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IN VITRO ANTIMICROBIAL ACTIVITY AND PHYTOCHEMICAL STUDIES OF *TERMINALIA CHEBULA* AGAINST THE MICROBES ISOLATED FROM FRUIT JUICES

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ABSTRACT

The present work has been conducted to evaluate the antimicrobial activity of *Terminalia chebula* against microorganisms associated with juices. Methanol, ethanol, acetone, and aqueous (hot and cold) extracts from fruits of *T. chebula* were tested for their antimicrobial activity through agar well diffusion method and minimum inhibitory concentration (MIC)/minimum bactericidal concentration (MBC) values were determined through the macrodilution broth method against *Bacillus cereus*, *Serratia* sp. and *Rhodotorula mucilaginosa*. Their total phenolic content and total tannin content were also evaluated. Organic and cold aqueous extracts displayed activity against all three tested microbes. There were highly positive relationship between antimicrobial activities and phenolic and tannin content of the tested extracts against each microorganism. Methanolic extract was found to be best against all tested microbes with lowest MIC of 0.78 mg/ml and MBC of 1.56 mg/ml and showed better antimicrobial activity than sodium benzoate. Therefore, methanolic extract of *T. chebula* has a biopreservative potential in fruit juices.

Keywords: *Terminalia chebula*, preservative, MIC, MBC

INTRODUCTION

Consumption of fresh fruit juices has increased in the last decades owing to antioxidants, vitamins and minerals that these juices provide to the people and help in reducing the risk of heart diseases, cancer and diabetes (**Mosqueda-Melgar *et al*., 2012**). These are considered as safe foods due to their low pH caused by naturally occurring organic acids which may prevent the growth of microbes (**Mosqueda-Melgar *et al*., 2008)**, but allow the growth of certain acid tolerant bacteria, yeasts and moulds. Spoilage of fruit juices is characterized by formation of CO2, alcohol, cloud loss and formation of fermented flavor attributed to pectin esterases (**ICMSF 2005; Lawlor *et al*., 2009; Tribst *et al*., 2009**). Fruit juices spoilage bacteria include acid tolerant bacteria such as acetic acid bacteria, lactic acid bacteria, *Clostridium*, *Bacillus*, members of Enterobacteriaceae family (*Klebsiella* sp., *Citrobacter* sp. and *Serratia* sp.) and some heat resistant bacteria such as *Alicyclobacillus acidoterrestris* and *Propionibacterium cyclohexinicum*. Among yeasts *Pichia*, *Candida*, *Saccharomyces* and *Rhodotorula* are commonly encountered genera responsible for spoilage of juices (**Lawlor *et al*., 2009; Raybaudi-massilia *et al*., 2009**; **Bevilacqua *et al*., 2011**).

Chemical preservatives such as sodium benzoate are widely used in foods and beverages, that naturally are in pH range below 4.5, in several countries and enjoy GRAS status up to a maximum permitted level of 0.1% in USA and 0.15-0.25% in Many countries (**Chipley, 2005**).

Consumer demand for minimally processed food products, with less use of chemical preservatives and at the same time without compromising food safety has increased interest in the use of natural antimicrobial compounds as biopreservatives in fruit juices (**Lawlor *et al*., 2009; Negi, 2012**). Natural antimicrobials such as organic acids, essential oils, plant extracts, and bacteriocins could be good alternative to ensure food safety (**Raybaudi- Massilia *et al*., 2009** ; **Burt, 2004; Tiwari *et al*., 2009).** The use of plant extracts, either as pure compounds or as standardized extracts, provides unlimited opportunities for control of microbial growth attributed to their chemical diversity.

Terminalia chebula is important medicinal plant widely distributed throughout India, Burma, Pakistan, Nepal, south-west China and Srilanka. It is commonly known as black myroblans and harad, belong to *Combretaceae* family. In Tibet, the fruit of *Terminalia chebula* is called the "king of medicine". It is medium size deciduous tree attaining a height of 25-30 m; with spreading branches and a broad roundish crown (**Sharma *et al*., 2012**; **Rathinamoorthy and Thilagavathi, 2014**).

