloid piperine (1-peperoyl piperidine) which is known to possess many pharmacological actions (Ahmad *et al*., 2012). Piperine exhibits diverse pharmacological activities like antihypertensive, anti-platelets, antioxidant, antitumor, antipyretic, analgesic, anti-inflammatory, antidiarrheal, antispasmodic, hepatoprotective, antibacterial, antifungal, antithyroids, antiapoptotic, anti-spermatogenic, insecticidal and larvicidal (Taqvi *et al*., 2008; Manoharan *et al*., 2009).

The World Health Organization (WHO) predicts that if no additional measures are taken, the annual death toll attributable to antimicrobial resistance may rise to 10 million and-exceed other causes such as cancer in 2050 (WHO, 2014). Therefore, it is a matter of global concern to take effective strategies to reduce antibiotic use to limit the spread of antimicrobial-resistant bacteria (ARBs) (Liu *et al*., 2016; Williams Nguyen *et al*., 2016). Due to concern about the development of AMR and the transfer of antibiotic resistance genes from animals to the human, it has led to the search for an alternative to antimicrobial drugs (Sweeney *et al*., 2018). Hence, the present study was planned to evaluate *in vitro* antibacterial sensitivity test and minimum inhibitory concentration of ethanolic and aqueous extracts of three plants *C. longa* (rhizome), *O. sanctum* (leaves) and *P. nigrum* (fruit) powders.

2. Materials and Methods

2.1 Collection of plant materials

Rhizome of *Curcuma longa* L. and Fruit of *Piper nigrum* L. (certified by FSSAI, Ministry of Health and Family Welfare, Government of India) were purchased from local market of Anand, Gujarat. Leaves of *Ocimum sanctum* L. were procured from Medicinal and Aromatic Plant Research Station, Anand Agricultural University, Anand. All the plants/plant materials were identified and authenticated by Botanist at Department of Genetics and Plant Breeding, B.A. College of Agriculture, AAU, Anand. *C. longa* rhizome, *O. sanctum* dried leaves and *P. nigrum* fruit were taken and dried under shade, then powdered by mechanical grinder and stored in air tight containers. The dried powder of *C. longa* rhizome, *O. sanctum* dried leaves and *P. nigrum* fruit were subsequently used for the preparation of ethanolic and aqueous extract.

2.2 Sources of test organisms for ABST and MIC

The test bacterial organisms were procured from National Collection of Industrial Microorganisms, National Chemical Laboratory, Pune. Bacterial strains of six bacterial species, namely; *Staphylococcus aureus* (ATCC 6538), *Streptococcus agalactiae* (ATCC 13813), *Bacillus cereus* (ATCC 11778), *Listeria monocytogenes* (ATCC 19111), *Escherichia coli* (ATCC 8739) and *Pseudomonas aeruginosa* (ATCC 9027) were selected. Purity and viability of the organisms were checked by morphological, cultural and biochemical tests and maintained by periodical subculture.

2.3 Preparation of ethanolic extracts

In Soxhlet extraction process, plant material (*C. longa* rhizome powder, *O. sanctum* dried leaves powder and *P. nigrum* fruit powder) was placed in a thimble-holder and filled with condensed fresh solvent ethanol from a distillation flask. When the liquid reaches the overflow level, a siphon aspirates the solution of the thimble-holder and unloads it back into the distillation flask, carrying extracted solutes into the bulk liquid. In the solvent flask, solute is separated from the solvent using distillation. Solute is left in the flask and fresh solvent passes back into the plant solid bed. The procedure is repeated until complete extraction is achieved. The solvents were later separated from the extract with the aid of rotary evaporator at 40°C (De castro and Garcia-ayuso, 1998). All three ethanolic extracts were labeled and stored at 4°C for further use.

2.4 Preparation aqueous extracts

For this purpose, about 100 g of the *C. longa* rhizome powder, *O. sanctum* dried leaves powder and *P. nigrum* fruit powder were soaked in 1 liter of distilled water with shaking thrice daily. The aqueous extracts obtained were concentrated in rotary evaporator at 50-60°C under reduced pressure leaving a residue. Aqueous extracts of *C. longa*, *O. sanctum* and *P. nigrum* powders obtained were transferred to a petri dish and kept over water bath (50°C) until the solvent gets completely evaporated. All three aqueous extracts were labeled and stored in air tight glass containers in refrigerator at 4°C for further experimental use.

2.5 Determination of antibacterial sensitivity by disk diffusion assay

2.5.1 Preparation of diffusion solution and different concentration

For the preparation of 10% dimethylsulfoxide (DMSO), 10 ml of DMSO was dissolved in 90 ml of distilled water in measuring cylinder.

