INNSPUB

International Journal of Biosciences | IJB |
ISSN: 2220-6655 (Print), 2222-5234 (Online)
http://www.innspub.net
Vol. 16, No. 4, p. 63-70, 2020

RESEARCH PAPER OPEN ACCESS

Synergistic effect of selected herb and spices extracts against bacterial strains causing food spoilage

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Key words: Herb, Spices, Biopreservation, Microorganisms.

http://dx.doi.org/10.12692/ijb/16.4.63-70 Article published on April 14, 2020

Abstract

Cinnamon, clove and mustard belongs to class of spices while oregano is herb. They are found to have the bioactive compounds. Phytochemical present in cinnamon is 70%-90% cinnamaldehyde, in clove eugenol content is around 95%, in mustard 71% allyl-isothiocyanate is present and oregano contains carvacrol and thymol 30% and 27% respectively along with many others phytochemicals. These plant derivatives possess antimicrobial activity against food borne pathogens and food spoilage bacteria. So, the present study was intended to analyze synergistic effect existed for various combinations of extracts used against selected food spoilage and pathogenic bacteria. For this purpose, the aqueous and ethanolic extracts of cinnamon, clove, mustard and oregano were prepared and concentrated by using shaking incubator and rotary evaporator. Various combinations were made out of these extracts and different concentrations of these extracts were used to analyze the antimicrobial potential against *Bacillus subtilis*, *Pseudomonas aeruginosa*, *Klebsiella pneumoniae* and *Escherichia coli* by using disc diffusion method. Ethanolic extract of combination of cinnamon, clove and mustard revealed the best zones of inhibition (13.87 ± 0.78 mm), (13.27 ± 0.38 mm), (13.47 ± 0.38 mm), (12.27 ± 0.56 mm) against *B. subtilis*, *P. aeruginosa*, *K. pneumoniae* and *E. coli*, respectively. The minimum zone of inhibition was observed in case of aqueous extract. The results revealed that cinnamon, clove, mustard and oregano extracts have significant results against these microorganisms. Conclusively, these extracts can be utilized as biopreservatives or to control spoilage in different food products after toxicological studies.

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Introduction

Food spoilage leads to the damage of food by changing texture, nutritional value and flavor of the food that makes the food unsuitable for consumption. The growth and activity of food-borne pathogens in foods can be controlled to acceptable levels through food preservation and safety measures (Cleveland *et al.,* 2001). In order to achieve food safety against such pathogens, food industry makes use of chemical preservatives. Synthetic chemical substances previously have been widely applied in the food industry, particularly in food preservation. Most commonly used synthetic preservatives in food industries are Sorbic acid (Dalton, 2002), Benzoic acid, Sulphites (McCann *et al.*, 2007) and Sodium EDTA.

Consumer awareness about the injurious health impacts of synthetic chemical additives such as toxic or even carcinogenic effects has increased the demand for high-quality, minimally processed foods with extended shelf-life, preferably free from or with a reduced level of added chemical antimicrobial agents (Zink, 1997).The increasing demand for safe food has increased the interest in replacing chemical additives by natural products, without injuring the host or the environment (Martinis, 2001).

In order to satisfy consumer demand with required safety measures, traditional methods of controlling food spoilage are gradually alternated by new innovative technology either in combination or used alone. Use of non-pathogenic microbes and their antimicrobial compounds to enhance shelf life of food and to ensure safety is another form of food preservation termed as bio preservation (Martinis, 2001; Nath *et al*., 2013).

Recent research has investigated the potential uses of plant based antimicrobial components that can be used in food preservation (Muhialdin *et al.,* 2011). Extracts of different herbs and spices that contain bioactive compounds are abundantly used for food preservation to replace synthetic compounds for food preservation (Fernandez *et al*., 2004). Cinnamon has been one of the most popular and widely used spices for hundred years (Wijesekera, 1997). Cinnamon is used against oxidant and microbial assay (Amirdivani, 2008). Cinnamaldehyde is the most represented component, with a content ranging from 62% to 90% in bark essential oil (Muchuweti *et al*., 2009). Oregano possesses anti-bacterial, antiviral, antifungal and anti-insecticidal properties (Harini, 2014).