The fruit of the tree possesses diverse health benefits and has been used as traditional medicine for household remedy against asthma, sore throat, vomiting, hiccough, diarrhea, bleeding piles, gout, and heart and bladder disease. It is good to increase appetite, digestive aid, liver stimulant, stomachic, gastrointestinal, and mild laxative (**Bag *et al*., 2013**). In *Terminalia chebula*, 33% of the total phytoconstituents are hydrolysable tannins and are responsible for pharmacological activity. Many researchers have reported 14 components of hydrolysable tannins like gallic acid, chebulagic acid, punicalagin, chebulanin, corilagin, neochebulinic acid, ellagic acid, chebulinic acid, 1,2,3,4,6-penta-O-galloyl- β-D-glucose, 1,6-di-o-galloyl-D-glucose, casuarinin, 3,4,6-tri-o-glloyl-D-glucose, terchebulin (**Juang *et al*., 2004; Sharma *et al*., 2012; Bag *et al*., 2013**)

The nature of the extracting solvent is most important factor in the extraction of antimicrobial compounds from plants. The most suitable solvents for plant extraction are polar solvents such as water, methanol, ethanol and acetone (**Chang and Lin, 2012**).

The main aim of the present study was to determine the *in vitro* antimicrobial activity of *T. chebula* fruits against the isolated microbes from fruit juices and to compare the effect of different solvents in the extraction method for antimicrobial activity. Quantitative phytochemical analysis of phenol and tannin content of different solvent extracts of *T. chebula* fruits was performed to establish a relationship between microbial inhibition and phytochemical constituents (Phenol and tannin).

MATERIALS AND METHODS

Plant collection

The fruits of *T. chebula* were obtained from the local market in Yamunanagar. The taxonomic identity of the plant was confirmed by Dr. B.D. Vashishta , plant taxonomist, Chairman of Botany Department, Kurukshetra University, Kurukshetra.

Extraction of plant material

The samples were carefully washed under running tap water followed by sterile distilled water and air dried at room temperature (35-40^{0}C) for 4-5 d, homogenized to a fine powder using a sterilized mixer grinder and stored in air tight bottles. Four different solvents, namely ethanol, methanol, acetone and aqueous (hot and cold), were used for extraction. A 10 g of fruits were separately

soaked in conical flasks each containing 100ml of acetone, ethanol, methanol (95%) and sterile distilled water.
Also, an equal amount (i.e., 10 g) of homogenized fruits was immersed separately in 100ml of hot sterile distilled water in conical flasks and allowed to stand for 30 min in a water bath (at 100^0C) with occasional shaking, followed by keeping all the flasks on rotary shaker at 200 rpm for 24 h. Each preparation was filtered through a sterilized Whatman No. 1 filter paper and finally concentrated to dryness under vacuum at 40^0C using a rota evaporator. The dried extract thus obtained was sterilized by overnight ultra violet-irradiation, checked for sterility on nutrient agar plates and stored at 4^0C in labelled sterile bottles until further use (**Sharma *et al*., 2012**).

Test microorganisms

Two bacteria, namely *Serratia* sp.(KC67407*), *Bacillus cereus* KRC1 (KC67408) and one yeast, *Rhodotorula mucilaginosa* (KC67409) were isolated from fruit juices. Bacterial strains were identified on the basis of gram staining, biochemical and molecular characteristics (16S rRNA sequencing) (Lawlor *et al*, 2009).Yeast was identified on the basis of staining, morphological, cultural characteristics and molecular characteristics (28S rRNA sequencing). The bacterial isolates were subcultured on nutrient agar and *R. mucilaginosa* on potato dextrose agar and incubated aerobically at 37^0C and 25^0C respectively. The media were procured from Hi Media Laboratory Pvt. Ltd., Bombay, India. (*Nucleotide sequence of all microorganisms has been submitted to GenBank database which provided the GenBank accession number, KC67407- KC67409).

