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Article *in* Journal of Evolution of Medical and Dental Sciences · January 2018

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ANTIMICROBIAL PROPERTIES IN BARK AND LEAF EXTRACTS OF FOUR CINNAMOMUM SPECIES

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ABSTRACT

BACKGROUND

Cinnamon has been recognised for its flavouring and medicinal properties since ancient times and is the second most important spice sold in the world market. The antibacterial activities of hexane, chloroform, methanol and water extracts of four Cinnamomum species were studied.

MATERIALS AND METHODS

Both bark and leaf extracts of C. verum, C. cassia, C. tamala and C. camphora was tested in vitro against 12 bacterial species by agar well diffusion assay and minimum inhibitory concentration (MIC) was determined. The bacterial species used in the study was Listeria monocytogenes, Vibrio cholerae, Escherichia coli ATCC 25922, Klebsiella pneumoniae ATCC 700603, Staphylococcus aureus ATCC 29213, Salmonella paratyphi, Salmonella typhi, Proteus mirabilis, Shigella boydii, Stenotrophomonas maltophilia ATCC 17666, Enterobacter hormaechei and Pseudomonas aeruginosa ATCC 27853.

RESULTS

The present study indicated that both bark and leaf extracts have the ability to inhibit Gram-positive and Gram-negative organisms. But bark extracts were more effective than leaf extracts in inhibiting the organisms. S. maltophilia was inhibited by all the tested bark extracts except methanol extracts of C. cassia and C. camphora. The diameter of zone of inhibition ranged from 16-51 mm. C. camphora hexane extracts showed least MIC value of 3.13 mg/mL with S. maltophilia. V. cholerae a potent pathogen was inhibited by C. camphora leaf chloroform extract at the MIC of 3.13 mg/mL.

CONCLUSION

From the present study, it could be concluded that selected extracts of cinnamon species have a remarkable potential in inhibiting the growth of major pathogenic bacteria.

KEYWORDS

Cinnamomum Species, Minimum Inhibitory Concentration, Agar Well Diffusion Assay.

HOW TO CITE THIS ARTICLE: Keloth S, Krishnamurthy KS, Janardhanan J, et al. Antimicrobial properties in bark and leaf extracts of four cinnamomum species. J. Evolution Med. Dent. Sci. 2018;7(05):683-689, DOI: 10.14260/jemds/2018/155

BACKGROUND

The need of exploring new methods of food preservation for the partial and total replacement of antimicrobial chemical additives are increasing nowadays. The new method of food preservation called 'natural antimicrobial system' was coined by Gould in 1995. His study emphasised the possible use of spices and their derivatives as an alternative for chemical antimicrobial food additives. Natural additives are safe, enhance flavour and they do not have any side effects.[1] Cinnamon has been recognised for its flavouring and medicinal properties since ancient times and is the second most important spice sold in the world market. Some economically important species of Cinnamomum are C. verum (Ceylon cinnamon naturally occurring in Sri Lanka, Southern India and Myanmar cultivated mainly for quills and bark oil, C. cassia (Chinese cassia occurring in South China, Vietnam, Laos and Myanmar cultivated for bark and leaf oil), C. tamala (the Indian cassia distributed in the forests of North Eastern India and Myanmar whose leaves are used in flavouring dishes), C. camphora (camphor tree, cultivated in Japan, Taiwan, China, Vietnam and Thailand, cultivated for camphor and camphor oil).[2] It was found that Cinnamon oil has marked antimicrobial activity against Escherichia coli, Staphylococcus aureus and Candida albicans.[3] Trans-cinnamaldehyde was observed as the major volatile compound in cinnamon and cassia bark oils. Brackman et al (2008) reported that cinnamaldehyde and cinnamaldehyde derivatives reduce virulence in Vibrio spp.[4] These compounds can interfere with biofilm formation, stress response and virulence in Vibrio spp. It possesses potent antibacterial, antifungal, antitermitic, larvicidal, nematicidal and insecticidal properties.[5] Camphor was found to be present at highest percentage in C. camphora bark and leaf essential oil. Methanol extracts of leaves and branches of C. camphora extracts were found to be effective in inhibiting Gram-positive bacteria such as Bacillus cereus, Bacillus subtilis and S. aureus.[6] Antibacterial activity of extracts of cinnamon essential oil was proved by different studies.