Oregano possesses strong antimicrobial properties because of special component present in it that is “cravacol”. Clove exhibits antimicrobial properties due to the presence of “eugenol” (Jirovetz *et al*., 2006). Mustard contains allyl isothio cyanates which inhibits food borne pathogens and is responsible for antimicrobial action (Tsao *et al.,* 2000). Synergistic effect of herbs and spices against food borne pathogens exhibits strong antimicrobial activity (Lambert *et al.,* 2001).

Therefore, the present study aimed to evaluate synergistic effect of selected herbs and spices extracts against food spoilage bacterial strains *viz. Bacillus subtilis*, *Pseudomonas aeruginosa*, *Klebsiella pneumoniae* and *Escherichia coli in vitro*.

Material and methods

Current research was performed at Food Microbiology Laboratory, Institute of Food Science and Nutrition, University of Sargodha, Sargodha, Pakistan.

The ethanolic and aqueous extracts of Cinnamon, Clove, Mustard and Oregano were prepared and later on were evaluated for antimicrobial potential against different pathogenic bacteria. All the experiments were performed in triplicate. The detail of materials and methods used is as under.

Collection and preparation of sample

Cinnamon, Clove, Mustard and Oregano were purchased from commercial market of Sargodha and transported to the Laboratory. The herb and spices were manually cleaned.

Preparation of extracts of cinnamon, clove, mustard and oregano

Ten grams (10g) of each of Cinnamon, Clove, Mustard and Oregano were taken by weighing on an electric balance (SHIMADZU), which were then transferred in 500mL capacity conical flasks. Distilled water (100mL) was added in each flask that already had sample. The process was repeated with 100mL ethanol to prepare flasks for each herb and spice. Then the flasks were placed in shaking incubator (SHING SAENG SKIR-601L) at 40°C and 120 RPM for 24 hours. The liquid extracts were filtered through Whatman No. 1 filter paper by using vacuum suction filtration assembly. The filtrates were transferred to a rotary evaporator (HEIDOLPH LABOROTA 4001) at 50°C to evaporate the solvent (water and ethanol) under vacuum to a final volume of 20mL.

Three combinations were made to assess synergy among selected herb and spices i.e. Cinnamon+Clove; Oregano+Clove+Cinnamon;

Mustard+Clove+Cinnamon. The extracts were combined in 50:50, 33:33:33 and 33:33:33, respectively.

Microbiological analysis

Microorganisms and culture media: Food borne pathogens (*Pseudomonas aeruginosa, Klebsiella pneumoniae, Bacillus subtilis*, and *Escherichia coli)* were obtained from Biochemistry Laboratory, Department of Chemistry, University of Sargodha, Sargodha (Pakistan). These cultures were maintained on nutrient agar plate by continuous sub-culturing after 10-15 days.

Assessment of antimicrobial activity: The antimicrobial effects of Cinnamon, Clove, Mustard and Oregano were evaluated against above mentioned strains by "Disc Diffusion Method" as suggested by Sadeghian *et al.* (2011) with some modifications.

Preparation of extracts impregnated paper discs: The nutrient agar medium was prepared and the test microorganisms were inoculated by "Pour Plate Method" on the entire surface of the plate. The paper discs made of Whatman No. 1 filter paper of 6.00mm diameter were placed on nutrient agar plates using a sterile pair of forceps.

Then different concentrations of extracts (20, 40, 60, 80 and 100µL) were absorbed in discs by micro-pipette. Then the plates were placed in incubator (MCO-15-AC) at 37˚C for 24 to 48hrs.

The zones of growth inhibition were measured by Caliper with an accuracy of 0.1mm.

