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DETERMINATION OF *IN VITRO* ANTIMICROBIAL ACTIVITY OF *STEVIA REBAUDIANA* (BERTONI) LEAF EXTRACTS AGAINST ANTIBIOTIC RESISTANT MICROORGANISMS

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Abstract – Antimicrobial activity of *Stevia rebaudiana (Bertoni*), well known as stevia plant which leaves extracts were investigated against a large number of microorganisms, but not yet evaluated its activity against resistant microorganisms. The aim of this study was to evaluate the antimicrobial proficiency of stevia against antibiotic resistant microorganisms (10 bacteria, 6 fungi and 16 yeast species) using Kirby-Bauer disc diffusion technique. Extracts were obtained from the stevia powder of leaves using different solvents like as n-hexane, petroleum ether, acetone, ethanol and water. Among different extracts of stevia, n-hexane, petroleum ether and acetone extracts were more potentially effective with variable efficiency against both gram positive and gram negative resistance bacteria compared to ethanolic and water extracts, respectively. Maximum zone of inhibition (21.0±0.5 mm) was observed with n-hexane extracts against *Bacillus cereus* and minimum zone of inhibition (7.5±0.5 mm) was specified by the water extracts against *Listeria monocytogenes*. The n-hexane extracts inhibited significant number of mycelial growth of tested fungi compared to other solvents extracts. Likely as, extracts with n-hexane exhibited more efficacies against most of the tested yeasts, particularly for *Candida* species. Minimum inhibitory concentration (MIC), minimum bactericidal concentration (MBC) and minimum fungicidal concentration (MFC) were also determined. The results of present study indicate that the stevia leaf extracts have inhibitory efficacy against microorganisms and further study will reveal the possibility of employing them in medicines for the treatment of infectious diseases caused by the test microorganisms.

INTRODUCTION

Expansions of antibiotic resistant microorganisms are one of major warning for the global health, at the same time considerably threatened to the existing antimicrobial therapy (Alanis, 2005). In recent years, infectious diseases increased to a great extent across the world and leading cause of death in both developing as well as developed countries (Luqman *et al*., 2005; Mothana and Lindequist, 2005). According to USA estimations, about 2.22 million hospitalized patients had adverse drug reactions and 1,06,000 patients died in every year (Joshi *et al*., 2011; Selvamohan *et al*., 2012). Researchers all over the world give effort to uncover new antimicrobial drugs but still we are facing multidrug resistance bacterial infections, moreover most of the present antibiotics are not devoid of side effects (Dowzicky and Park, 2008; Rudrappa and Bais, 2008). Within this situation search for new antimicrobial compounds are urgent that supposed to same efficacy with superior security. To explore the new antimicrobial agent bacteria, fungi, algae, symbiotic lichens, mosses and higher plants are used from the widen period (Bhatia and Narain, 2010; Joshi *et al*., 2011). A gigantic number of medicinal plants have been acknowledged as valuable provisions of natural antimicrobial compounds as a substitute that can potentially be feasible in the treatment of these erratic bacterial infections (Val *et al*., 2001; Chandra

et al., 2017). Following the information of the World Health Organization (WHO), medicinal plants would be the ideal resource to obtain a variety of remedies (Regginato *et al*., 2020).

A vast number of plants have been recognized as precious sources of natural antimicrobial compounds as an alternative which can potentially be effective in the microbial infections (Iwu *et al*., 1999; Selvamohan *et al*., 2012). The present work was therefore designed to investigate the antimicrobial effects of important medicinal plant namely *Stevia rebaudiana (Bertoni*) (commonly known as Stevia) against a range of gram positive and gram negative pathogenic bacteria as well as different fungal and yeast species. *S.rebaudiana* belongs to family Asteraceae and synonymously termed as natural sweetener. The leaves of stevia are significant to control blood pressure, cholesterol and diabetes as well as have antimicrobial properties (Jayaraman *et al*., 2008); Shamima *et al*., 2019). The leaves of stevia plant also known as honey leaf or sweet leaf and contain various metabolites like alkaloids, terpenoids, glycosides, tannins and flavonoids (Liu and Li, 1995; Chalapathi *et al*., 1999). The major compound of stevia green leaves are stevioside that are non-caloric in nature (Savita *et al*., 2004). The bioactive substances of this plant have a wide range of biological functions; however, pharmacological studies are very limited. At the same time, available studies have assessed antimicrobial activity against few microorganisms. This study aimed to evaluate the antimicrobial activity of this important medicinal plant against antibiotic resistant isolates of gram positive and gram negative bacteria, in addition to pathogenic fungi and yeast, also performing minimum inhibitory concentration (MIC), minimum bactericidal concentration (MBC) and minimum fungicidal concentration (MFC).