Though there are a few reports available on antibacterial activity of solvent extracts of some cinnamon species, studies

'Financial or Other Competing Interest': None.
Submission 27-11-2017, Peer Review 14-01-2018,
Acceptance 20-01-2018, Published 29-01-2018.
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DOI: 10.14260/jemds/2018/155

on comparison among different Cinnamomum species is very less and hardly there are any studies on leaf extracts. The main objective of the present study was to compare antibacterial properties of four Cinnamomum species in both bark as well as leaf extracts and to determine minimum inhibitory concentration (MIC) of extracts to inhibit the bacteria and compare the antimicrobial potential of extracts with standard antibiotics.

MATERIALS AND METHODS

In-Vitro study was conducted to compare antibacterial properties of four Cinnamomum species in both bark as well as leaf extracts and to determine MIC of extracts to inhibit the bacteria and compare the antimicrobial potential of extracts with standard antibiotics.

Plant Materials

Bark and leaf samples of 4 cinnamon species were collected from ICAR-IISR experimental farms, Peruvannamuzhi and Chelavoor, Kozhikode, Kerala. The cinnamon species used for the studies were C. verum, C. cassia, C. tamala and C. camphora.

Reagents and Materials

Solvents such as hexane, chloroform, methanol, and dimethyl sulfoxide (DMSO) were supplied by Sisco Research Laboratory Ltd. (SRL). Mueller-Hinton agar and antibiotic discs such as IC 005, IC 002, IC 003, HX 027, HX 063 and HX 001 were supplied by Hi-Media.

Microorganisms and Culture

A total of 12 bacteria were kindly provided by the Department of Molecular Biology and Diagnostics, Malabar Institute of Medical Sciences, Kozhikode, Kerala. They are Listeria monocytogenes, Vibrio cholerae, Escherichia coli ATCC 25922, Klebsiella pneumoniae ATCC 700603, Staphylococcus aureus ATCC 29213, Salmonella paratyphi, Salmonella typhi, Proteus mirabilis, Shigella boydii, Stenotrophomonas maltophilia ATCC 17666, Enterobacter hormaechei, Pseudomonas aeruginosa ATCC 27853. The strains were preserved as stock culture on nutrient agar.

Preparation of Crude Extract

Cinnamon samples (leaf and bark) collected from the farm were thoroughly cleaned, dried in an oven (Memmert make) at 45°C to a constant weight and then powdered. Powdered samples were extracted with solvents in the order of increasing polarity such as hexane, chloroform, methanol and water. The filtrates were vapourised by Rotavap (Buchi, Germany) and dried. The extracts were dissolved in DMSO to the final concentration of 25 mg/mL and stored at 40°C until further use.

Determination of Antibacterial Activity

Antibacterial activity of cinnamon extracts was determined by agar well-diffusion method.[7] The bacterial samples were taken from stock culture and suspended in sterile nutrient broth at a density equivalent to that of 0.5 McFarland standards. The tubes were kept for incubation at 37°C for 4 hours. Sterile Mueller-Hinton agar plates were prepared. A sterile cotton swab was dipped into the standardised bacterial suspension and used to evenly inoculate the entire surface of Mueller-Hinton agar plates. Wells of 8 mm diameter were cut on the agar surface. 100 µL of extracts (dissolved in DMSO, 25 mg/mL) were added to the well. A well-containing 100 µL DMSO alone has served as control. The inoculated plates were incubated overnight at 37°C. After incubation, diameter of zone of inhibition were measured and recorded in mm.

Determination of Minimum Inhibitory Concentration (MIC)

The combination of extract and bacteria showing good zone of inhibition was selected for determination of minimum inhibitory concentration. A stock solution of extract at a concentration of 25 mg/mL was prepared. 0.5 mL of extract was mixed with 0.5 mL of nutrient broth in a test tube. The tubes were mixed well and 0.5 mL of extract with nutrient broth were taken from the tube and mixed with another test tube with 0.5 mL nutrient broth, and the serial dilution continued to get a final concentration of 1.56 mg/mL in the last test tube. 0.5 mL of the standardised bacterial suspension was inoculated to all the test tubes and incubated overnight at 37°C. After incubation a sterile cotton swab was dipped into the test tubes containing extracts and bacterial suspension, and used to evenly inoculate the entire surface of Mueller-Hinton agar plates. The plates were again incubated overnight. The MHA plates showing lowest and highest bacterial density were selected. The plate having bacterial density adjacent to the highest density was considered for determination of MIC.