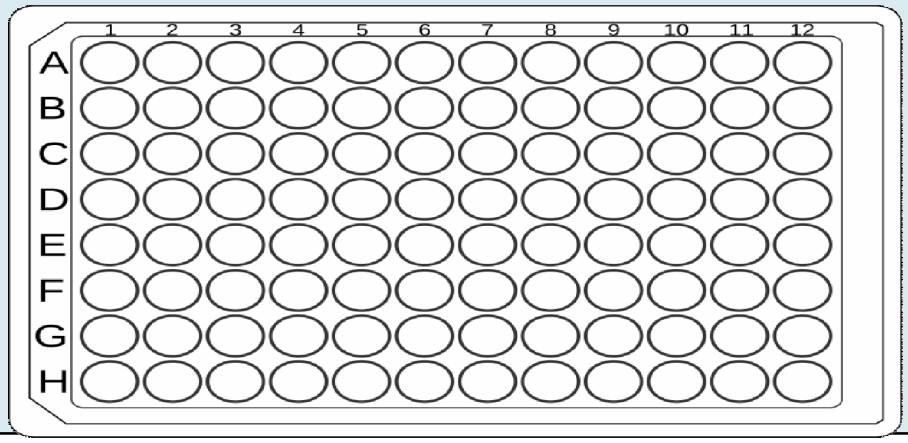
For the preparation of diffusion solution, 0.5 ml of tween 80 was dissolved in 99.5 ml of 10% DMSO. The preparation of various dilutions of *C. longa*, *O. sanctum* and *P. nigrum* ethanolic and aqueous extracts is shown in Table 1.

Table 1: Schedule for the preparation of *C. longa*, *O. sanctum* and *P. nigrum* ethanolic and aqueous extracts dilutions

	Concentration (%)	*C. longa* (g)	*O. sanctum* (g)	*P. nigrum* (g)	Diffusion solution
A	50	0.5	0.5	0.5	0.5 ml
B	20	0.2	0.2	0.2	0.8 ml
C	10	0.1	0.1	0.1	0.9 ml
D	05	0.05	0.05	0.05	0.95 ml

Table 2: Microdilution technique of MIC using 96 well micro titer plate

		Row											
Column	Concentration of plant Extract (% W/V)	25	12.5	6.25	3.12	1.56	0.78	0.39	0.19	0.10	0.05	PC	GC
	No. of Wells	1	2	3	4	5	6	7	8	9	10	11	12
	A												
	B												
	C												
	D												
	E												
	F												
	G (NC)												
	H (VC)												



PC-Positive control (Standard drug); GC-Growth control (Bacterial suspension) NC-Negative control (BHI broth); VC-Vehicle control (DMSO + Tween 80); A-*Staphylococcus aureus*; B-*Streptococcus agalactiae*; C-*Bacillus cereus*; D-*Listeria monocytogenes*; E-*Escherichia coli*; F-*Pseudomonas aeruginosa*.

2.5.2 Procedure for disk diffusion assay

Screening of *C. longa*, *O. sanctum* and *P. nigrum* ethanolic and aqueous extracts for antibacterial activity was done by the disc diffusion method. Four gram-positive and two gram-negative strains of bacteria were tested. It was performed using an 18 h culture at 37°C in 10 ml of Mueller Hinton agar. The test suspension was standardized to match 0.5 McFarland turbidity standard which corresponds to approximately 1.5×10^8 CFU/ml with sterile saline solution. Five hundred microliters of the suspensions were spread over the plates containing Mueller-Hinton agar (for *Streptococcus agalactiae* 5% defibrinated sheep blood was added) using a sterile cotton swab in order to get a uniform microbial growth on both control and test plates. A diluent containing 10% dimethylsulfoxide (DMSO) and tween 80 (99.5 ml DMSO mixed with 0.5 ml tween 80) was prepared to facilitate diffusion of *C. longa*, *O. sanctum* and *P. nigrum* extracts. Various concentration of *C. longa*, *O. sanctum* and *P. nigrum* extracts (50%, 20%, 10% and 5%) were prepared with diluent. It was sterilized by filtration through a 0.22 mm membrane filter. Under aseptic condition, empty sterilized discs (Whatman No. 5, 6 mm diameter) were impregnated with 50 ml of different concentrations (50%, 20%, 10% and 5%) of the respective *C. longa*, *O. sanctum* and *P. nigrum* ethanolic and aqueous extracts and placed on the agar surface (Wayne, 2002). Sterile disc moistened with diluent was placed on the seeded petriplate as a vehicle control. A standard disc of gentamicin, tetracycline, cefpirome and ampicillin were used as reference control. All petri plates were sealed with sterile laboratory parafilm to avoid eventual evaporation of the test samples. The plates were left for 30 min at room temperature to allow the diffusion of oil and then they were incubated at 37°C for 18 h. After the incubation period, the zone of inhibition was measured with a vernier caliper. All the dilutions of *C. longa*, *O. sanctum* and *P. nigrum* ethanolic and aqueous extracts were tested in triplicate manner against each bacterium.

2.6 Determination of MIC by microbroth dilution technique

Minimum inhibitory concentration (MIC) of *C. longa*, *O. sanctum* and *P. nigrum* ethanolic and aqueous extracts was determined by microbroth dilution technique with some modifications (Wiegand *et al*., 2008).

