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Antimicrobial activity of *Stevia rebaudiana* against antibiotic resistant ESBL producing uropathogens and evaluation of its antioxidant activity

Darshana Raut and Aruna K*
Department of Microbiology, Wilson College, Mumbai-400007
*Corresponding author: **Dr. K Aruna,**
Head, Department of Microbiology, Wilson College, Mumbai-400007, India.
E-mail: *arunasam2000@yahoo.co.in*

Abstract

Worldwide, about 150 million people are diagnosed with urinary tract infections (UTI) annually. Treatment of these common infectious diseases has become difficult due to the emergence of antibiotic resistant strains like ESBL (extended spectrum β-lactamase) producers. Pathogenic organisms are unlikely to develop resistance against herbs that are blend of active compounds. The plant used in present study was *Stevia rebaudiana* which is traditionally used as a source of natural sweetener. Antibacterial activity of aqueous extract and four solvent extracts (petroleum ether, chloroform, methanol and ethanol) of *Stevia rebaudiana* leaves were investigated against 49 ESBL producing uropathogens. The methanol extract showed zone of inhibition in the range of 18-24mm as compared to aqueous extract which showed negligible zone of inhibition against the uropathogens. Minimum bactericidal concentration (MBC) was found to be in range of 10 mg/ml - 20mg/ml. Methanolic extract of *Stevia rebaudiana* also showed synergistic activity along with ampicillin, thereby reducing the MBC of ampicillin from 10mg/ml to 200-300µg/ml. The antioxidant activity of *Stevia rebaudiana* was checked by DPPH method. The IC_{50} values of methanolic extract of *Stevia rebaudiana* and standard ascorbic acid were found to be 32.765 µg/ml and 6.474µg/ml respectively. The phytochemicals present in *Stevia rebaudiana* were analysed by HR-LCMS and confirmed the presence of eight major components including terpenes, flavanoids and Quinones that might be responsible for its antibacterial activity.

Keywords: ESBL, *Stevia rebaudiana*, MBC, Synergy, antioxidant activity, HR-LCMS.

Introduction

Infliction of UTI has been deleterious to mankind affecting 35% of healthy individuals at some stages in their life. Moreover, the emergence of ever- increasing multi drug resistant microbial strains make the UTI's more severe (Singhal et al., 2014). The most common pathogens causing UTI belong to *Enterobacteriaceae* family and among these the Extended Spectrum Beta Lactamases (ESBL) producing bacteria are the major agents of UTI both in hospitalized and outpatients (Mendelson et al., 2005). ESBL are plasmid mediated enzymes that are capable of hydrolyzing the amide bond of the four-membered characteristic β-lactam ring, thus rendering the resistance against β-lactam antibiotics (Laurent et al., 2010).

ESBL producing uropathogens are difficult to treat, requiring alternative medications or higher doses of existing antibiotics which would be expensive and

dangerous too. Scientists are shifting their attention from antibiotics to herbal extracts that contains secondary metabolites like alkaloids, terpenes, sterols, flavanoids, carotenoids etc have been found to have antimicrobial activities (Suchita et al., 2010). Apart from antimicrobial activity, interest has been considerably increased in finding naturally occurring antioxidants for therapeutic usage to replace synthetic antioxidants which are strictly prohibited due their carcinogenicity.

Stevia rebaudiana belongs to genus *Stevia.* It has been used by native people as a sweetener and also for therapeutic purposes. Basically, it is a shrub that originally existed in Paraguay and Brazil (Gamboa et al., 2012).The leaves of *Stevia rebaudiana* have been proved to contain sweet diterpene glycosides, flavonoids, alkaloids, hydroxycynnamic acid, few amino acids, lipids, essential oils and trace elements (Tadhani et al., 2006; Abou-Arab et al., 2010). In addition to sweetening property, *Stevia rebaudiana* also possess antioxidant activity. Antioxidant delays the oxidation of lipid molecules by inhibiting the initiation or propagation of scavenging oxidative chain reactions. *Stevia rebaudiana* also exhibit antihyperglycemic, anticancerous and also antihypersensitive effect (Jeppensen et al., 2003). It has also been reported that *Stevia rebaudiana* has ability to maintain glucose level in diabetic's patients (Curi et al., 1986).There is no toxic or genotoxic activity found in *Stevia rebaudiana* extracts (Sekihashi et al., 2002)

In the present study, crude extracts of *Stevia rebaudiana* leaves in different solvents were used to evaluate antimicrobial activity against 49 antibiotic resistant ESBL producing uropathogens. The methanolic extract of *Stevia rebaudiana* was also checked for its synergistic activity with ampicillin. The radical scavenging activity of *Stevia rebaudiana* was studied by DPPH method using ascorbic acid as standard. HR-LCMS analysis of *Stevia rebaudiana* extract was also carried out.