MATERIALS AND METHODS

Sample collection

Disease free fresh leaves of stevia were collected from Khagrachari district, Bangladesh. Samples were labeled and stored at 4 °C temperature before processing. Fresh stevia leaves were washed, dried under shade condition for two weeks and cut into small pieces, crushed with a blender to make fine powder. Coarse parts were discarded and only fine powder was stored in air tight bags at 4 °C for use in extraction process.

Plant extracts preparation

The powdered plant materials obtained from stevia leaves were treated consecutively solvents like as n-hexane, petroleum ether, acetone, ethanol and water to afford corresponding fractions (Dabur *et al*., 2007). Five different extracts were prepared by dissolving 1g of stevia leaf powder in total of 100 mL of each solvent separately to make 10 mg/mL final concentration. This whole procedure was done in three separate segments. Firstly, 1 g of stevia powder was soaked in 40 mL of each solvent for 2 hours and then filtered with whatman filter paper (Sigma-Aldrich). Secondly, the remaining solid part was treated again with same procedure with 30 mL of each solvent for another 2 hours and then filtered. Finally, the solid part was treated with another 30 mL of each solvent for 18-20 hours with the same procedure and then filtered. All filtrate of same solvent from three separate segments for each were collected in same bottle that the total amount of solution of each solvent became 100 mL (40 mL + 30 mL + 30 mL). This process was done at room temperature and prepared extracts of stevia leaves with different solvents were stored at 4 °C in air tight bottles for further experiments.

Test microorganisms

This study was handled with 10 resistant human pathogenic bacteria such *Bacillus cereus*, *Staphylococcus aureus*, *Enterobacter faecalis*, *Listeria monocytogenes*, *Pseudomonas aeruginosa*, *Vibrio parahaemolyticus*, *Salmonella typhimurium*, *Shigella flexneri*, *Escherichia coli* and *Klebsiella pneumoniae* were used to check the inhibition capacity of stevia plants leaves extracts. Subsequently, 6 resistant fungi such as *Aspergillus flavus*, *Cladosporium herbarum*, *Penicilliumro queforti*, *Trichoderma viride*, *Fusarium chlamydosporum* and *Macrophomina phaseolina* were also used. Furthermore, 16 resistant yeast species (previously identified with Biolog™ identification system, Laboratory species) were also used to verify the efficiency of stevia leaves. These resistant yeast species were *Candida shehatae*, *Candida maltosa*, *Candida incommunis*, *Candida zylanoids*, *Candida parapsilosis*, *Rhodotorulaa cheniorum*, *Rhodosporidium diobovatum*, *Trichosporon beigelii*, *Zygosaccharomyces bailii*, *Clavispora lusitaniae*, *Kluyveromyces delphensis*, *Hanseniaaspora osmophila*, *Debryomyces hansenii*, *Kluyveromyces thermotolerans*, *Schizosaccharomyces octosporus* and *Kluyveromyces marxianus*. A series of morphological (growth on suitable media), physiological and biochemical tests as well as

microscopic observation were carried out to identify the selected isolates (Cheesbrough, 1999). Antimicrobial susceptibility test to all microorganisms were performed according to modified Kirby-Bauer Disk diffusion method following the Clinical and Laboratory Standards Institute (CLSI; Wayne, PA, USA) guideline (Hudzicki, 2009). Antibiotic resistant isolates were defined as those isolates that are resistant to one or more classes of antibiotics (Clinical and Institute, 2015).

Antimicrobial susceptibility assays

Antibacterial activity

Various extracts of Stevia leaves were subjected to antibacterial susceptibility assay using the agar disc diffusion method (Sahraei *et al*., 2014) by using Mueller Hinton Agar (MHA) medium (Hi Media). Ciprofloxacin (500 mg) disk was used as positive control and 10% DMSO soaked filter paper disk was used as the negative control, respectively. Incubation period was maintained for 18-24 hours at 37±2 °C. After incubation, plates were observed for the formation of a zone around the well that corresponds to the antimicrobial activity of tested extracts. The zone of inhibition (ZOI) formed on the medium were evaluated in mm using a scale. The experiment was carried out in triplicates.

Antifungal activity

Poisoned food technique was employed to determined antifungal activities (Grover and Moore, 1962). Rose Bengal (RB) agar (Hi-Media) medium was used for the culture of fungi. All the results were compared with the standard antifungal antibiotic Nystatin (100 ppm). The zone of inhibition (ZOI) formed on the medium were evaluated in mm using a scale. Fungus growth was measured up to 5 days of incubation at 30±2 °C. The percentage of inhibition was calculated as follows:

$$I = (C-T)/C \times 100$$

Where, I = percentage of inhibition; C = diameter of the fungal colony in the control and T = diameter of the fungal colony in treatment.