The antimicrobial activity was assessed by measuring diameter of the zone of inhibition around the disc. All disc diffusion tests were performed in triplicate for each pathogen, and the antibacterial activity was expressed as the mean of the inhibition zone diameter (mm). The results were also compared with commercial antibiotics (Amoxicillin& Ciprofloxacin) by using antibiotic impregnated discs.

Minimum inhibitory concentration (mic): For MIC fresh growth of pathogenic strains (*Staphylococcus aureus, Escherichia coli, Bacillus subtilis* and *Salmonella typhi*) were taken in broth medium. Milk was taken in sterilized beakers and kept at room temperature and after that strains were inoculated in milk with the ratio of 2%, separately, and in that milk Combination extract of Cinnamon, Clove and Mustard was added with 0%, 2%, 4%, 6% and 8% concentration for each strain. Then TPC was performed to check the microbial count at 0 time and then after every 2 hours up to 24 hours of total incubation.

Statistical analysis

Results obtained from different parameters were subjected to statistical analysis using Analysis of Variance (ANOVA) technique under Factorial Design to evaluate the antimicrobial effect of Cinnamon, Clove, Mustard and Oregano Extracts by following methods as described by (Steel and Torri, 1997). Statistical analysis was performed using SPSS v.18 Chicago.

Results and discussion

Results obtained in the present study confirmed that aqueous and ethanolic extracts of Cinnamon, Clove, Mustard and Oregano possessed varied antibacterial activity against *P. aeruginosa, K. pneumoniae, B. subtilis* and *E. coli.*

Antimicrobial activity of individual extracts of selected herb and spices as well as three different combinations of selected herb and spices were explored. The extracts of Cinnamon, Clove and Mustard exhibited notable antibacterial activities toward all examined bacteria with different potentialities. On the other hand, the extracts of Oregano showed weak antibacterial activities against most of the tested strains. Lowest zone of inhibition was exhibited by aqueous extract of Cinnamon against *K. pneumoniae* and *B. subtilis*; and by aqueous extract of Mustard against *K. pneumoniae* while greatest zone of inhibition was observed against *E. coli* for aqueous and ethanolic extracts of Clove; *P. aeruginosa* for ethanolic extracts of Cinnamon and Mustard; and *K. pneumoniae* for ethanolic extracts of Cinnamon indicating that Cinnamon, Clove and Mustard were the most effective spices in inhibiting the microbial growth (Table 1).

Table 1. Diameter of zone of inhibition of individual extracts against pathogens.