Materials and Methods

Test organisms:

ESBL producing Gram-negative pathogens were collected and characterized in our previous study (Aruna and Tariq, 2012; Tariq and Aruna, 2016). Forty nine ESBL producing uropathogens which were used in current studies include 10 representative isolates of each of the following genera, i.e., *Klebsiella*, *Escherichia*, *Pseudomonas*, *Proteus* and 9 isolates of *Citrobacter*. The isolates were maintained on Luria-Bertani (LB) Agar slants supplemented with 100µg/ml of ampicillin and stored at 4^0C.

Preparation of Extract:

Dried *Stevia rebaudiana* leaf powder was provided by Elixir Company, Mumbai. The Bioactive components from *Stevia rebaudiana* were extracted using five different solvents such as water, methanol, chloroform, petroleum ether and ethyl alcohol. 50 gm of leaf powder was extracted in 200ml solvents using Soxhlet apparatus for period of 8 hrs.The extracts were further concentrated at 40^0C in water bath to obtain semi solid mass. The concentrated extracts were preserved at 4^0C until further use.

Sterility testing of Extracts:

A loopful of each solvent extract of *Stevia rebaudiana* was streaked on Nutrient Agar (NA) and Sabouraud's Agar (SAB) plates to check the sterility of all the extracts. The plates were incubated at 30^0C for 24hrs. The plates were checked for absence or presence of growth (Sule et al., 2008).

Qualitative evaluation of antibacterial activity of *Stevia rebaudiana* extracts against ESBL test uropathogens:

The antibacterial activity of each solvent extract against 49 ESBL producing uropathogens was checked by Agar well diffusion method (Toda et al., 1989). Sterile 20 ml of molten NA butt was cooled at around 40^0C and seeded with 0.4ml of 24h old test cultures (0.1 OD at 540 nm). The seeded NA butt was poured into sterile petri plates. After solidification of the medium, wells were punched using sterile cork borer (8mm diameter). 50 µl of the solvent extracts was added to the wells and incubated at 37^0C for 24h to observe the zones of inhibition. Control wells were also set up using the solvents for each isolate. Out of the five solvents used for extraction, the solvent extract showing maximum zone of inhibition against test pathogens was selected for further study.

Quantitative evaluation of antibacterial activity of *Stevia rebaudiana* extract against ESBL test uropathogens by Agar dilution method:

The determination of minimum bactericidal concentration (MBC) of *Stevia rebaudiana* extract was done by Agar dilution method. Sterile molten NA

butts were supplemented with different concentrations of solvent extract of *Stevia rebaudiana* (5-20 mg/ml with an interval of 5mg/ml). After solidification of medium, 5µl of test pathogen was spot inoculated and plates were incubated at 37^0C for 24h. The lowest concentration of *Stevia rebaudiana* that completely inhibited the growth of test culture was considered as its MBC (Wayne et al., 2006).

Determination of synergistic activity

The agar dilution method was used to determine synergy between methanolic extract of *Stevia rebaudiana* and ampicillin. The sub-lethal concentration i.e. 1/2 of MBC of methanolic extract was incorporated into molten NA butt along with 100-500µg/ml of ampicillin with an interval of 100µg/ml (Wayne et al., 2006).

Antioxidant activity of *Stevia rebaudiana* by DPPH method:

2, 2-Diphenyl-1-picrylhydrazyl (DPPH) assay is aimed in measuring the scavenging activity of any antioxidant towards DPPH, which is stable organic nitrogen radical. The electrons in DPPH get paired off with suitable antioxidant and lose its colour. The disappearance of violet colour of DPPH is proportional to antioxidant activity. A volume of 1.5 ml of various diluted aliquots of *Stevia rebaudiana* extract and ascorbic acid (control) was mixed with 1.5ml of 0.2mM DPPH in methanol. The mixture was kept in dark for 30min after vigorous shaking. The absorbance of DPPH was measured at 520nm using spectrophotometer (Bendaoud et al, 2009). Ascorbic acid was used as Standard antioxidant (Soni et al., 2012**).** The capability to scavenge the DPPH radical of each solution was calculated using following equation:

Scavenging effect (% inhibition) =

$$[(A_{control} - A_{test})/ A_{control})] \times 100.$$

The IC_{50} was calculated by plotting a graph of the %inhibition versus various concentration of extract (µg/ml) (Ruiz et al., 2015.)