Table 3: Antibacterial activity of *C. longa*, *O. sanctum* and *P. nigrum* ethanolic extract and standard antibiotic discs against various bacteria

Groups	Zone of inhibition (mm)					
	Staphylococcus aureus	***Streptococcus agalactiae***	***Bacillus cereus***	***Listeria monocytogenes***	***Escherichia coli***	***Pseudomonas aeruginosa***
CL A (50%)	11.33 ± 0.33	-	10.67 ± 0.33	12.33 ± 0.67	-	-
CL B (20%)	10.67 ± 0.33	-	8.67 ± 0.33	11.00 ± 0.58	-	-
CL C (10%)	9.67 ± 0.33	-	8.33 ± 0.33	9.00 ± 1.00	-	-
CL D (5%)	9.33 ± 0.67	-	8.00 ± 0.00	0.00	-	-
OS A (50%)	11.00 ± 0.58	7.33 ± 0.33	11.00 ± 0.58	12.67 ± 0.67	-	-
OS B (20%)	10.00 ± 0.00	-	8.67 ± 0.88	-	-	-
OS C (10%)	8.33 ± 0.33	-	7.00 ± 0.58	-	-	-
OS D (5%)	6.33 ± 0.33	-	5.67 ± 0.33	-	-	-
PN A (50%)	8.67 ± 0.33	-	11.00 ± 1.53	7.33 ± 0.33	-	-
PN B (20%)	7.67 ± 0.67	-	9.00 ± 1.00	-	-	-
PN C (10%)	6.67 ± 0.33	-	8.67 ± 1.20	-	-	-
PN D (5%)	5.67 ± 1.45	-	6.67 ± 0.33	-	-	-
Gentamicin	24.33 ± 0.88	27.67 ± 0.88	19.33 ± 0.33	24.00 ± 0.58	18.33 ± 0.33	25.00 ± 0.58
Tetracycline	23.67 ± 0.88	33.67 ± 0.88	25.67 ± 0.88	28.33 ± 0.33	22.67 ± 0.88	17.33 ± 0.33
Cefpirome	17.00 ± 0.58	17.00 ± 0.58	13.67 ± 0.88	30.00 ± 1.15	29.00 ± 0.58	29.33 ± 0.33
Ampicillin	30.33 ± 0.88	10.00 ± 1.15	10.33 ± 0.33	10.33 ± 0.33	21.67 ± 0.88	-

CL = *C. longa*, OS= *O. sanctum*, PN = *P. nigrum* ethanolic extract

Table 4: Antibacterial activity of *C. longa*, *O. sanctum* and *P. nigrum* aqueous extract and standard antibiotic discs against various bacteria

Groups	Zone of inhibition (mm)					
	Staphylococcus aureus	***Streptococcus agalactiae***	***Bacillus cereus***	***Listeria monocytogenes***	***Escherichia coli***	***Pseudomonas aeruginosa***
CL A (50%)	-	-	-	11.33 ± 0.3	-	-
OS A (50%)	12.00 ± 1.00	-	11.00 ± 0.58	8.67 ± 0.33	10.67 ± 0.33	-
OS B (20%)	-	-	8.67 ± 0.33	-	-	-
OS C (10%)	-	-	-	-	-	-
Gentamicin	24.33 ± 0.88	27.67 ± 0.88	19.33 ± 0.33	24.00 ± 0.58	18.33 ± 0.33	25.00 ± 0.58
Tetracycline	23.67 ± 0.88	33.67 ± 0.88	25.67 ± 0.88	28.33 ± 0.33	22.67 ± 0.88	17.33 ± 0.33
Cefpirome	17.00 ± 0.58	17.00 ± 0.58	13.67 ± 0.88	30.00 ± 1.15	29.00 ± 0.58	29.33 ± 0.33
Ampicillin	30.33 ± 0.88	10.00 ± 1.15	10.33 ± 0.33	10.33 ± 0.33	21.67 ± 0.88	-

CL = *C. longa*, OS = *O. sanctum*, PN = *P. nigrum* aqueous extract. CL B (20%), CL C (10%), CL D (05%), OS D (05%), PN A (50%), PN B (20%), PN C (10%) and PN D (05%) concentrations were evaluated for ABST but not showed inhibitory effects against the tested organisms.

Table 5: MIC of *C. longa, O. sanctum* and *P. nigrum* ethanolic extract against various bacteria

Sr. No	Bacterial species	MIC (mg/ml)		
		C. Longa	*O. Sanctum*	*P. Nigrum*
1	*Staphylococcus aureus*	36.43 ± 13.79	10.40 ± 2.60	41.63 ± 10.43
2	*Streptococcus agalactiae*	7.80 ± 0.00	0.47 ± 0.00	5.20 ± 1.30
3	*Bacillus cereus*	20.80 ± 5.20	7.80 ± 0.00	36.43 ± 13.79
4	*Listeria monocytogenes*	26.00 ± 5.20	10.40 ± 2.60	62.50 ± 0.00
5	*Escherichia coli*	10.40 ± 2.60	3.23 ± 0.67	36.43 ± 13.79
6	*Pseudomonas aeruginosa*	125.0 ± 0.00	125.0 ± 0.00	125.0 ± 0.00

Table 6: MIC of *C. longa, O. sanctum* and *P. nigrum* aqueous extract against various bacteria