Extract	Conc	Aqueous Extract				Ethanol Extract			
		P.aeruginosa	*K.pneumoniae*	*B.subtilis*	*E.coli*	*P.aeruginosa*	*K.pneumoniae*	*B.subtilis*	*E.coli*
Cinnamon	20	6.6±0.7^{a}	5.0±0.6^{a}	5.0±0.6^{a}	6.3±0.8^{e}	6.3±0.3^{a}	6.7±0.9^{ef}	7.3±0.9^{a}	8.7±0.3^{cd}
	40	8.0±1.1^{a}	8.3±0.9^{de}	7.3±0.9^{a}	7.3±0.3^{de}	10.3±0.9^{a}	9.3±0.9^{cd}	8.3±1.2^{a}	9.7±0.3^{bc}
	60	10.7±0.7^{a}	7.7±0.9^{de}	9.3±0.9^{a}	10.7±0.3^{ab}	12.3±0.3^{a}	11.3±0.7^{bc}	9.0±1.2^{a}	10.3±0.3^{a}
	80	11.0±0.6^{a}	8.3±0.3^{de}	10.3±0.3^{a}	11.3±0.3^{a}	13.0±0.6^{a}	13.3±0.9^{b}	11.0±0.6^{a}	11.7±0.3^{a}
	100	12.0±0.6^{a}	10.7±0.9^{c}	11.0±0^{a}	11.7±0.4^{a}	16.0±0.6^{a}	16.0±0.6^{a}	11.7±0.9^{a}	11.7±0.4^{a}
Clove	20	9.7±0.3^{a}	5.3±0.3^{d}	6.7±0.7^{a}	6.7±0.9^{a}	9.7±0.7^{a}	5.3±0.3^{d}	6.3±0.3^{a}	8.7±1.2^{a}
	40	11.3±0.3^{a}	6.7±0.7^{d}	9.7±0.9^{a}	10.0±1.0^{a}	11.7±0.7^{a}	8.7±0.3^{c}	8.7±0.7^{a}	13.7±0.3^{a}
	60	12.3±0.3^{a}	8.3±0.3^{c}	11.0±0^{a}	14.7±0.3^{a}	13.0±0.6^{a}	11.7±0.7^{ab}	12.0±0.6^{a}	14.7±0.3^{a}
	80	12.7±0.3^{a}	8.7±0.3^{c}	11.3±0.3^{a}	14.7±0.9^{a}	13.3±0.9^{a}	11.7±0.3^{ab}	13.0±0.6^{a}	15.3±0.3^{a}
	100	14.0±0.6^{a}	10.3±0.9^{b}	12.7±0.3^{a}	16.0±0.6^{a}	14.0±0.6^{a}	13.0±0.6^{a}	12.0±1.2^{a}	16.0±1.1^{a}
Mustard	20	6.7±0.3^{a}	5.0±0.6^{a}	5.7±0.7^{a}	6.0±0.5^{e}	8.7±0.3^{a}	7.3±0.3^{a}	6.7±0.9^{a}	7.3±0.3^{e}
	40	8.3±0.3^{a}	7.7±0.9^{a}	6.7±0.7^{a}	7.0±0.4^{e}	11.0±0.4^{a}	8.7±0.3^{a}	8.7±0.3^{a}	8.0±0.3^{de}
	60	11.7±0.7^{a}	9.7±0.9^{a}	9.7±0.3^{a}	11.0±0.3^{bc}	13.3±0.3^{a}	9.7±0.7^{a}	9.0±0.6^{a}	10.0±0.6^{cd}
	80	14.0±0.6^{a}	11.0±0.6^{a}	10.0±0.6^{a}	12.3±0.4^{ab}	14.3±0.3^{a}	11.3±1.2^{a}	10.0±0.6^{a}	9.7±0.4^{cd}
	100	15.0±0.6^{a}	13.0±0.6^{a}	10.7±0.9^{a}	14.0±0.3^{a}	16.0±0.6^{a}	12.7±0.9^{a}	11.0±0.6^{a}	10.3±0.7^{bc}
Oregano	20	7.7±0.3^{ef}	7.7±0.9^{a}	8.3±0.8^{a}	7.0±0.6^{a}	7.0±0.6^{f}	6.7±1.2^{a}	7.3±0.3^{a}	6.3±0.9^{a}
	40	9.0±0.6^{de}	11.7±0.3^{a}	9.7±1.2^{a}	8.0±0.3^{a}	8.7±0.3^{de}	10.0±0.6^{a}	9.3±0.3^{a}	8.7±0.3^{a}
	60	8.3±0.3^{def}	12.7±0.3^{a}	10.0±1.0^{a}	10.0±0.6^{a}	9.3±0.3^{cd}	9.7±0.9^{a}	10.7±1.3^{a}	10.0±1.0^{a}
	80	8.0±0.6^{def}	13.7±0.3^{a}	12.0±0.0^{a}	11.7±0.9^{a}	12.0±0.6^{ab}	14.3±0.3^{a}	11.7±0.3^{a}	9.7±1.7^{a}
	100	10.7±0.9^{bc}	15.7±0.9^{a}	12.7±1.2^{a}	12.7±1.4^{a}	12.7±0.3^{a}	14.7±1.4^{a}	13.0±0.9^{a}	11.7±0.9^{a}

The results are closely related to the findings of Hoque *et al.* (2008) who reported antimicrobial activity of extracts of cinnamon and clove against different pathogens. Results are also supported by the findings of Danlami *et al.* (2016) and Chaudhary *et al.* (2007) that is significant antimicrobial effect of mustard and oregano extracts (Aqueous), respectively. They recorded10.8 to 23.0 mm zones of inhibition against *Pseudomonas aeruginosa* and *K. pneumoniae* at different concentrations of aqueous extracts of mustard and oregano.