Screening for antimicrobial activity

The acetone, methanol, ethanol, hot and cold aqueous solvent extracts of *T. chebula* fruits were used for evaluation of antimicrobial activity by the agar well diffusion method. In this method, a pure isolate of bacteria and yeast was grown on NA and PDA plates and incubated at 37^0C and 25^0C for 24 h and 72h respectively. One plate of each microorganism was taken and colonies were transferred into normal saline (0.85%) under aseptic conditions. Density of each microbial suspension was adjusted to be equal to that of 10^6cfu/mL (standardized by 0.5McFarland standard) and used as the inoculum for performing an agar well diffusion assay. One hundred microliter (100 µL) of the inoculum of each test organism was spread onto the agar plates so as to achieve a confluent growth. The agar plates were allowed to dry and 8 mm wells were made with a sterile borer in the inoculated agar plates. The lower portion of each well was sealed with molten agar medium. The dried extract samples of *T. chebula* fruits was dissolved in dimethylsulphoxide (DMSO) to the final concentration of 100 mg/mL for the bioassay analysis. A 100 µL volume of each extract was propelled directly into the wells (in triplicate) of the inoculated agar plates for each test organism. The plates were allowed to stand for 1 h at room temperature (40^0C) for diffusion of the extract into agar and incubated at 37^0C and 25^0C for 24 h and 72h respectively. Sodium benzoate (100mg/ml) was used as positive reference standards to determine the sensitivity of each microbial species tested. Sterile DMSO served as the negative control. The antimicrobial activity, indicated by an inhibition zone surrounding the well containing the extract, was recorded if the zone was greater than 8 mm. The experiments were performed in triplicate and the mean values of the diameter of inhibition zones ± standard deviations were calculated (**Shan *et al*., 2007; Aneja *et al*., 2011**).

Determination of minimum inhibitory concentration

Minimum inhibitory concentration (MIC) for each test organism was determined by the macrodilution broth method. A twofold serial dilution of each extract was prepared by first reconstituting the dried extract (100 mg/mL) in DMSO, followed by dilution in Mueller-Hinton broth (bacteria) and potato dextrose broth (yeast) to achieve a decreasing concentration range of 50 mg/mL to 0.39 mg/mL. Each dilution was seeded with 100 µL of the standardized microbial inoculum (1.5×10^6 cfu/mL). The inoculated culture tubes of bacteria were incubated at 37^0C for 24 h and yeast at 25^0C for 72h. A set of tubes containing only broth was kept as control. Afterwards, incubation tubes were examined for changes in turbidity as an indicator of growth. The lowest concentration that did not permit any visible growth was considered as MIC (**Das *et al*., 2010**).

Determination of minimum bactericidal concentration

Minimum bactericidal concentration (MBC) is the lowest concentration of antimicrobial agent that will not allow the growth of an organism after subculturing on antibiotic free media. MBC was determined by subculturing the preparations that did not show any bacterial growth in the MIC determination. A 100 µL aliquot from the selected tube (showing MIC) was spread over the MHA plate and incubated at 37^0C for 24 hours and examined for bacterial growth. The MBC, the lowest concentration of the plant extract giving 99.9% reduction of the bacterial growth of various plants parts against the bacterial pathogens, was recorded (**Ncube *et al*., 2008; Sharma *et al*., 2012**).

Determination of minimum fungicidal concentration

A loopful of culture from each set of tubes that did not show any visible growth of the yeast in MIC determination was subcultured on to fresh plates of PDA and incubated at 25^0C for 72 hours. Minimum fungicidal concentration for each plant extracts against the tested yeast was recorded as the lowest concentration that did not yield any fungal growth on the solid medium (**Aneja *et al*., 2011**).

Determination of total phenolic content

Total phenolic content was estimated using the Folin- Ciocalteu colorimetric method. The appropriate dilutions of the extract sample (0.2 ml) were oxidized 4 min with 1 ml of 0.5M Folin-Ciocalteu reagents and then the reaction was neutralized with saturated sodium carbonate (75 g/l) 1 mL. The absorbance of the resulting blue color was measured at 760 nm with a spectrophotometer after incubation for 30 min. at room temperature. Quantification was done based on a standard curve of gallic acid. Results were expressed as milligram of expressed in milligram gallic acid equivalents (mg gallic acid/g extract). All tests were performed in triplicate (Shan *et al*., 2007).