Sr. No.	Bacterial species	MIC (mg/ml)		
		C. longa	*O. sanctum*	*P. nigrum*
1	*Staphylococcus aureus*	15.60 ± 0.00	62.50 ± 0.00	52.07 ± 10.43
2	*Streptococcus agalactiae*	6.50 ± 1.30	23.40 ± 7.80	3.23 ± 0.67
3	*Bacillus cereus*	13.00 ± 2.60	83.33 ± 20.83	31.20 ± 0.00
4	*Listeria monocytogenes*	41.63 ± 10.43	62.50 ± 0.00	83.33 ± 20.83
5	*Escherichia coli*	10.40 ± 2.60	15.60 ± 0.00	41.63 ± 10.43
6	*Pseudomonas aeruginosa*	166.67 ± 41.67	104.17 ± 20.83	125.00 ± 0.00

2.6.1 Preparation of drug stock solution

Stock solution of *C. longa, O. sanctum* and *P. nigrum* ethanolic and aqueous extracts (250 mg/ml) was prepared using 99.5 ml DMSO mixed with 0.5 ml tween 80 as an emulsifying agent, mixed well by shaking vigorously. The concentration of *C. longa, O. sanctum* and *P. nigrum* ethanolic and aqueous extracts was decided based on their density. Chloramphenicol stock solution (250 mg/ml) was prepared in sterile water to use as positive control.

2.6.2 Preparation of bacterial suspension

After overnight incubation, all bacterial cultures were prepared to Mcfarland 0.5 standard equivalent to 1.5×10^8 cfu/ml. Final dispensing inoculums were prepared in sterile test tubes by adding 2 ml bacterial suspension (1.5×10^8 cfu/ml) of respective organisms into 198 ml sterile broth. Final dispensing inoculum concentrations were 1.5×10^6 cfu/ml.

2.6.3 Procedure for microbroth dilution technique

Sterile 96 well microtiter plates with sterile lid were used. Sterile broth of 100 µl was added in each wells except first well of the row. Then, 200 µl extract from stock solution was added in first well of first column. Followed by, 100 µl extract from well number 1 was taken and added to well number 2 and then it was serially diluted (two-fold dilution) up to well number 10. From 10 number well, discarded 100 µl extract. Stock solution of chloramphenicol (100 µl) was added in column number 11 as positive control. Serial dilution of extract kept starting concentration 25 % in first well whereas 0.05 % in 10^{th} number well. At lastly, 100 µl bacterial suspensions were dispended in well number 1 to 12 of row. The 12^{th} well was kept as a growth control where drug. G' row of 96 well plate was kept as negative control where only BHI broth added and 'H' row of 96 well plate was kept as vehicle control where only vehicle added. Microtiter plate was incubated at 37ºC for 16-20 h. Details of microdilution technique of MIC using 96 well micro titer plate are showed in Table 2.

All microtiter plates in agar plates where incubated at 37° for 16 to 20 h. After incubation, freshly prepared 30 µl of iodonitro tetrazolium chloride (INT) dye (1 mg/ml) was dispended in all wells of micro-titer plate. Plate was again incubated for 30 min for development of purple color, which indicated presence of live bacteria. MIC for each bacterial species was observed as absence of color development. This protocol was done in triplicate for each test drug.

3. Results

3.1 Antibacterial sensitivity test of ethanolic extracts

The antibacterial activity of ethanolic extracts of four different concentrations (50%, 20%, 10% and 5%) of *C.longa, O. sanctum* and *P. nigrum* have been presented in Table 3. Results revealed that many of the gram-positive and gram-negative tested bacteria were sensitive to the *C. longa, O. sanctum* and *P. nigrum* ethanolic extracts. There was no inhibition in growth of bacteria with the vehicle control. Four antibacterial drugs (gentamicin, tetracycline, cefpirome and ampicillin) were found active against test bacteria.

Result of the present study on antibacterial sensitivity test revealed that ethanolic extracts of *C.longa* at 50%, 20%, 10% and 5% concentrations showed antibacterial activities against *Staphylococcus aureus* and *Bacillus cereus. C. longa* ethanolic extract at 50%, 20%

and 10% concentrations showed antibacterial activity against *Listeria monocytogenes*. No antibacterial activity of *C. longa* ethanolic extract was found against *Streptococcus agalactiae, Escherichia coli* and *Pseudomonas aeruginosa*. Ethanolic extracts of *O. sanctum* at 50%, 20%, 10% and 5% concentrations showed antibacterial activities against *Staphylococcus aureus* and *Bacillus cereus* while at 50% concentration showed inhibitory effect against *Streptococcus agalactiae* and *Listeria monocytogenes.* No antibacterial activity of *O. sanctum* ethanolic extract was found against *Escherichia coli* and *Pseudomonas aeruginosa*. Representative photographs of the zones of inhibition against test bacteria for ethanolic extracts are shown in Figures 1 to 6.