Activity against yeast

The various extracts of stevia leaves activity against resistant yeasts were also assayed by the disk diffusion method (Hudzicki, 2009). Sabouraud Dextrose Broth (SDB) medium (Hi Media) was used to culture yeast species. Incubation period was sustained for 24 hours at 30±2 °C. After incubation period, plates were observed for the formation of a zone around the well that corresponds to the activity of yeast of tested plant extracts. The zone of inhibition (ZOI) was assessed in mm.

Assessment of minimum inhibitory concentration (MIC), minimum bactericidal concentration (MBC) and minimum fungicidal concentration (MFC)

The minimum inhibitory concentration (MIC) was considered as the lowest concentration of the extract that completely inhibits the bacterial growth and minimum bactericidal concentration (MBC) is the lowest concentration of an antibacterial agent required to kill a particular bacterium (Mishra *et al*., 2017). The broth microdilution method was used to determine the MIC and MBC according to CLSI guideline (Hudzicki, 2009). Serial dilutions of the plant extracts were prepared with 10% DMSO of the original extract. Inoculums of microorganism were prepared in Mueller Hinton Broth (MHB) medium (Hi Media) to give a final concentration of 5×10^5 CFU/mL in each well. 100 mL of plant extract was added to each well of the 96-well microplate. Fifty (50) mL of bacterial suspension was added to each well except the negative controls. Ciprofloxacin (500 mg) was used as positive control and 10% DMSO soaked filter paper disk was used as the negative control, respectively. The plates were incubated at 37±2 °C for 18-24 hours. Antimicrobial activity was assessed by measuring absorbance at 690 nm of wave length. MFC values of plant extracts against six tested fungi were also determined by micro and macro dilution broth technique using RB medium (Hi-Media) (Jones, 1985).

Statistical analysis

Analysis of variance (ANOVA) was used for data analysis. The significance of the differences among treated samples was evaluated using the least significant difference (LSD) test for multiple comparisons of the means of the growth diameter. Each experiment has done in three replicates and presented as mean of inhibition zone (mm) ± SD.

RESULTS

Determination of antimicrobial potentiality of stevia

Antibacterial assay

The *In Vitro* antimicrobial activity of n-hexane,

petroleum ether, acetone, ethanol and water extracts of dried stevia leaves are shown in Fig. 1. The result depicted that the stevia extracts with different solvent exhibited a varying degree of antibacterial activity against majority of tested organisms. At the end of 24 hours, the extracts showed statistically