Determination of total tannin content

Analysis of total tannin was determined by titrimetric method. Zinc ion reacts with tannin compounds in alkali solution, to form complexes. Residual zinc ion is then titrated with EDTA, and zinc complexed tannin is determined from EDTA consumption and total zinc content. The acetone, methanol, ethanol, hot and cold aqueous extracts (1 mg each) were each placed into glass vials and dissolved with 1mL of deionized water. The vials were warmed in a water bath for 5 min at 35 ± 2◦C. ZnAc (1 M, 0.4mL) and NH_3 (0.28mL) were mixed together, and the warmed 1-mL extract solutions were added. The solutions were replaced in the water bath for 30 min at 35 ± 2◦C. Deionized water (8.92 mL) was added to make the final volume up to 10.6mL. After careful filtering, sample solutions were obtained. The solutions (0.8 mL) were further diluted with 5.2mL of deionized water, and 0.5mL of NH3–NH4Cl buffer (pH 10) was added. Finally, the mixture was titrated with 0.05M EDTA. The blank was detected without addition of the extract. The total tannin content (%/mg extract) of each extract was calculated as follows: $\{[0.1556 \times (V_{blank} - V_{extract})]/W_{extract}\} \times 100\%$. V_{blank} and $V_{extract}$ represent the EDTA titration volumes (mL) recorded for the respectiveblank and extract solutions. W represents the weight (mg) of each extract (Shan *et al*., 2007).

Statistical analysis

The experimental results were repeated thrice in triplicate each time and expressed as mean ± SD and results were statistically evaluated using Dennett's *T*-test. *P* value less than 0.05 was considered significant.

RESULTS

Extraction yield

A perusal of data in table 3 represent the yield, total phenolic and tannin content of the four extracts. The yield of the four extracts varied from 13.6% to 19.6%.

Total phenolic content

Total phenolic content was determined for the extracts of *T. chebula* which show the best antimicrobial activity from a linear gallic acid standard curve. The total phenolic content of four extracts varied from 416.3 mg to 720 mg gallic acid/g extract. The greatest concentration of phenolic content was observed in methanolic extract of *T. chebula* fruits.

Tannin content

The total tannin content of the four active extracts varied from 15.3.6 to 29.3%. This result suggests that the methanolic extract provided the greatest concentration of tannin content of the four extracts.

Effect of solvent on antimicrobial activity

The antimicrobial activity of *T. chebula* fruits extracts differed in various organic (methanol, ethanol, acetone) and aqueous (hot and cold) extracts. Positive control showed significantly sized inhibition zone against the tested bacteria (ranging between16.8-21.7) and the yeast (with zone of inhibition 14.7) and negative control did not produce inhibitory effect against all tested microbes. A perusal of the data in table 2 reveals that all organic and cold aqueous solvents of fruit extracts possessed antibacterial and antiyeast activity against tested microbes. Hot aqueous extracts of fruits of *T. chebula* lacked antimicrobial activity against *Serratia* sp. and *R. mucilaginosa*. Methanolic extract of fruits was found most effective against *B. cereus* (25.6 mm) followed by *Serratia* sp. (23.3mm). The

antimicrobial activity of organic extracts of fruits was found better than sodium benzoate.

The MIC values ranged between 0.78 mg/ml and 50 mg/ml for the different fruit juice associated microorganisms. MBC values ranged between 1.56 mg/ml and 50mg/ml. Among all the tested fruits solvent extracts, methanolic extract of fruits was the best solvent where the lowest MIC of 0.78 mg/ml and MBC of 1.56 mg/ml was observed against *B. cereus* that followed by MIC value of 6.25 mg/ml against *R. mucilaginosa* and MIC was 12.5 mg/ml for *S. marcescens* (Table 3). All the obtained results were statistically significant as they showed ($P< 0.05$) compared with control (Table 2).

Relationship between antimicrobial activity, total phenolic content and total tannin content

The correlation between antimicrobial activity and phenolic content are shown in figure 1. The R^2 values were between 0.99 and 0.88 and decreased in the following order *Serratia*> *B. cereus*> *R. mucilaginosa*.

The relationship between antimicrobial activity and total tannin content was also calculated (Fig 2). The R^2 values were between 0.70 and 0.95 and decreased in the following order *B. cereus*> *Serratia*> *R. mucilaginosa*.