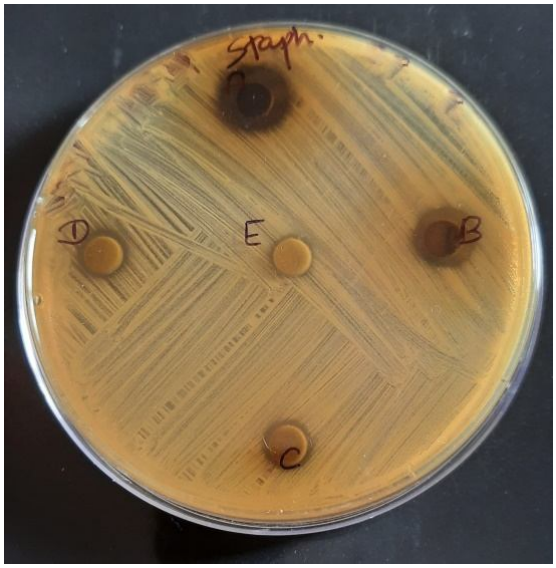


Figure 1: Antibacterial sensitivity test of ethanolic extract of *C. longa* concentrations against *Staphylococcus aureus* (A=50%, B=20%, C=10%, D=5%, E=Vehicle).

Figure 2: Antibacterial sensitivity test of ethanolic extract of *O. sanctum* concentrations against *Staphylococcus aureus* (A=50%, B=20%, C=10%, D=5%, E=Vehicle).

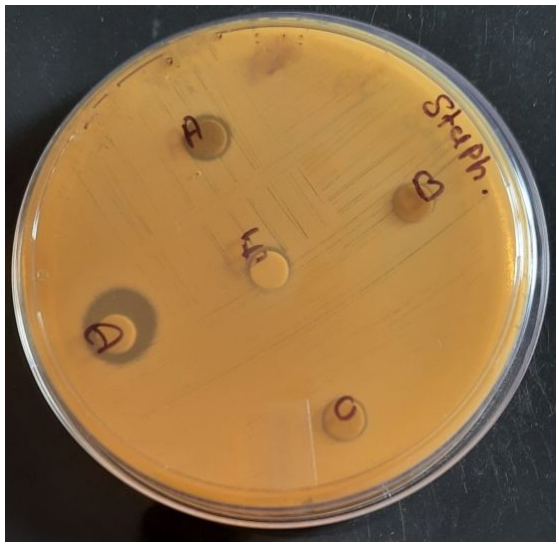


Figure 3: Antibacterial sensitivity test of ethanolic extract of *P. nigrum* concentrations against *Staphylococcus aureus* (A=50%, B=20%, C=10%, D=5%, E=Vehicle).

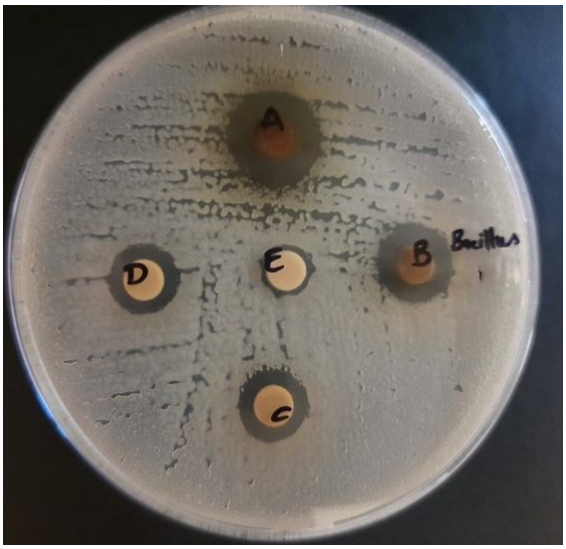


Figure 4: Antibacterial sensitivity test of ethanolic extract of *C. longa* concentrations against *Bacillus cerus* (A=50%, B=20%, C=10%, D=5%, E= Vehicle).

3.2 Antibacterial sensitivity test of aqueous extracts

The antibacterial activity of aqueous extracts of four different concentrations (50%, 20%, 10% and 5%) of *C. longa, O.sanctum* and *P. nigrum* have been presented in Table 4. Result of the present study on antibacterial sensitivity test revealed that aqueous extracts of *C. longa* at 50% concentration showed antibacterial activity against *Listeria monocytogenes* whereas no antibacterial activity was found against *Staphylococcus aureus, Streptococcus agalactiae, Bacillus cereus, Escherichia coli* and *Pseudomonas aeruginosa.* Aqueous extracts of *O. sanctum* at 50% concentration showed antibacterial activity against *Staphylococcus aureus, Listeria*

monocytogenes and *Escherichia coli* while at 50% and 20% concentrations showed antibacterial activity against *Bacillus cereus*. No antibacterial activity of *O. sanctum* aqueous extract was found against *Streptococcus agalactiae* and *Pseudomonas aeruginosa*. No antibacterial activity of *P. nigrum* aqueous extract was found against all tested organisms at all concentrations.

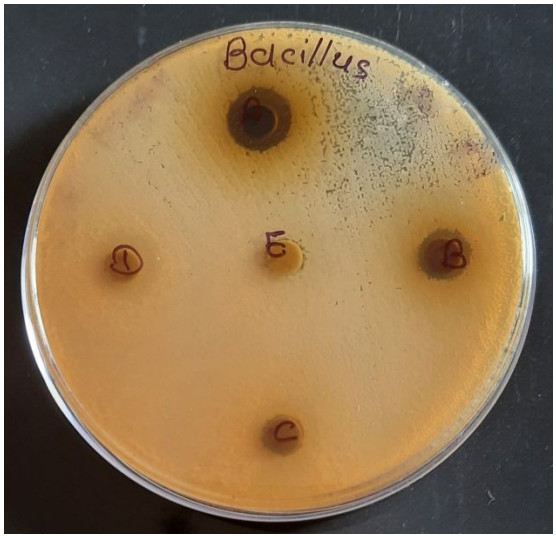


Figure 5: Antibacterial sensitivity test of ethanolic extract of *O. sanctum* concentrations against *Bacillus cerus* (A=50%, B=20%, C=10%, D=5%, E= Vehicle).