Comparison of Antibacterial Potential of Extracts with Antibiotics

Combination of antibiotics specific for Gram positive, Gram negative and Pseudomonas species were used for testing. Sterile MHA plates were swabbed with standardised bacterial suspension and antibiotic rings were placed on the agar surface using sterile forceps and incubated overnight at 37°C. The diameter of zone of inhibition in mm was measured and recorded. The concentrations of each antibiotic with the concentration of extracts were analysed by comparing diameter of zone of inhibition of both extract and antibiotic for a specific organism by paired t-test.

RESULTS

Extracts -bark	L. monocytogenes	V. cholerae	E. coli	K. pneumoniae	S. aureus	S. paratyphi	S. typhi	P. mirabilis	S. boydii	Steno. maltophilia	E. hormaechei	P. aeruginosa
C. verum												
hexane	21	30	20	15	28	24	24	27	24	41	18	20
chloroform	ND	ND	ND	ND	ND	ND	ND	ND	ND	21	13	ND
methanol	11	ND	ND	ND	ND	ND	ND	ND	ND	25	ND	22
water	12.5	ND	ND	ND	ND	ND	ND	14	ND	16.5	13	14
C. cassia												
hexane	26	28	25	19	17	22	25	29	26	51	ND	21
chloroform	17	18	13	13	ND	19	15	13	ND	35	12	19
methanol	10	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	17
water	12	13	ND	ND	12	13	ND	16	ND	13	12.5	16
C. tamala												
hexane	14.5	ND	10	ND	ND	12	12	25	12	25	ND	14
chloroform	11	ND	11	ND	ND	15	ND	ND	11	22	ND	30
methanol	ND	ND	ND	ND	ND	ND	ND	ND	ND	25	ND	ND
water	13	ND	ND	ND	ND	12.5	ND	13	ND	17	12	16
C. camphora												
hexane	11.5	ND	12	ND	ND	12	12	12	13	26	ND	22
chloroform	20	ND	ND	ND	ND	ND	ND	ND	12	16	ND	16
methanol	9	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	22
water	12	ND	ND	ND	ND	12	ND	15	ND	13	ND	ND
DMSO control	No ZOI	No ZOI	No ZOI	10	No ZOI	No ZOI	No ZOI	No ZOI	No ZOI	No ZOI	13	18

Table 1. Diameter of Zone of Inhibition (mm) of Bark Extract

- ND- not detected.
- NO ZOI- no zone of inhibition.

Extracts – leaf	L. monocytogenes	V. cholerae	E. coli	K. pneumoniae	S. aureus	S. paratyphi	S. typhi	P. mirabilis	S. boydii	Steno. maltophilia
C. verum										
Hexane	16	ND	12	12.5	13	17	14	14.5	11.5	20
chloroform	15.5	ND	14	12	15	15	13	14	ND	26
methanol	12	12	15	12	13	15	13	ND	ND	16
Water	12	13	ND	ND	ND	12	ND	16	12.5	15
C. cassia										
Hexane	21	12	13	15	14	16	11.5	14	13	24
chloroform	14	12.5	13	13	15	17	15	16	16	29
methanol	12	12	12	12	ND	13	ND	ND	ND	19
Water	12	14	ND	ND	14	13.5	ND	17	ND	16
C. tamala										
Hexane	ND	29	13	12	ND	13	ND	ND	11.5	20
chloroform	12.5	ND	ND	13.5	ND	ND	ND	ND	ND	20
methanol	12	13	12	13	ND	ND	ND	ND	ND	19
Water	11.5	ND	ND	ND	12	ND	14	14	ND	14
C. camphora										
Hexane	14	30	14	13.5	ND	ND	ND	ND	13	21
chloroform	12	31	ND	13	ND	ND	ND	ND	ND	ND
methanol	12	19	ND	ND	12	ND	ND	12.5	ND	ND
water	ND	12.5	ND	13	12	12	ND	15	ND	15
DMSO control	No ZOI	No ZOI	No ZOI	10	No ZOI	No ZOI	No ZOI	No ZOI	No ZOI	No ZOI

Table 2. Diameter of Zone of Inhibition (mm) of Leaf Extract

Species	Extract	bacteria	MIC (mg/ml)
C. verum	Hexane	S. aureus S. typhi V. cholera S. boydii E. coli	6.25 12.5 6.25 12.5 12.5
	Methanol	P. aeruginosa	12.5
C. cassia	Hexane	S. typhi S. boydii S. maltophilia E. coli	12.5 12.5 3.13 25
	Chloroform	S. maltophilia	12.5
C. tamala	Chloroform	P. aeruginosa	6.25
	Methanol	S. maltophilia	12.5
C. camphora	Hexane	P. aeruginosa S. maltophilia	6.25 12.5
	Methanol	P. aeruginosa	6.25