Table 1 Extraction yield, total phenolic content and total tannin content of the four extracts of *Terminalia chebula*

Extract	Extraction yield[a]	Total phenolic content[b]	Total tannin content[c]
Acetone	17.2±1.8	556.6±7.6	21.0±2.64
Methanol	19.6±2.6	720.0±9.5	29.3±2.5
Ethanol	15.4±2.4	600.3±8.3	26.6±3.2
Cold aq	13.6±1.2	416.3±5.2	15.3± 3.1

The data are presented as mean±SD for three replicates
[a] %w/w
[b] mg gallic acid /g extract
[c] %mg extract

Table 2 Antimicrobial activity of *Terminalia chebula* fruits extracts against juice associated bacteria and yeast by agar well diffusion method

Solvent extracts mg/ml	Bc	Sm	Rm
Acetone	19.3[a]±0.57[b]	18.6±1.15	17.6±0.57
Methanol	25.6±0.57	23.3±0.57	19.3±0.57
Ethanol	22.6±0.57	19.3±0.57	16.6±0.57
Cold aq	17.3±0.57	14.6±1.15	14.3±0.57
Hot aq	14.3±0.57	-	-
Control	20±1	15.6±0.57	14.6±0.57
Dmso	-	-	-

Bc= *Bacillus cereus*, Sm= *Serratia marcescens*, Rm= *Rhodotorula mucilaginosa*, (-) = no activity, [a]Values, including diameter of the well (8mm), are means of three replicates, [b]± Standard deviation, data were analyzed by one way analysis of variance followed by Dunnett"s test.

Table 3 Minimum inhibitory concentration, minimum bactericidal concentration and minimum fungicidal concentration of *Terminalia chebula* leaves and fruits extracts against juice associated bacteria and yeast

Solvent extracts	mg/ml	Bc	Sm	Rm
Acetone	MIC	1.56	6.25	25
	MBC	3.12	12.5	50
Ethanol	MIC	1.56	25	12.5
	MBC	3.12	50	25
Methanol	MIC	0.78	12.5	6.25
	MBC	1.56	25	12.5
Hot aqueous	MIC	50	Nt	Nt
	MBC	>50	Nt	Nt
Cold aqueous	MIC	50	25	25
	MFC	>50	50	50

Bc= *Bacillus cereus*, Sm= *Serratia marcescens*, Rm= *Rhodotorula mucilaginosa*, MIC= Minimum inhibitory concentration;MBC= minimum bactericidal concentration; MFC= minimum fungicidal concentration.