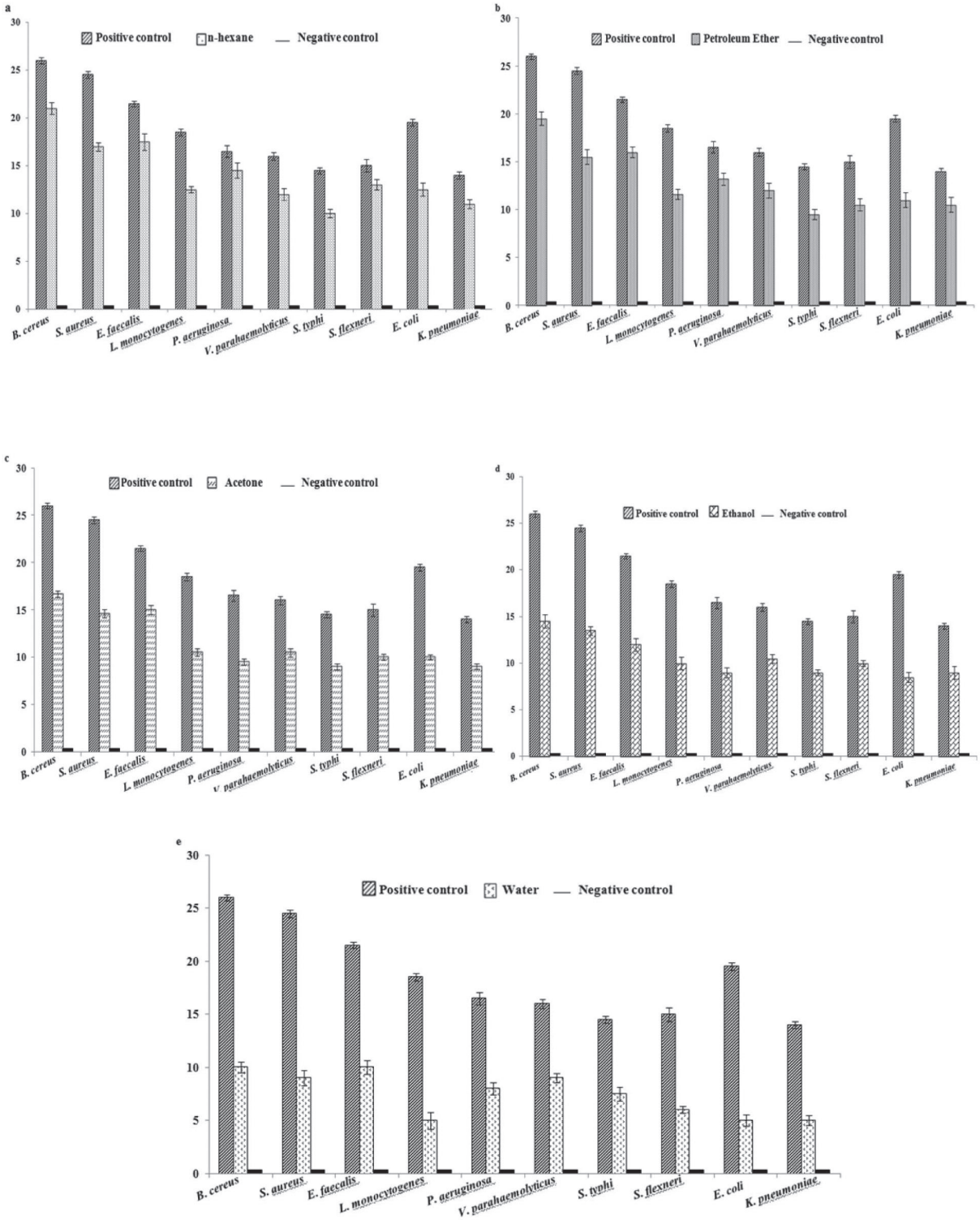


Fig. 1. Antibacterial activity of stevia leaves with different solvent extracts, (**a**) n-hexane extracts, (**b**) Petroleum ether extracts, (**c**) Acetone extracts, (**d**) Ethanol extracts and (**e**) Water extracts.

significant antibacterial activity. Among different extracts of stevia observed in this study, non-polar solvents like as n-hexane, petroleum ether, acetone were found to be effective against both gram positive and gram negative bacteria. The polar solvents like as ethanol and water extracts exhibit less inhibition capability against various bacterial isolates. The antibacterial activities of extracts according to the zone of inhibition ranged between 7.5±0.5 to 21.0±0.5 mm. Maximum zone of inhibition (21.0±0.5 mm) was observed with n-hexane extract against *B. cereus* and minimum zone of inhibition (7.5±0.5 mm) was specified by the water extracts against *L. monocytogenes*. The extracts with n-hexane showed significantly higher activity compared to the petroleum ether, acetone, ethanol and water extracts against both gram positive and gram negative bacterial isolates. The water extracts of stevia leaves did not show any considerable activity against tested bacteria, especially with *S. flexneri*, *E. coli* and *K.pneumoniae* and *L. monocytogenes*. The positive control ciprofloxacin showed significantly higher activity compared to all the extracts. The results of the present study indicated that gram positive bacteria were more susceptible to stevia leaf extract than gram negative bacteria. The low susceptibility of gram negative bacteria could be attributed to the presence of hydrophobic lipopolysaccharide in their outer membrane which provides protection against different agents (Zhao *et al*., 2001). The extracts of stevia exhibited antagonistic activity towards indicator microorganisms that may suggest their ability to synthesize a broad spectrum compounds. Previous antibacterial studies of Tadhani and Subhash (2006) reported that the extract of stevia had high antibacterial activity against *S. aureus*, *L. monocytogenes*, *B. cereus* and *E. coli*, zones of inhibition was found to be 8.33, 8.67 and 10.00 mm for ethanol extract, 10.00, 11.00 and 8.33 mm for ethyl acetate extract, respectively (Tadhani and Subhash, 2006).

Antifungal activity

Antifungal activity of different solvents extracts were recorded and presented in the Fig. 2. The obtained results demonstrated that the n-hexane extract of the stevia inhibit significant number of mycelial growth of tested fungi, compared to the extracts with petroleum ether, acetone, ethanol and water. In fact, the extract with water exhibit little inhibition potentiality and almost shows no antifungal activity against tested fungal strains. In previous study, Tadhani and Subhash (2006) stated about the antifungal activity similar to our study (Tadhani and Subhash, 2006). Moreover, the research of Jayaraman *et al.* (2008) also documented the comparable results as present study (Jayaraman *et al*., 2008).