Table 3. Minimum Inhibitory Concentration (MIC) of Bark Extract

Species	Extract	Bacteria	MIC (mg/ml)
C. verum	Chloroform	P. aeruginosa	12.5
		S. maltophilia	6.25
C. cassia	Chloroform	S. paratyphi	12.5
		S. maltophilia	6.25
C. tamala	Hexane	V. cholerae	6.25
C. camphora	Hexane	V. cholerae	6.25
	Chloroform		3.13

Table 4. Minimum Inhibitory Concentration (MIC) of Leaf Extracts

Bacteria	IPM	CIP	TOB	MO	OF	SPX	LE	NX	COT	CL	NA	AMC	K	GAT	GEN	AK	S	CTR	CPD	TI
P. mirabilis	30	27	33	29	30	25	35	33	27	16	20	22	31	22	25	24	30	33	30	35
S. boydii	25	16	25	20	15	16	20	17	ND	12	ND	11	24	12.5	29	23	14	30	20	23
E. coli	30	40	30	30	30	30	37	36	35	20	33	ND	30	35	29	22	27	37	22	35
K. pneumoniae	27	18	23	22	22	20	21	20	30	ND	19	ND	27	20	24	21	20	22	12	ND
V. cholerae	25	26	36	29	29	32	27	30	12	20	18	29	25	24	32	22	30	32	21	30
S. typhi	25	21	29	34	15	27	32	27	44	ND	19	22	30	28	29	20	20	ND	12	23
S. paratyphi	29	16	31	20	20	21	25	ND	19	12	SZ	SZ	30	22	26	17	15	17	17	20
E. hormaechei	32	33	25	31	12	31	29	25	35	15	24	12	25	30	24	24	22	25	14	30
P. aeruginosa				AT		AZ			TCC	PIT	GAT	CPZ	NET	CB			MZ		PI	
	33	28	35	26	14	38	28	29	27	27	26	23	28	26	23	25	32	25	26	21

Table 5. Antibiotic sensitivity pattern of Gram negative bacteria (mm)

IPM-imipenem, CIP- ciprofloxacin, TOB- tobramycin, MO- moxifloxacin, OF- ofloxacin, SPX- sparfloxacin, LE- levofloxacin, NX- norfloxacin, COT- co-trimoxazole, CL- colistin, NA- nalidixic acid, AMC- augmentin, K- kanamycin, GAT- gatifloxacin, GEN- gentamicin, AK- amikacin, S- streptomycin, CTR- ceftriaxone, CPD- cefpodoxime, TI- ticarcillin, CB- carbenicillin, PI- piperacillin, AT- aztreonam, AZ- azlocillin, TCC- ticarcillin, PIT- piperacillin, GAT- gatifloxacin, CPZ- cefoperazone, NET- netillin, MZ- mezlocillin.

Bacteria	CEP	CD	COT	E	GEN	OF	P	VA	AMP	C	OX	LZ	AZM	AK	CLR	TEI	MET	AMC	NV	TE
S. aureus	33	35	20	30	31	30	20	19	20	23	24	19	22	30	22	19	31	23	33	19
L. monocytogenes	ND	21	ND	23	26	22	ND	20	ND	23	ND	20	20	29	21	22	ND	ND	21	21
S. maltophilia	Sensitive (ZOI > 35mm)																			

Table 6. Antibiotic Sensitivity Pattern of Gram Positive Bacteria (mm)

CEP- cephalothin, CD- clindamycin, COT- Co-trimoxazole, E- erythromycin, GEN- gentamicin, OF- ofloxacin, P- penicillin, VA- vancomycin, AMP- ampicillin, C- chloramphenicol, OX- oxacillin, LZ- linezolid, AZM- azithromycin, AK- amikacin, CLR- clarithromycin, TEI- teicoplanin, MET- methicillin, AMC- amoxyclav, NV- novobiocin, TE- tetracycline.