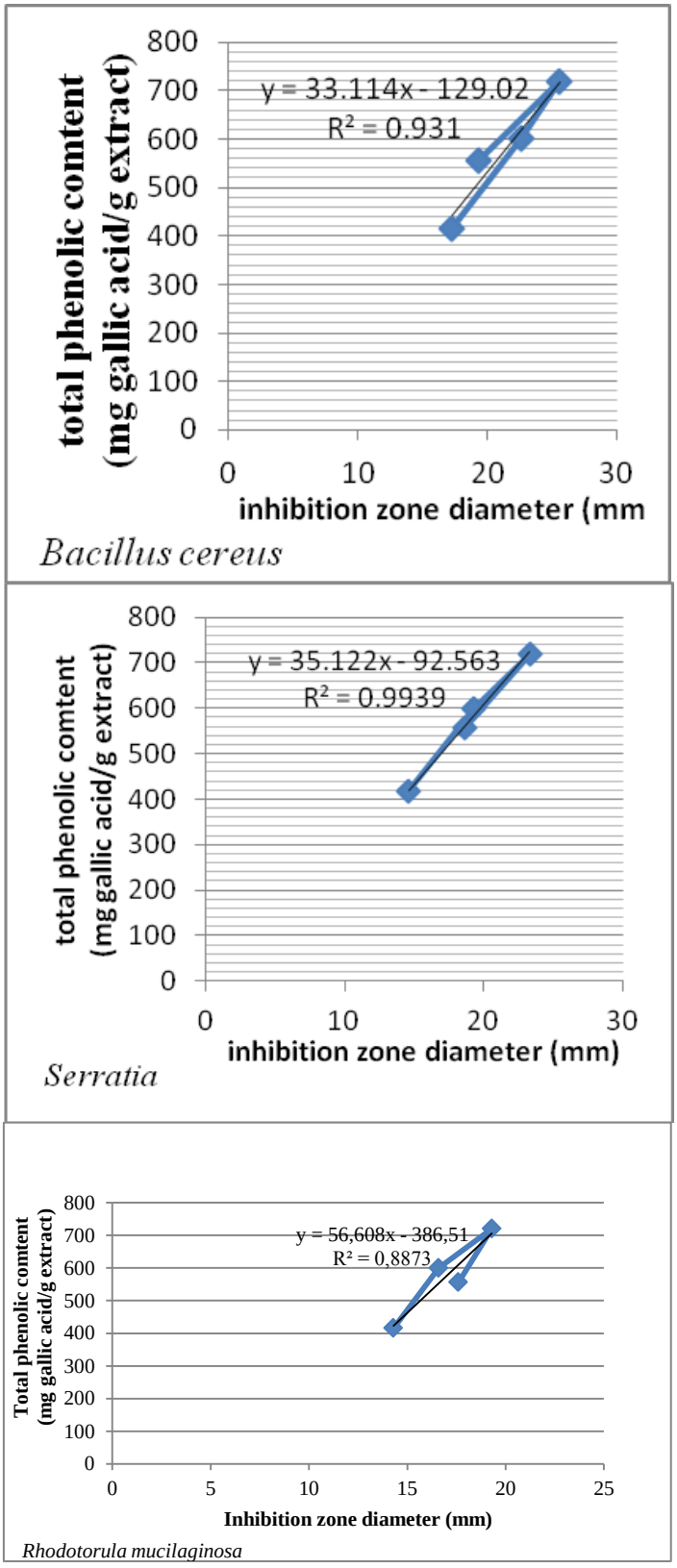

Figure 1 Relationship between inhibition zone diameter (mm) and total phenolic content (mg gallic acid equivalents/ g extract) of 4 solvent extracts from *T. chebula* fruits for microbes associated with juices

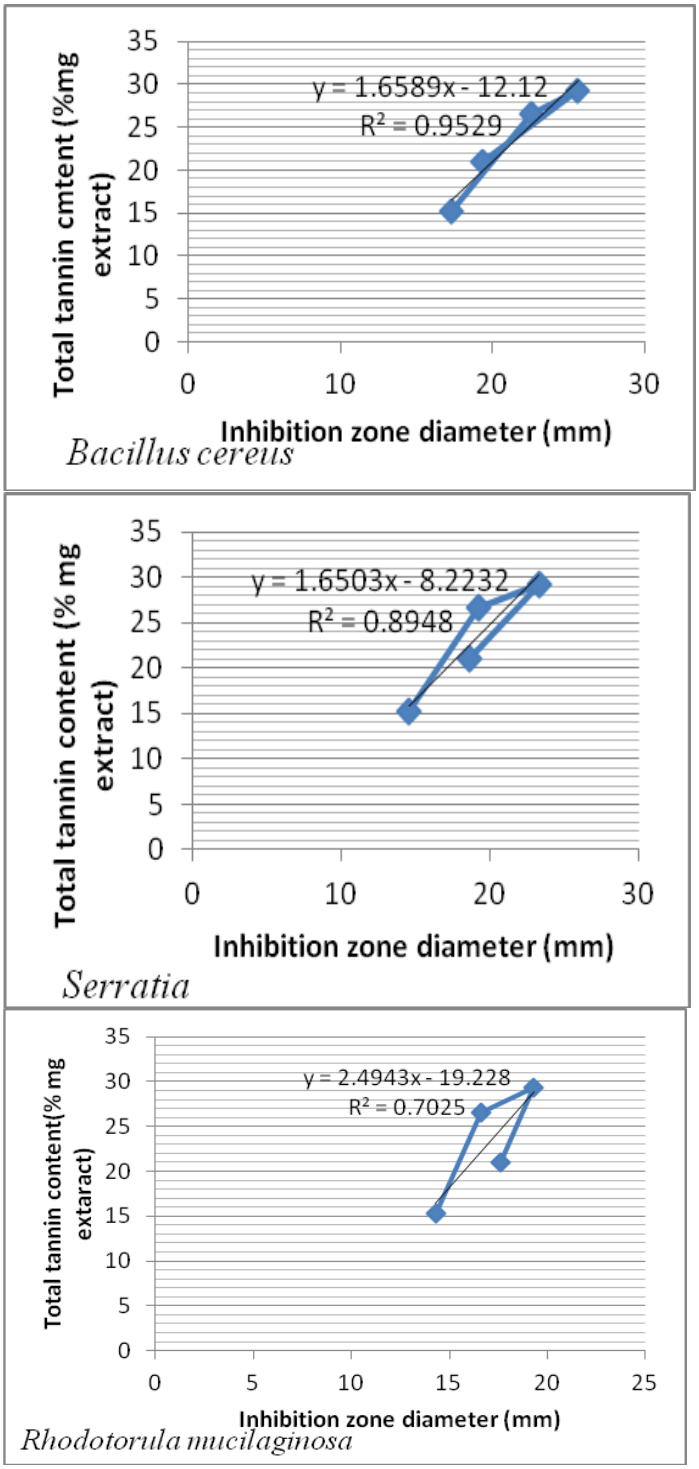

Figure 2 Relationship between inhibition zone diameter (mm) and total tannin content (% mg extract) of 4 solvent extracts from *T. chebula* fruits for microbes associated with juices