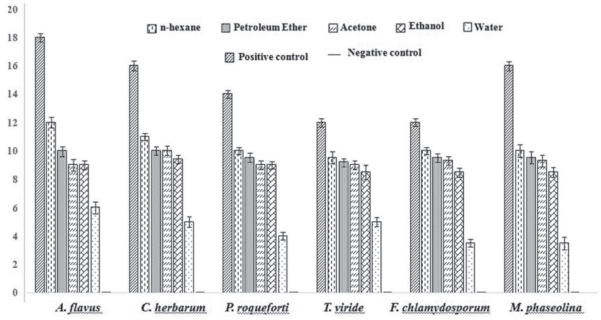


Fig. 2. Antifungal activity of different solvent extracts of stevia leaves.

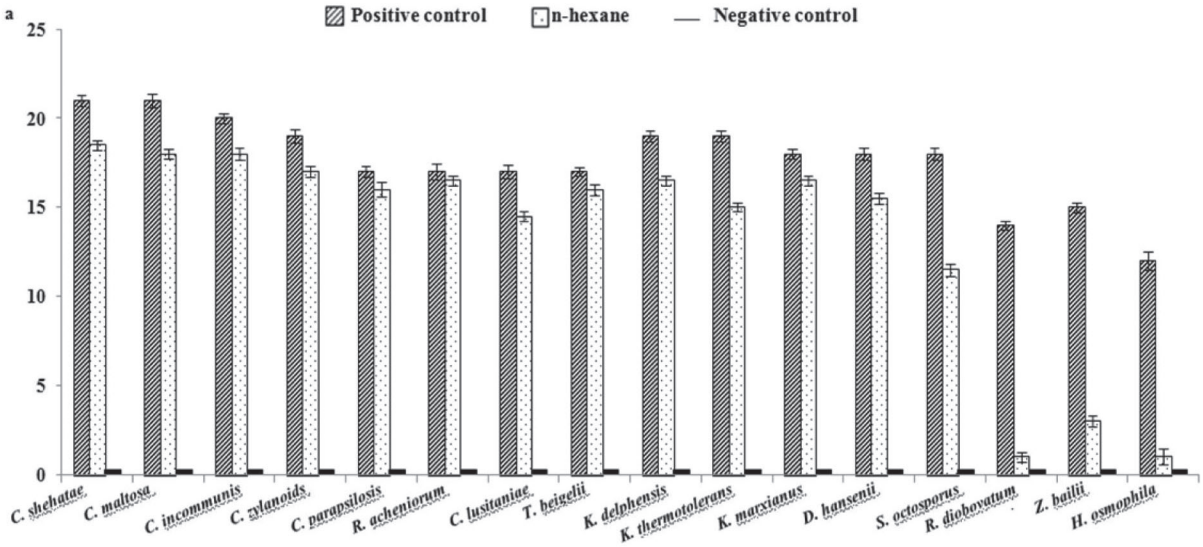
Activity against yeast species

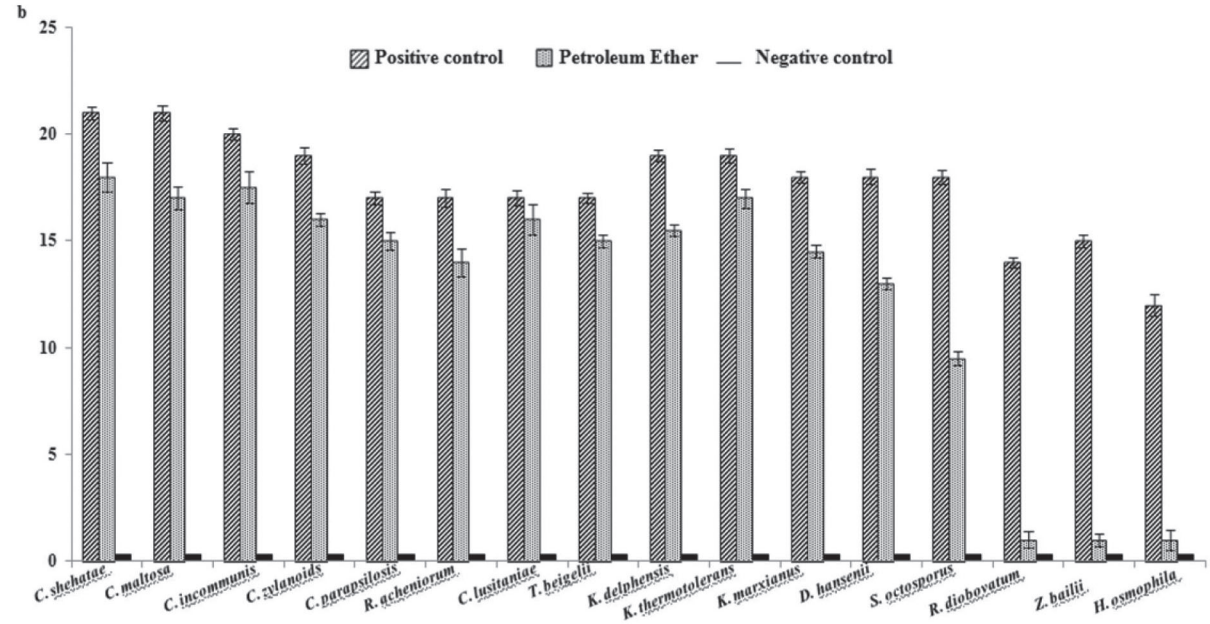
A number of yeast species can become an opportunistic pathogen causing disease in immuno suppressive hosts. For these reasons the antagonistic activity of stevia leaf extracts were tested with a range of yeast species. The extract with different solvent exhibited different inhibition profiles. Results are expressed in Fig. 3. The obtained findings indicated that the extracts with n-hexane are more active against most of the yeasts, particularly for *Candida* species. The extracts with petroleum ether gives more or less similar results compared to the extracts with n-hexane. The extracts with acetone showed significant antagonistic activity exclusively towards *C. shehatae*, *C. maltosa* and *S. octosporus.* The extracts with ethanol have very strong activity against *Candida* species as well as enable to inhibit *T. beigelii* and *Z. bailii.* By contrast the extracts with water displayed considerable sensitivity against tested yeast species. However, no inhibitory effect on the growth of *R. diobovatum*, *Z. bailii* and *H. osmophila* were observed in the case of all extracts from stevia. The extracts with n-hexane showed a significantly higher activity compared to petroleum ether, acetone, ethanol and water extract, respectively.

Determination of MIC, MBC and MFC value of plant extracts

Assessment of MIC value was performed for only that extracts which showed a significant zone of

inhibition to the tested microorganisms in the previous antimicrobial assay by Disk diffusion method. Among all plant extracts tested, the extract with n-hexane was found to show strong antimicrobial activity. Table 1 point up the MIC and MBC value of the n-hexane extract of stevia. The results indicated that higher concentration activity was remarkable in comparison to lower one. The present findings were consistent with the previous report on the extracts of stevia (Ghosh *et al*., 2008). Inhibitions of fungal radial mycelial growth were recorded against the all six test fungi at a concentration of 200 mg/mL, which was comparable to standard nystatin. The MIC values were found to vary from 200 to 300 mg/mL against the tested fungi (Table 2). The n-hexane extract of stevia exhibited the significant MIC values against *A. flavus*, *C. herbarum* and *T. viride*. The MFC values were found