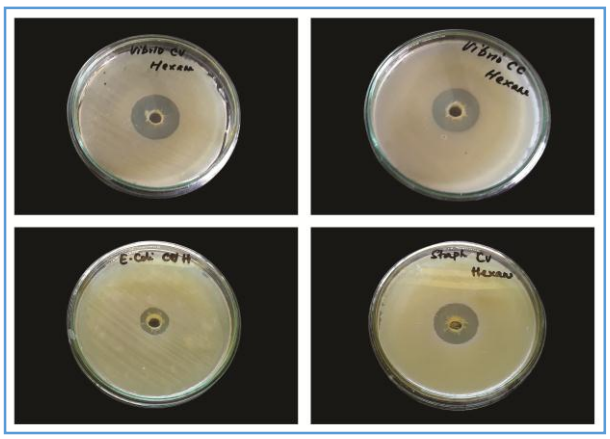

Figure 1. Hexane and Chloroform extract of Cinnamon Leaf Extract against Bacteria

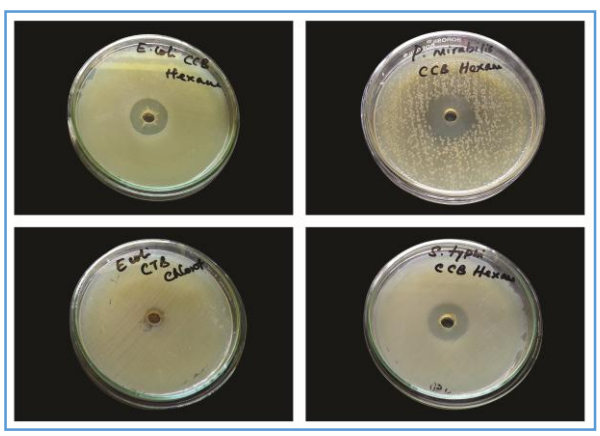

Figure 2. Hexane and Chloroform extract of Cinnamon bark extract against Bacteria

DISCUSSION

Antibacterial Activity of Bark Extracts

Antibacterial activity of bark extracts of four cinnamon species against twelve pathogenic bacteria is summarised in Table 1. Bark extracts were shown to be more efficient than leaf extract in inhibiting the tested organisms. Most of the bark extracts were potent enough to inhibit the growth of both Gram-positive and Gram-negative bacteria. Stenotrophomonas maltophilia is an emerging multidrug-resistant global opportunistic pathogen. It causes nosocomial and community-acquired infections in immune-compromised individuals.[8] Present study reveals that Stenotrophomonas maltophilia was inhibited by all of the tested bark extracts, except methanol extract of C. cassia and C. camphora. The diameter of zone of inhibition (ZOI) towards sensitive extracts ranged from 16 - 51 mm. S. maltophilia was found to be most sensitive among the tested organisms. Studies showed that cinnamon essential oil can inhibit the pathogens causing respiratory infections including S. maltophilia.[9] To the best of our knowledge, this study is the first report on the antibacterial activity of solvent extracts of four cinnamon species against S. maltophilia. Vibrio cholera, a potent and important enteric pathogen was found to be inhibited by hexane extract of both C. verum and C. cassia (ZOI 30 and 28 mm). Enterotoxin secreted by V. cholerae was an important virulence factor of the organism, which was responsible for a fatal secretory diarrhoea called cholera. All the bacteria, analysed in the antimicrobial activity testing were inhibited by C. verum and C. cassia hexane extract except Enterobacter hormaechei, which showed resistance to C. cassia hexane extract. The genera Escherichia, Klebsiella, Enterobacter (collectively called coliform bacilli) and Proteus are members of normal intestinal flora; also they can act as an opportunistic pathogen. They can cause nosocomial infections of urinary tract, surgical sites, blood stream and pneumonia. P. mirabilis is the most frequent cause of infection related to kidney stones. K. pneumoniae causes severe pneumonia.[10] S. aureus (causative agent of superficial skin infections such as boils, furuncles, styes and impetigo in humans) was highly resistant to most of the extracts. But it showed good zone of inhibition with C. verum hexane extract (28 mm). These results indicate that bark of both C. verum and C. cassia serve as an excellent anti-bacterial agent and can inhibit a range of bacteria. All the bacteria tested were clinically significant organisms. Most of them are enteric pathogens. They are E. coli, K. pneumoniae and E. hormaechei, P. mirabilis, S. typhi, S. paratyphi, V. cholerae, S. boydii and L. monocytogenes. As the cinnamon is used in routine culinary purposes and is able to inhibit these enteric pathogens increases the relevance of the present study which highlights the nutraceutical properties of cinnamon.