The inhibition zones increased with increasing concentration of extracts. At high concentrations, ethanolic extracts exhibited marked inhibition activity against bacteria. The ethanolic solutions of the extracts were found to have potent antimicrobial activity against all the Gram-positive and Gram-

negative organisms, i.e., *P. aeruginosa, E. coli, K. pneumonia* and *B.subtilis*, tested by the disc diffusion method.

It was revealed that the inhibition of pathogens was significantly better in case of use of different combinations of various extracts rather than the use of individual extracts. The combination (50:50) of aqueous extract of Cinnamon and Clove exhibit lowest activity 5.0mm against *Pseudomonas aeruginosa* while significant activity of 20.0mm was observed by combination (33:33:33) of ethanolic extracts of Cinnamon, Clove and Mustard against *Bacillus subtilis* (Table 2). The results are coincident with the findings of Baljeet *et al.* (2015) who also observed that the combinations of extracts from different herbs and spices gave better results for the inhibition of different pathogens.

Table 2. Diameter of zone of inhibition of combination extracts against pathogens.

Extract	Conc	Aqueous Extract				Ethanol Extract			
		P.aeruginosa	*K.pneumoniae*	*B.subtilis*	*E.coli*	*P.aeruginosa*	*K.pneumoniae*	*B.subtilis*	*E.coli*
Cinnamon+Clove (1:1)	20	5.0±0.6^{a}	5.7±0.4^{a}	5.3±0.3^{a}	5.7±0.3^{a}	8.3±0.3^{a}	6.7±0.2^{a}	8.3±0.7^{a}	7.3±0.3^{a}
	40	7.3±0.3^{a}	8.0±0.6^{a}	7.7±0.9^{a}	7.0±0.6^{a}	10.7±0.7^{a}	8.3±0.4^{a}	9.7±0.9^{a}	9.0±0.6^{a}
	60	11.0±0.6^{a}	9.7±0.4^{a}	10.7±0.9^{a}	9.3±0.6^{a}	14.3±0.3^{a}	10.0±0.3^{a}	12.7±0.3^{a}	10.0±0.6^{a}
	80	12.8±0.3^{a}	10.3±0.2^{a}	12.0±0.6^{a}	10.0±0.6^{a}	14.7±0.9^{a}	11.7±0.3^{a}	14.0±0.6^{a}	11.3±0.6^{a}
	100	14.7±0.7^{a}	12.3±0.3^{a}	13.3±0.7^{a}	13.0±0.3^{a}	16.0±0.6^{a}	12.7±0.3^{a}	14.7±0.3^{a}	14.0±0.3^{a}
Cinnamon+Clove+ Mustard (1:1:1)	20	6.0±0.6^{a}	9.0±0.6^{a}	8.0±0.6^{f}	9.0±0.6^{a}	8.3±0.9^{a}	10.67±0.9^{a}	8.3±0.3^{f}	9.3±0.7^{a}
	40	10.0±0.3^{a}	10.7±0.3^{a}	9.7±0.3^{ef}	10.3±0.7^{a}	11.7±0.0^{a}	11.0±0.0^{a}	9.3±0.3^{ef}	10.3±0.7^{a}
	60	10.7±0.7^{a}	11.3±0.7^{a}	11.3±0.3^{de}	10.7±0.3^{a}	13.7±0.7^{a}	14.3±0.7^{a}	14.3±0.9^{c}	11.7±0.3^{a}
	80	12.0±0.7^{a}	13.7±0.7^{a}	13.3±0.3^{cd}	12.7±0.7^{a}	15.3±0.0^{a}	15.0±0.0^{a}	17.3±1.2^{cd}	14.0±0.6^{a}
	100	17.0±0.3^{a}	14.3±0.3^{a}	13.7±0.7^{c}	14.3±0.3^{a}	17.3±0.3^{a}	16.3±0.3^{a}	20.0±1.2^{a}	16.0±0.6^{a}
Cinnamon+Clove+ Oregano (1:1:1)	20	6.3±0.9^{a}	6.0±0.6^{a}	5.7±0.9^{f}	5.7±0.3^{a}	8.7±0.3^{a}	10.3±1.3^{a}	11.3±0.9^{de}	9.3±0.9^{a}
	40	9.0±0.6^{a}	8.3±0.9^{a}	7.0±0.6^{f}	8.0±0.6^{a}	9.0±0.6^{a}	10.7±0.3^{a}	13.7±1.5^{bcd}	11.3±0.9^{a}
	60	10.0±0.6^{a}	10.7±0.3^{a}	10.7±0.9^{e}	9.7±0.9^{a}	12.3±0.7^{a}	13.0±0.6^{a}	14.0±0.6^{abc}	12.7±0.9^{a}
	80	10.7±0.3^{a}	12.0±0.6^{a}	12.3±0.9^{cde}	11.3±0.3^{a}	13.0±1.0^{a}	14.3±0.6^{a}	13.3±0.3^{bcd}	13.7±0.9^{a}
	100	12.0±0.6^{a}	13.3±0.3^{a}	15.3±0.9^{ab}	14.0±0.6^{a}	14.0±0.6^{a}	15.3±0.3^{a}	16.3±0.9^{a}	15.3±0.3^{a}