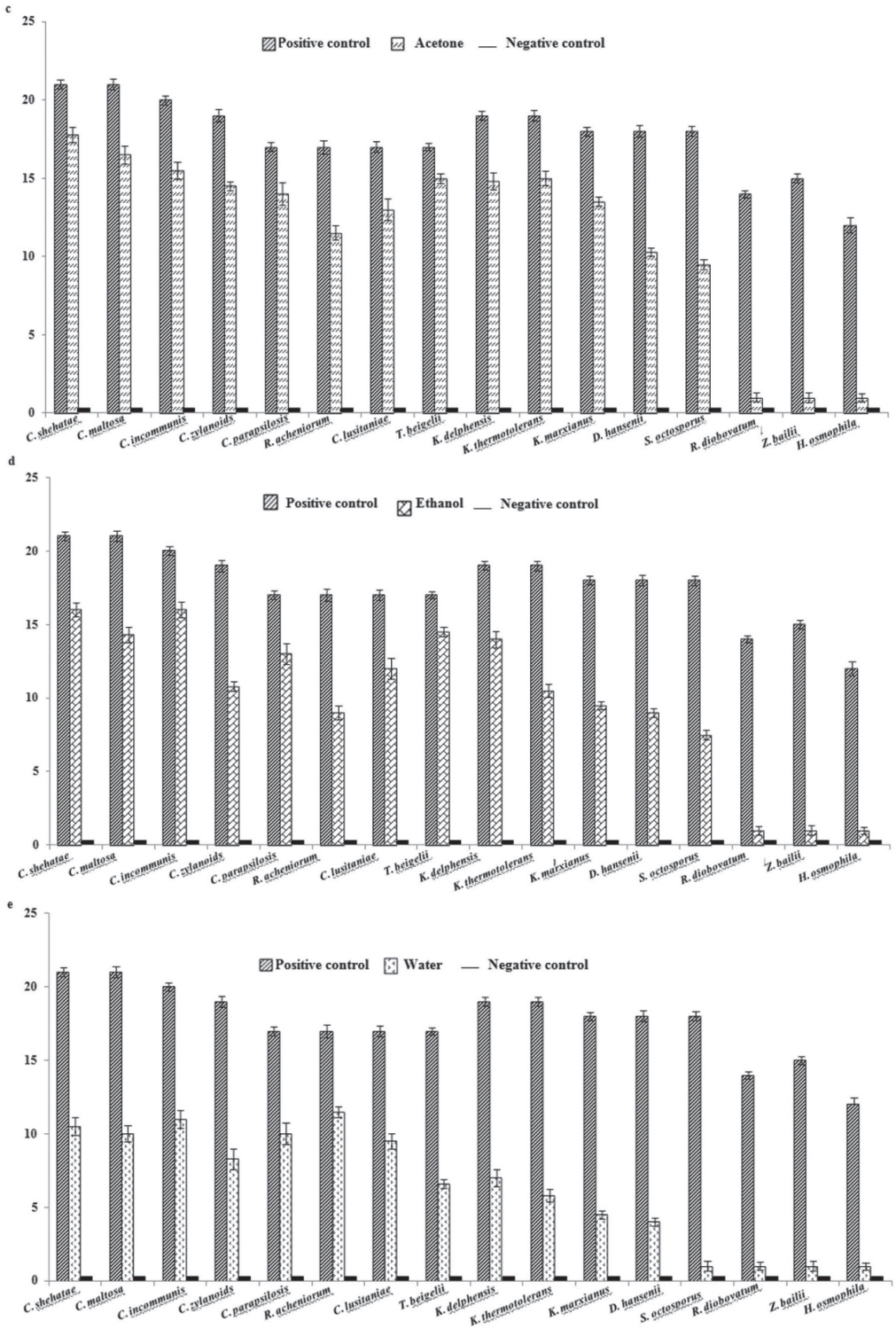


Fig. 3. Inhibitory effects of stevia leaves against different yeast species. (**a**) n-hexane extracts, (**b**) petroleum ether extracts, (**c**) acetone extracts, (**d**) ethanol extracts and (**e**) water extracts

Table 1. MIC and MBC value of the n-hexane extract of stevia leaves against microorganisms (mg/ml).

Test organism	Bacterial growth in Muller-Hinton medium (Plant extract concentration)							
	50	100	200	300	400	500	MIC (mg/ml)	MBC (mg/ml)
B. cereus	+	+	-	-	-	-	200	300
S. aureus	+	+	-	-	-	-	200	300
E. faecalis	+	+	+	-	-	-	300	400
L. monocytogenes	+	+	-	-	-	-	200	300
P. aeruginosa	+	+	+	-	-	-	300	400
V. parahemolyticus	+	+	+	-	-	-	300	400
S. typhi	+	+	+	+	-	-	200	300
S. flexinery	+	+	+	+	-	-	400	500
E. coli	+	+	+	+	-	-	400	500
K. pneumoniae	+	+	+	+	-	-	400	500

(+) means growth appears, (-) means no growth

Table 2. MIC and MFC value of the n-hexane extract of stevia leaves against microorganisms (mg/ml).

Test organism	Fungal growth (Plant extract concentration)							
	50	100	200	300	400	500	MIC (mg/ml)	MFC (mg/ml)
A. flavus	+	+	-	-	-	-	200	300
C. herbarum	+	+	-	-	-	-	200	300
P. roqueforti	+	+	+	-	-	-	300	400
T. viride	+	+	-	-	-	-	200	300
F. chlamydosporum	+	+	+	-	-	-	300	400
M. phaseolina	+	+	+	+	-	-	400	500

(+) means growth appears, (-) means no growth.

to vary between 300 and 500 mg/mL among all.