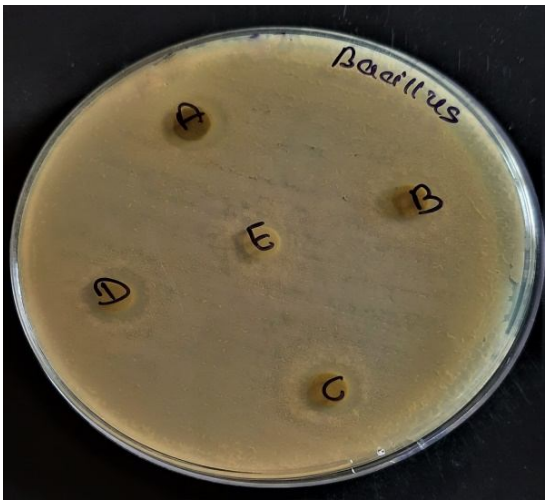


Figure 6: Antibacterial sensitivity test of ethanolic extract of *P. nigrum* concentrations against *Bacillus cerus* (A=50%, B=20%, C=10%, D=5%, E= Vehicle).

3.3 MIC of ethanolic and aqueous extracts

MIC of *C. longa*, *O. sanctum* and *P. nigrum* ethanolic and aqueous extract at 250, 125, 62.5, 31.2, 15.6, 7.8, 3.9, 1.9, 0.95 and 0.47 mg/ml concentration with two fold serial dilution in six different gram positive and gram-negative organisms in triplicate manner was tested. The MIC values (Mean ± S.E.) of ethanolic & aqueous extracts of *C. longa*, *O. sanctum* and *P. nigrum* against different bacterial species (*Staphylococcus aureus*, *Streptococcus agalactiae*, *Bacillus cereus*, *Listeria monocytogenes*, *Escherichia coli* and *Pseudomonas aeruginosa*) have been presented in Table 5 and 6, respectively. Representative photographs of 96 well plates used for determination of MIC for ethanolic extract of *O. sanctum and* aqueous extract of *C. longa* against different bacterial species are presented in Figures 7 and 8.

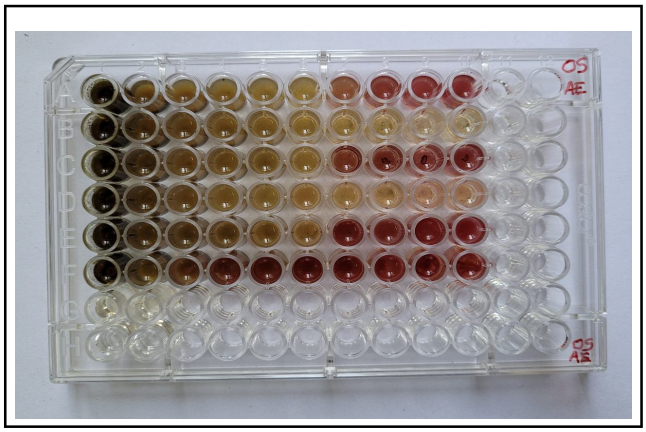


Figure 7: Microtiter plates showing MIC of ethanolic extract of *O. sanctum* against different bacterial species (A= *Staphylococcus aureous*, B= *Streptococcus agalactiae* C= *Bacillus cereus*, D= *Escherichia coli*, E= *Listeria monocytogenes*, F= *Pseudomonas aeruginosa*).

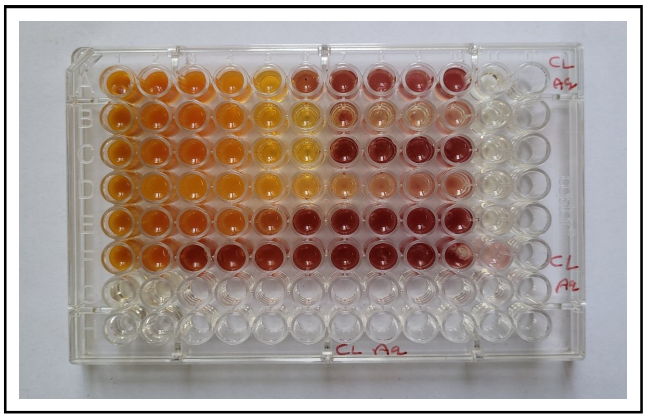


Figure 8: Microtiter plates showing MIC of aqueous extract of *C. longa* against different bacterial species (A= Staphylococcus aureous, B= Streptococcus agalactiae C= Bacillus cereus, D= Escherichia coli, E= Listeria monocytogenes, F= Pseudomonas aeruginosa).