The antimicrobial activity of herbs and spices might be due to presence of a variety of phenolic and essential oils which possess a wide spectrum of activity against bacteria and fungi (Tassou *et al.,* 2004). It could also be due to the presence of bioactive compounds in the selected spices and herbs. The results are in accordance with the findings of Chang *et al.* (2001) and Dorman and Deans (2000) who also illustrated that antimicrobial potential of herbs and spices is due to the presence of bioactive compounds like cinnamaldehyde (cinnamon), eugenol (clove) and Carvacrol (oregano). The results are also justified by the investigations of a number of other scientists who also reported presence of bioactive compounds in spices and herbs like in Cinnamon, in Clove, in Oregano and in Mustard; these compounds have high antimicrobial activity (Vernin *et al.*, 2001; Batra, 2003; Jirovetz *et al.,* 2006).The inhibition of pathogens might arise due to the breakdown of cell wall of microorganism by the bioactive compounds; also reported by Dorman and Deans (2000).

Experiment was conducted to observe the MIC of Combination of Cinnamon, Clove and Mustard against the following tested pathogens (*S. aureus, E. coli, S. typhi* and *B. subtilis*) (Fig 1. a,b,c,d).It was evident from the results that the lag phase was prolonged up to 8 hours, 12 hours and 22 hours in case of 4%, 6% and 8% extract concentration, respectively whereas in control and 2% concentration of extract the cultures attained logarithmic phase of growth after 6 hours. It was concluded that 4% concentration of extract was found to be effective for

MIC against above pathogens. The results are contrasted with that of Cui *et al.* (2016) who also mentioned 0.025% MIC value of cinnamon oil against a number of pathogens. Ashraf *et al.* (2017) also worked on MIC of clove extract. His findings are also similar to that of present study; clove exhibited 11.4mm and 9.2mm zones of inhibition against *S. aureus P. aeruginosa,* respectively.