High Performance Liquid chromatography mass spectroscopy (HR-LCMS):

The bioactive components of *Stevia rebaudiana* were analyzed by HR-LCMS G6550A system (Agilent technologies). The method used for Chromatography was 30 mins ± ESI 10032014. The Gas temperature used for analysis was 250°C. The theoretical mass of protonated compound was for identification in this analysis. The HR-LCMS was carried out in IIT Bombay, Mumbai 400076. The compounds were identified by comparison of their retention time (RT) and mass with stored library available with IIT, Bombay.

Results

Sterility testing of Extracts:

The five extracts of *Stevia rebaudiana* leaves were subjected to sterility check. The NA and SAB agar plates showed absence of growth after incubation at 30^0C for 24hrs. Thus, all the five extracts were found to be sterile as it was free from the bacterial and fungal microorganisms.

Qualitative evaluation of antibacterial activity of various solvents extracts of *Stevia rebaudiana* against ESBL pathogens:

The antibacterial activity of aqueous, methanol, ethyl alcohol, petroleum ether and chloroform extracts showed significant difference in activity. The aqueous extract did not show any inhibition against ESBL pathogens. Methanol, ethyl alcohol, petroleum ether and chloroform extract showed variable antibacterial effect as shown in figure 1. Among these four solvents, methanol exhibited maximum antibacterial activity against uropathogens followed by chloroform, ethyl alcohol and then petroleum ether.

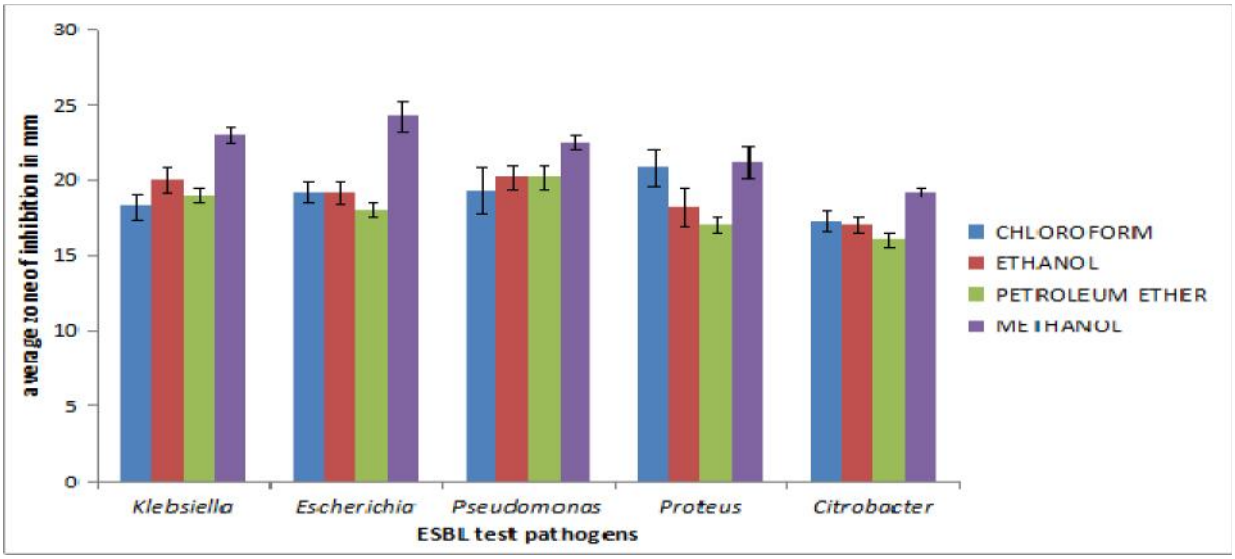

Figure 1: Antibacterial activity of four solvent extracts by agar well diffusion method.