Antibacterial Activity of Leaf Extracts

Antibacterial activity of leaf extracts of four cinnamon species against twelve pathogenic bacteria is summarised in Table 2. The diameter of zone of inhibition of leaf extracts was less compared to bark extracts indicating that leaf extracts are less sensitive compared to bark extracts. But P. aeruginosa showed the ZOI of 30 mm with C. verum chloroform extract. P. aeruginosa is an opportunistic pathogen, a major threat to hospitalised patients those who were affected with cancer and burns.[11] V. cholerae showed the ZOI of 29, 30 and 31 mm with C. tamala hexane extract, C. camphora hexane and chloroform extract and S. maltophilia had a ZOI of 29 mm with C. cassia chloroform extract respectively. The ZOI of other extracts with the twelve bacteria tested were in the range of 11.5 - 26 mm. Earlier work using agar well diffusion method suggested that leaf oleoresin can inhibit Penicillium citrinum, leaf volatile oil and oleoresin have shown better results in comparison with bark volatile oil, oleoresin and commercial bactericide.[12] Study of essential oil from leaves of Cinnamomum osmophloeum oils had an excellent inhibitory effect on bacteria.[13] Results from the antifungal tests conducted demonstrated that cinnamaldehyde possessed the strongest antifungal activity compared to the other constituents of the essential oils.[14]

Determination of Minimum Inhibitory Concentration (MIC)

The extracts showing a ZOI greater than 15 mm were selected for determination of minimum inhibitory concentration. The MIC values of different extracts against test bacteria are listed in Table 3. C. cassia hexane extract showed a least MIC value with S. maltophilia (3.13 mg/mL). Most of the bark extracts and leaf extracts showed the MIC in the range of 12.5 - 6.25 mg/mL. Interestingly, V. cholera a potent pathogen was inhibited by C. camphora leaf chloroform extract at the MIC of 3.13 mg/mL. The highest MIC was obtained for E. coli with C. cassia bark hexane extract (25 mg/mL). Previous studies reported that in comparison with crude extracts, essential oil of cinnamon have lower MIC value. This could be due to the fact that crude extracts contain both volatile and non-volatile

contents, but essential oil contain higher levels of volatile components such as cinnamaldehyde than crude extract.[15] In another study conducted revealed that both essential oil and pure cinnamaldehyde have an equal potential in inhibiting Gram-positive, Gram-negative bacteria, yeasts and molds.[16]

Comparison of Antibacterial Potential of Extracts with Antibiotics

Antibiotic sensitivity pattern of Gram-positive, Gram-negative and Pseudomonas species were checked and was compared with some selected extracts which showed good antibacterial activity. From the results, it was demonstrated that the antibacterial activity of tested extracts was on par with the antibiotics. Even though the extracts were in crude form when compared to highly purified antibiotics, they showed a diameter of zone of inhibition similar to the antibiotics. S. maltophilia was found to be sensitive to all the tested antibiotics.[17] Present work also showed that S. maltophilia was inhibited by many of the tested extracts giving highest diameter of zone of inhibition and statistically significant (p-0.005). E. coli tested in the study was resistant to Augmentin, which is in accord with those found in the studies.[18] Majority of the organisms used in the present study comes under the family enterobacteriaceae. From the results, it is shown that all of them are susceptible to Imipenem, a carbapenem. Because these organisms have the ability to produce β-lactamase enzyme, which give resistance to penicillin and cephalosporin. So carbapenems become the drug of choice.[19]

CONCLUSION

From the present study, it could be concluded that selected extracts of cinnamon species have a remarkable potential in inhibiting the growth of major pathogenic bacteria. Activity of the extract was comparable with the activity of the antibiotics in inhibiting the pathogens. Though both bark and leaf extracts of cinnamon species showed antimicrobial property, bark extracts are found to be better than leaf extracts. Further purification of the extracts from their crude form would definitely enhance their antimicrobial efficiency. Incorporation of these extracts in purified form adds a new dimension in food preservation as a very safe alternative with a very appealing odour and a very high consumer preference. To our knowledge, this is the first report on the comparison of antibacterial property of extracts from different Cinnamomum sp. and also the comparison between leaf and bark extracts on their antibacterial activity.

ACKNOWLEDGEMENTS

The authors thank the Director, ICAR- Indian Institute of Spices Research for providing facilities. The authors express their sincere thanks to the Principal, Co-operative Institute of Health Sciences, Thalassery, Kerala for providing lab facilities for this research work.

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