Ethanolic extract of *O. sanctum* showed best MIC against *Staphylococcus aureus*, *Streptococcus agalactiae*, *Bacillus cereus*, *Listeria monocytogenes* and *Escherichia coli* than *C. longa* and *P. nigrum* ethanolic extracts. Ethanolic extracts of all three test plants were not found activity against *Pseudomonas aeruginosa*. Aqueous extract of *C. longa* showed best MIC against *Staphylococcus aureus*, *Bacillus cereus*, *Listeria monocytogenes* and *Escherichia coli* than *O. sanctum* and *P. nigrum* aqueous extract. *P. nigrum* showed best

MIC against *Streptococcus agalactiae* than *C. longa* and *O. sanctum* aqueous extract. Aqueous extracts of all three test plants were not found activity against *Pseudomonas aeruginosa.*

4. Discussion

The results with respect to antibiotic sensitivity test were in accordance with Kim *et al.* (2005), who reported significant inhibitory effect of *C. longa* ethanolic extract against *Staphylococcus aureus* (14.00 mm) and no significant inhibitory effect of *C.longa* aqueous extract against *Staphylococcus aureus.* Shan *et al.* (2007) reported the no antibacterial effects of ethanol extracts of *Piper nigrum* against *Escherichia coli*. Niamsa and Sittiwet (2009) reported antibacterial activity of *C. longa* aqueous extract against *Escherichia coli* (15.00 mm) and *Staphylococcus aureus* (18.00 mm). Harikrishnan *et al.* (2010) reported significant inhibitory effect of *O. sanctum* ethanolic extract against *Staphylococcus aureus* (12.00 mm). Pundir and Jain (2010) reported antibacterial activity of *C. longa* ethanolic extract against *Escherichia coli* (22 to 24 mm) and *C. longa* aqueous extract against *Escherichia coli* (17 to 18 mm) and *Staphylococcus aureus* (26 to 28 mm). Similarly, Butkhup and Samappito (2011) reported antibacterial activity of *C.longa* methanolic extract against *Bacillus cereus* (6.00 mm). They also reported antibacterial activity of *P. nigrum* methanolic extract against *Bacillus cereus* (10.00 mm), *Staphylococcus aureus* (7.00 mm), *Escherichia coli* (11.00 mm) and *Salmonella typhi* (7.00 mm). Goyal and Kaushik (2011) reported antibacterial activity of *O.sanctum* ethanolic extract against *Staphylococcus aureus* (13.83 ± 0.58) and *Escherichia coli* (12.50 ± 0.50) and *O. sanctum* aqueous extract against *Escherichia coli* (11.93 ± 1.01mm) and *Staphylococcus aureus* (15.23 ± 0.40 mm). Zarai *et al.* (2013) reported antibacterial activity of ethanolic extracts of *P. nigrum* against *Bacillus subtilis* (13.8 ± 0.3 mm) and *Staphylococcus aureus* (10.7 ± 1.5 mm) and *aqueous extracts of P. nigrum against Bacillus subtilis* (9.3 ± 0.6 *mm), Staphylococcus aureus* (8.5 ± 0.5 mm) and no antibacterial activity against *Escherichia coli.* Dhiman *et al.* (2016) reported inhibitory effect of *C. longa* ethanolic extract against *Bacillus cereus* (17.30 ± 1.52 mm) and no inhibitory effect of *C. longa* aqueous extract against *Bacillus cereus.* Gul and Bakht (2017) reported the antibacterial potentials of turmeric methanolic extracts against *Staphylococcus aureus* (13.5 mm), *Escherichia coli* (10.0 mm) and *Salmonella typhi* (8.5 mm) and turmeric aqueous extracts against *Escherichia coli* (7.0 mm) and *Salmonella typhi* (7.0 mm) and no activity against *Staphylococcus aureus.* Mittal *et al.* (2018) reported the antibacterial effects of ethanol extracts of *O. sanctum* against *Staphylococcus aureus* (21.00 mm) at maximum concentration used. Shafi *et al.* (2018) found antibacterial activity of *O. sanctum* ethanolic extract against *Escherichia coli* (17.38 ± 0.92 mm) and *Streptococcus* spp (10.88 ± 0.58 mm) while testing bovine mastitis isolates. Kachhawa *et al.* (2019) reported the antibacterial activity of ethanolic extracts of *P. nigrum* against *Staphylococcus aureus* (10.50 ± 0.10 mm), *Streptococcus agalactiae* (8.43 ± 0.08 mm) and *Escherichia coli* (8.26 ± 0.03 mm). Adhikari *et al.* (2020) reported antibacterial activity of *O. sanctum* methanol extract against *Staphylococcus aureus* (8.00 ± 0.50) and *Escherichia coli* (8.00 ± 1.04). Similarly, for other plant extracts also reported antibacterial activities, *in vitro* antibacterial activity of acetone extract of banana fruit peel investigated against *Staphylococcus epidermidis.* Results showed 17 mm zone of inhibition and 32 µg/ml MIC against *Staphylococcus epidermidis* (Kumari *et al.*, 2020). Similarly, antibacterial activity of *Euphorbia hirta* leave extract was carried out using the agar well diffusion method and it was found effective against *Bacillus subtilis* (14.2 ± 0.01), *Staphylococcus aureus* (11.4 ± 0.13) and *Escherichia coli* (8.27 ± 0.05) (Khan *et al.*, 2021).