Quantitative evaluation of antibacterial activity of methanolic extract of *Stevia rebaudiana* against ESBL test uropathogens by Agar dilution method

The agar dilution method was used to determine the minimum bactericidal concentration (MBC). The MBC (mg/ml) values of methanolic extract of *Stevia rebaudiana* leaves are reported in table 1.

Table 1: MBC of methanolic extract of *Stevia rebaudiana*

Test organisms	No. of isolates	MBC of methanolic extract of *Stevia rebaudiana.*
Klebsiella	10	15mg/ml
Escherichia	10	10mg/ml
Pseudomonas	10	15mg/ml
Proteus	10	15mg/ml
Citrobacter	09	10mg/ml

Determination of synergistic activity:

ESBL producing test pathogens showed high MBC for ampicillin (>10mg/ml). The MBC of ampicillin was reduced significantly to 200-400µg/ml when used in combination with methanolic extract of *Stevia rebaudiana* leaves (table 2). This clearly indicates synergy between ampicillin and methanol extract.

Table 2: MBC values of ampicillin in synergy with sub-MBC of methanolic extrcat of *Stevia rebaudiana* leaves

Test organisms	MBC of Ampicillin	Synergism: MBC of ampicillin in combination with sub-MBC of *Stevia rebaudiana* leaf extract.
Klebsiella (10)	>10mg/ml	200µg/ml
Escherichia (10)		200µg/ml
Pseudomonas (10)		300µg/ml
Proteus (10)		300µg/ml
Citrobacter (09)		200 µg /ml

Antioxidant activity by DPPH method:

Antioxidant activity of methanolic extract of *Stevia rebaudiana* was carried out by DPPH method using ascorbic acid as standard. DPPH is a purple coloured stable radical of organic nitrogen with a maximum absorbance at 517 nm. When the odd electron becomes paired off in the presence of a free radical scavenger to form hydrazine, the absorption reduces and the DPPH solution is decolourised from deep violet to light yellow. The degree of reduction in absorbance measurement indicates the radical scavenging (antioxidant) effect of the extract. IC_{50} is the concentration of extract that brings about 50% loss of DPPH and is used to compare the antioxidant power (Shekhar et al., 2014). In the present study, the IC_{50} values of methanolic extract of *Stevia rebaudiana* and standard ascorbic acid were found to be 32.765 µg/ml and 6.474µg/ml respectively. The scavenging effect (%) against concentration graph is shown in fig 2 and 3.

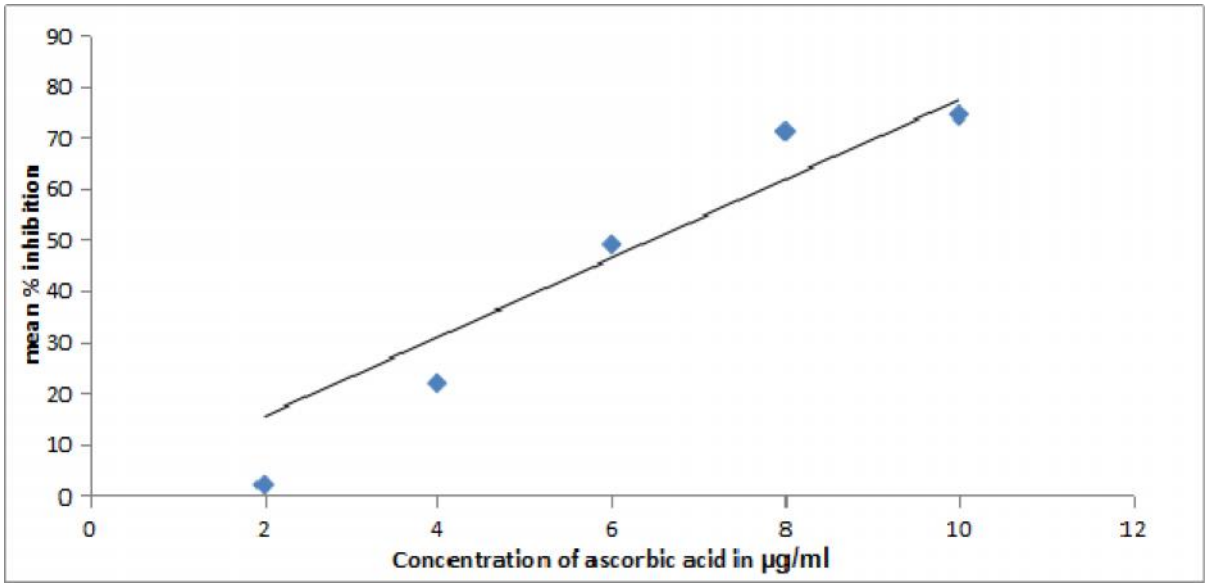

Figure 2: Radical scavenging effect (%inhibition) of Ascorbic acid.