DISCUSSION

Antibiotic resistant microorganisms (bacteria, fungi and yeast) are one of the foremost threats to the global health care, not only mankind also for other animals (Cabello *et al*., 2001; Bhatia and Narain, 2010; Rhimi *et al*., 2020). This state of affairs leads the researchers to search for a new source of antimicrobial substances such as plants as medicinal plants have been extensively studied as alternative agents for the prevention of infections. At present world, both in developing and developed countries, antibiotic resistance are major problem that sustains to challenge the healthcare division. The origin and expansion of multidrug resistant pathogens have substantially threatened to the present antibacterial therapy. Medicinal plants are considered as an affluent source of essential elements which can be used in development and synthesis of drugs (Vuorela *et al*., 2004). The compounds found in plants are of different kinds, but major biochemical classes are alkaloids, polyphenols, glycosides and terpenes. This has necessitated a query for a new paternity of antimicrobial substances such as some plants consider as important source of nutrition while these plants also contain variety of bioactive compounds of known therapeutic characteristics. Microorganisms are invisible and manikin in size but dangerous enemies to mankind. They can cause intense damage to human body as well as other living organisms. The agents having the capacity of killing the microbes or arresting the multiplication are called antimicrobial agents. There are a lot of antimicrobial drugs of which some are known but some are not established and still hidden in nature. Hence, the last decade spoke for an enhancement in the investigations on plants as an origin of human disease management and more natural antimicrobials have guided scientists to go through the research about the effectiveness and utility of inhibitory compounds such as plant extracts (Aiyelaagbe, 2001; Mounnissamy *et al*., 2002). There are various reports for antibiotics resistance of human pathogens to attainable antibiotics. Bioactive

compounds of plant origin appear to be one of the choices for the control of these antibiotic resistant human pathogens (Woldemichael *et al*., 2003; Ghosh *et al*., 2008). Microbial resistance to antimicrobial agents is a matter of great concern because a valuable drug may become useless if sensitive strains are annihilated by resistant ones. Resistance may become more dominant in a human body by spread of microorganisms containing resistance genes. The only groups of therapeutic agents which can deflect the evidential diseases suffered by infected individuals by microorganisms are called antibiotics (Aínsa, 2003; Sambasivaraju and Fazeel, 2016). According to WHO, nontoxic plants can be used as therapeutic agents. Stevia plant is safe to human use and also has antimicrobial and therapeutic properties (Jayaraman *et al*., 2008; Silva *et al*., 2008). So the present study dealt with antimicrobial activity of stevia plant extracts with different pathogens. Stevia plant extracts were prepared in different solvent, i.e. aqueous (polar), ethanol (polar), acetone (non-polar), petroleum ether (non-polar) and n-hexane (non-polar).

Among different extracts of stevia, n-hexane, petroleum ether and acetone extracts showed effectiveness against both gram positive and gram negative bacteria at the end of 24 hours where extracts with ethanol and water exhibit less inhibition capability against various bacterial isolates. Antibacterial activity with ten (10) common pathogenic bacteria, antifungal activity with six (06) fungi and activity against yeast with a number (16) of yeasts were assayed to determine antimicrobial potentiality of stevia plant extracts. Stevia plant extracts with different solvent showed different antibacterial activity towards different tested bacterial isolates. Some exhibited a significant antibacterial activity while others exhibited a limited activity against the tested bacterial isolates as judged by their MIC values. In this study, water extracts showed less antibacterial activity compared to other solvents. The previous study also summarized that water extracts of plants showed less antibacterial activity than other solvent extracts of stevia which also matched with present findings (Selvamohan *et al*., 2012). The MIC and MBC value of n-hexane extract of stevia indicated that higher concentration activity was significant than the lower one. The n-hexane extract of stevia also exhibit significant inhibition against tested fungal and yeast species. The results of the present study specifies that the five different extracts of stevia leaves exhibited significant antimicrobial potency, among them the extracts with different solvent were more and water is less efficient. These investigations may be due to polarity of the compounds, where the solvents were the non-polar compounds and water acts as a polar solvent among the extracts inspected. Therefore, our results revealed the importance of non-polar extracts of stevia leaves to inhibit resistant microorganisms.

CONCLUSION

Antibiotic resistant microorganisms are constantly increasing at present time. This situation leads to explore for new antimicrobial substances to control resistant microorganisms. On the basis of obtained findings from this study, non-polar extracts of stevia leaves exhibit strongest antimicrobial activity against test microorganisms and showed significant MIC value. Further the extract of stevia exhibited antifungal activity. Moreover, the findings on the activity against fungus and yeast indicated that the extracts have strong activity. Therefore, our results revealed the importance of stevia leaves to control resistant bacteria.

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Competing Interests

The authors declare no conflict of interest.

Data Availability Statement

The experimental data used to support the findings of this study are available from the corresponding author upon request.

***Authors Contribution**

Sadia Afrin and Kazi Asma Ahmed Shamima contributed equally to this work and are regarded as joint first authors.

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