DISCUSSION

The use of dietary herb and spices as food additives in foods to improve the sensory characteristics of food but also increase the shelf life by reducing or eliminating survival of pathogenic bacteria (**Tajkarimi *et al*., 2010**). Numerous studies have been done *in-vitro* to evaluate the antimicrobial activity of plant extracts; very few studies are available for food products owing to the use of crude extracts in most studies (**Negi, 2012**). *T. chebula* is an important medicinal plant always listed at the top of the list of 'Ayurvedic Materia Medica' attributed to its extraordinary power of healing and traditionally used in the treatment of various ailments for human beings (**Bag *et al*., 2012**). Therefore, in the present study, different organic (ethanol, methanol, acetone) and aqueous (hot and cold) fruit extracts of this plant were evaluated for their antibacterial and antifungal potential for the first time against the microbes associated with fruit juices.

In our observations, the organic fruit extracts of *T. chebula* were found to be the most active in inhibiting the growth of all the tested microbes compared to aqueous extracts. Our investigation was confirmed by earlier observations on an alcoholic extract that exhibited greater activity than the aqueous extracts against bacteria (**Ahmad *et al*., 1998; Sharma *et al*., 2012; Bag *et al*., 2012**). Several other works has also been reported about the antibacterial activity of *T. chebula* fruit extracts against *E. coli*, *Helicobacter pylori*, *Staphylococcus aureus*, *Salmonella typhi*, *S. epidermidis*, *Bacillus subtilis*, *Proteus vulgaris* and *Pseudomonas aeruginosa* (**Chattopadhyay and Bhattacharyya, 2007; Kannan *et al*., 2009; Sharma *et al*., 2012**). *T. chebula* fruit extracts in organic solvents also exhibit antifungal activity against *R. mucilaginosa* in present study and same observations were confirmed by several workers (**Dutta *et al*., 1998; Mehmood *et al*., 1999; Bonjar, 2004).**

In the present study, among the three microorganisms tested, *B. cereus* was the most sensitive to the five different extracts of *T. chebula* fruits. The highest sensitivity of *B. cereus* may be due to its cell wall structure and outer membrane (**Shan *et al*., 2007; Negi, 2012**).

A majority of the described antimicrobial effects of *T. chebula* extracts have been attributed to their secondary metabolites, notably tannins, gallic acid, chebulic acid, chebulagic acid, corilagin, mannitol and other compounds **(Bag *et al*., 2012, 2013**). Phenols and tannins are the most common active constituents of plant extracts possess antimicrobial activities (**Ahmed and Beg, 2001**). Other researchers have also reported that phenolic and tannin content from plant sources could inhibit various food borne pathogens (**Shan *et al*., 2005**). The current study investigated a highly linear correlation between phenolic content and antimicrobial activity of the extracts of *T. chebula* fruits. Same results have been showed between the total tannin content and antimicrobial activity of extracts of *T. chebula* fruits. **Shan *et al* (2007**) observed the highly positive correlation between phenolic content and antimicrobial activities of spice and herbs. The present work shows that antimicrobial activity of the extracts of *T. chebula* fruits is closely related to the concentration of phenolic and tannin content of the extracts. The fruit extracts of *T. chebula* in organic solvents possess better antimicrobial activity than sodium benzoate, showing great potential to be developed as a natural preservative to control the spoilage and pathogenic bacteria in fruit juices.

CONCLUSION

T. chebula is one of the most versatile plants having a wide spectrum of pharmacological and medicinal activities. This versatile medicinal plant is the unique source of various types of compounds having diverse chemical structure. The present study revealed that *T. chebula* possesses promising antimicrobial activity and can serve as an alternative to synthetic antimicrobials. However, further experiments, *in vivo* studies of these constituents in fruit juices to check the survival of microorganisms and examine its effect on physical, chemical properties of juices.

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