According to the findings of the present study, *C. longa*, *O. sanctum* and *P. nigrum* ethanolic extracts have varied degrees of antibacterial activity in terms of zone of inhibition against tested gram-positive and gram-negative bacteria except *Pseudomonas aeruginosa*. Although, the mechanisms related with the antibacterial activities of extracts are not fully understood, several mechanisms of actions have been hypothesized. It is stated that the potent antibacterial activity of the *C. longa*, *O. sanctum* and *P. nigrum* ethanolic extracts may be due to presence of high concentration of active principal like curcumin, eugenol and piperine, respectively.

The results with respect to MIC of ethanolic and aqueous extracts of *C. longa*, *O. sanctum* and *P. nigrum* were in accordance with Kim *et al.* (2005) who reported significant inhibitory effect of *C. longa* ethanolic extract against *Staphylococcus aureus* (2.0 mg/ml) and *C. longa* aqueous extract against *Staphylococcus aureus* (64.0 mg/ml). Erturk (2006) reported MIC of ethanolic *P. nigrum* extracts against *Escherichia coli* (15.00 mg/ml), *Staphylococcus aureus* (12.50 mg/ml) and *Pseudomonas aeruginosa* (5.00 mg/ml). Joshi *et al.* (2009) reported significant MIC of *O. sanctum* ethanolic extract against *Staphylococcus aureus* (10.00 mg/ml), *Salmonella typhi* (2.5 mg/ml) and *Bacillus cereus* (10.0 mg/ml). Niamsa and Sittiwet (2009) reported 4.00 mg/ml and 6.00 mg/ml MIC for *Escherichia coli* and *Staphylococcus aureus*, respectively. Harikrishnan *et al.* (2010) reported significant MIC of *O. sanctum* ethanolic extract against *Staphylococcus aureus* (4.28 mg/ml). Butkhup and Samappito (2011) reported MIC of *C. longa* methanolic extract against *Bacillus cereus* (128 µg/ml), *Salmonella typhi* (64 µg/ml) and *Escherichia coli* (64 µg/ml). Goyal and Kaushik (2011) reported MIC of *O.sanctum* ethanolic extract against *Escherichia coli* (4.10 mg/ml) and *Staphylococcus aureus* (2.05 mg/ml) and for *O. sanctum* aqueous extract against *Staphylococcus aureus* (2.05 mg/ml). Shanmugapriya *et al.* (2012) reported the MIC of aqueous extracts of *P. nigrum* against *Bacillus subtilis* (100 µg/ml), *Escherichia coli* (200 µg/ml) and *Staphylococcus aureus* (200 µg/ml). Zarai *et al.* (2013) reported the MIC of ethanolic extracts of *P. nigrum* against *Bacillus subtilis* (2.5 mg/ml), *Escherichia coli* (2.5 mg/ml) and *Staphylococcus aureus* (1.25 mg/ml). Dhiman *et al.* (2016) reported the ethanolic extract of *C. longa* appeared effective with MIC 25 mg/ml against *Bacillus cereus.* Okmen *et al.* (2017) reported the MIC of *P. nigrum* ethanolic extract against *Staphylococcus aureus* (6.5 mg/ml). Kachhawa *et al.* (2019) reported the MIC of ethanolic extracts of *P. nigrum* against *Staphylococcus aureus* (62.50 mg/ml), *Streptococcus agalactiae* (31.25 mg/ml) and *Escherichia coli* (31.25 mg/ml). Likewise, *in vitro* antibacterial effect of catechin was evaluated by determining MIC using microbroth dilution method against various gram-positive and negative typed culture and results found the MIC ranges from 1.25 to 10.0 mg/ml (Varia *et al.*, 2021).

5. Conclusion

From the findings of the present studies on antibacterial sensitivity and MIC, it could be concluded that *C. longa, O. sanctum* and *P. nigrum* ethanolic extracts evinced antibacterial activity against *Staphylococcus aureus, Bacillus cereus* and *Listeria monocytogenes. O. sanctum* aqueous extract exhibited antibacterial activity against *Staphylococcus aureus, Bacillus cereus, Listeria monocytogenes* and *Escherichia coli*, while *C. longa* aqueous extract showed antibacterial activity against *Listeria monocytogenes*. Ethanolic extract of *O. sanctum* had lower MIC than *C. longa* and *P. nigrum* ethanolic extracts against *Staphylococcus aureus*, *Streptococcus agalactiae*, *Bacillus cereus*, *Listeria monocytogenes* and *Escherichia coli.* Aqueous extract of *C. longa* had lower MIC than *O.sanctum* and *P. nigrum* aqueous extract against *Staphylococcus aureus*, *Bacillus cereus*, *Listeria monocytogenes* and *Escherichia coli. P. nigrum* aqueous extract showed lower MIC against *Streptococcus agalactiae* than *C. longa* and *O. sanctum* aqueous extract. Extracts of selected plant inhibited growth of tested gram-positive and gram-negative bacteria at various concentrations indicating it possesses antibacterial activities. Furthermore, these extracts showed lower antibacterial activity as compared to tested standard antibacterial drugs.

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Conflict of interest

The authors declare no conflicts of interest relevant to this article.

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