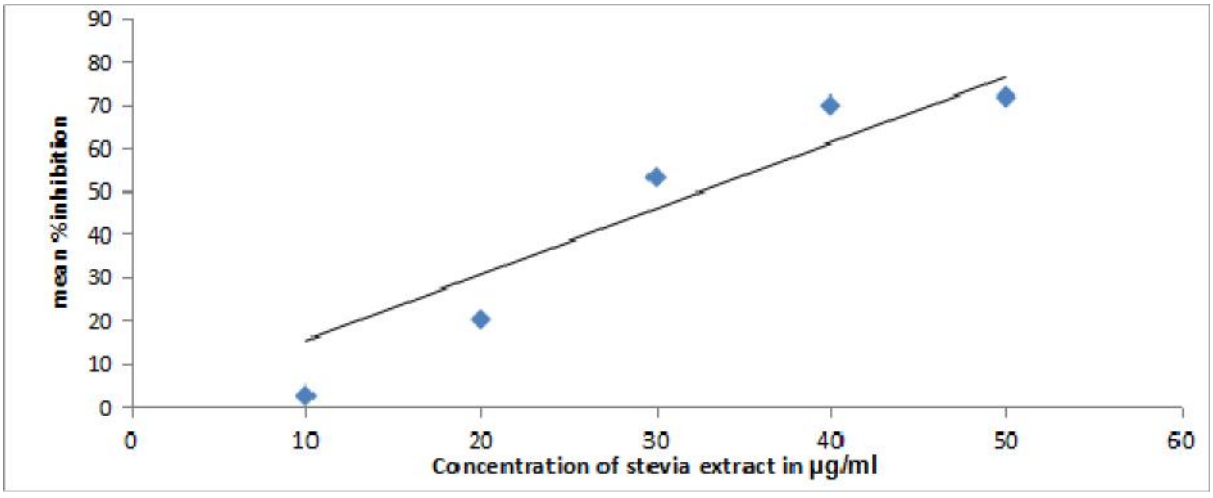

Figure 3: Radical scavenging effect (%inhibition) of methanolic extract of *Stevia rebaudiana.*

HR-LCMS:

HR- LCMS analysis of methanolic leaf extract showed presence of many antibacterial compounds such as Rutin, Quercitrin, and Dihydrodeoxystreptomycin that contributes to inhibitory action of *Stevia rebaudiana* (Li et al., 2009). Along with antibacterial compounds, antioxidant molecules were also found such as Delphinidin, rosmarinic acid etc. Few glycosides were also found such as sarmentoside B. The retention time and mass of identified compounds are reported in table 3. The chromatogram of methanolic extract is shown in figure 4.