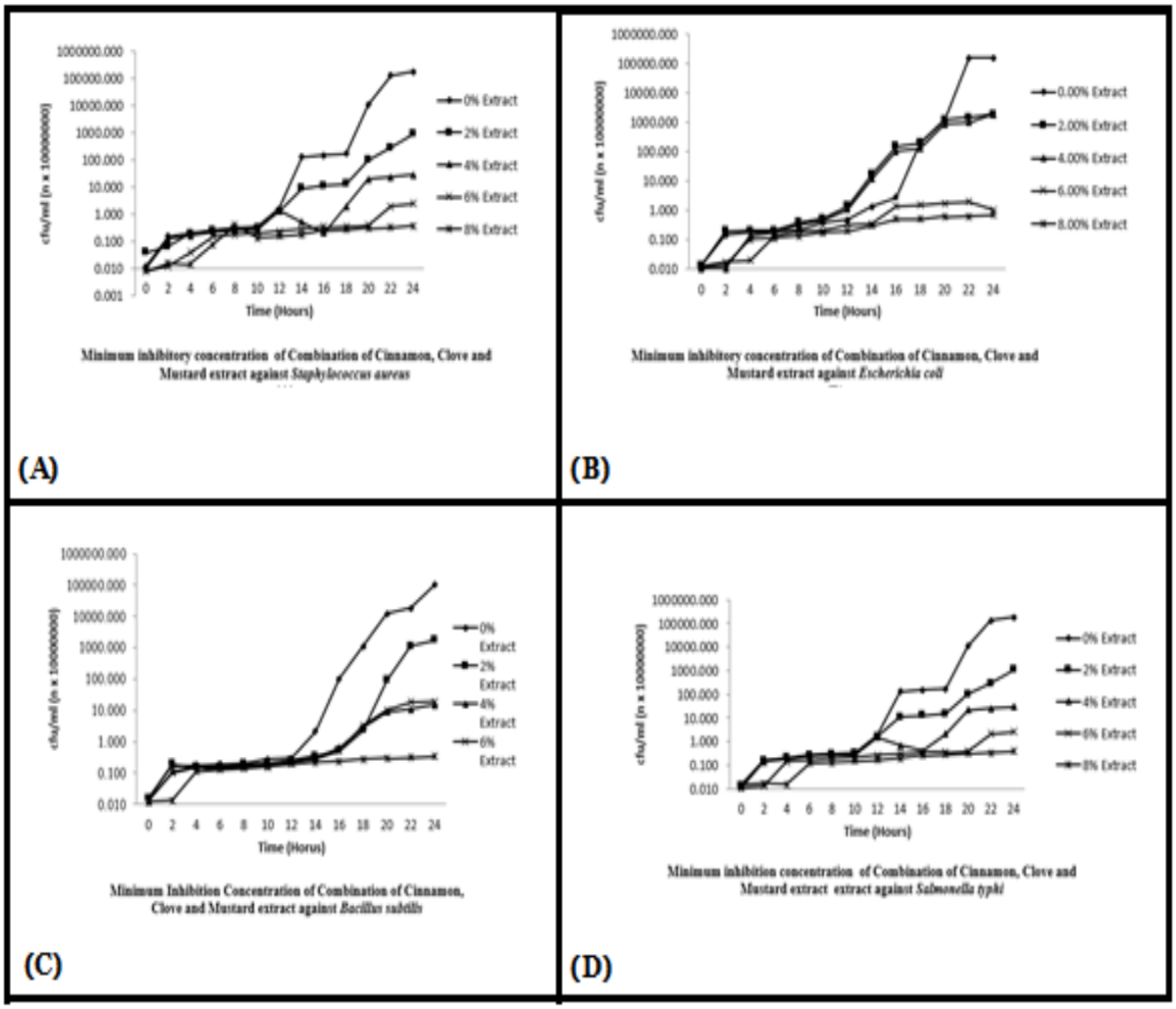

Fig. 1. Minimum inhibitory concentration of Combination of Cinnamon, Clove and Mustard extract against various pathogens.

Conclusion

From these results it was concluded that the extracts of cinnamon, clove, oregano and mustard possessed significant antimicrobial potentials against food borne pathogens due to the presence of various bioactive compounds. Synergy was observed in extracts when used in combination. The best antimicrobial activity was observed in case of ethanolic extracts. Keeping in view the antibacterial potential of extracts it was concluded that the extracts of cinnamon, clove, mustard and oregano can be effectively used as bio-preservative and treatment of food borne infectious disease. However, there is need of *in-vivo* studies and identification of bioactive compounds in the extracts. Moreover, there is also need of research work on other spices and herbs as well.

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