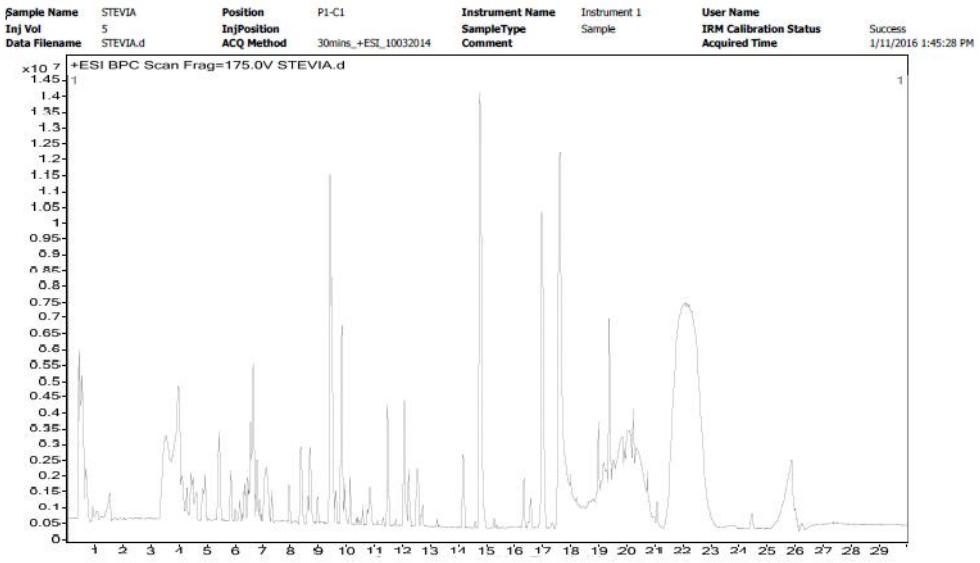

Figure 4: Chromatogram of *Stevia rebaudiana* extract.

Table 3: HR-LCMS analysis of *Stevia rebaudiana* extract

	Retention Time (mins)	Mass	Compound name.
1.	5.871	610.1538	Rutin
2.	6.379	448.1013	Quercitrin
3.	6.585	303.0517	Delphinidin
4.	8.976	664.3076	Sarmentoside b
5.	9.414	567.29	Dihydrodeoxystreptomycin
6.	9.472	360.0854	Rosmarinic acid
7.	17.661	526.2598	Khayasin C
8.	25.998	149.1065	Trolamine

Discussion

Plant extracts and their active constituents are being increasingly reported for their antibacterial activity from different parts of the world (Nayan et al., 2011). The study of phytochemicals and its effectiveness against multidrug resistant organisms is less studied. Many scientists have reported antibacterial, antifungal and antioxidant activity of *Stevia rebaudiana* and its various parts (Preethi et al., 2011), but no data is published about its activity against antibiotic resistant uropathogens. Thus, the present study was focused on antibacterial as well antibiotic resistance reversal activity of *Stevia rebaudiana* leaf extract.

Qualitatively the antibacterial activity was checked by agar well diffusion method. The solubility of bioactive components varies with solvent used. Among the five solvents used for extraction, methanol extract was very effective. It showed prominent zone of inhibition in range of 18-24mm against ESBL producing uropathogens. Similar studies were carried out with methanol extract. *Stevia rebaudiana* which showed its effectiveness against *Bacillus subtilis* NCIM 2708 and *E. coli* DM 4100 (Singh et al., 2012). Similar study was done using ethanol and methanol solvent extracts of *Stevia rebaudiana* where ethanol extract was observed to be effective against Gram positive

and few Gram negative organisms (Sunitha et al., 2015). Other studies have also shown that the acetone and ethanol extracts of *Stevia rebaudiana* were effective against Gram positive organisms and bacteria causing wound infection respectively (Jayaraman et al., 2008; Pugalvendhan et al., 2012). The plant extract is chemically complex in nature and all components might be cumulatively responsible for its antibacterial activity and therefore bacteria cannot develop resistance against the plant extract (Tariq et al., 2014)

Quantitatively, the inhibitory concentration of methanolic extract of *Stevia rebaudiana* was evaluated by determining its MBC. It showed complete inhibition of test pathogens at concentration ranging from 10-20 mg/ml. Other study showed that methanolic extract of *Stevia rebaudiana* (100mg/ml) was effective against *Streptococcus mutans* (Maryam et al., 2012). The efficacy of extract can be further increased by either modifying the extraction method or using higher grade solvents (Tariq et al., 2014).

Ampicillin is a preferred drug for common infections including UTI, due to its broad spectrum, low cost and almost negligible side effects (Timothy et al., 2002). The resistance developed by ESBL producing uropathogens against beta lactam antibiotics limits its usage.

The MBC of ampicillin was reduced from 10mg/ml to 200-300µg/ml when used in combination with sub-MBC of *Stevia rebaudiana* extract. This resistance reversal of ampicillin indicated the synergistic activity which could be an effective therapy against uropathogens. But this is needed to be confirmed by in-vivo experiments. The phenolic compounds present in *Dalea versicolor* exhibited synergistic activity against *Staphylococcus aureus* and *Bacillus cereus* (Belofsky et al., 2004). Similar studies on medicinal plant in combination with amoxicillin showed to lower the MBC of amoxicillin (Hanan et al., 2012). Previous study done on Green tea extract in combination with ampicillin showed to inhibit the metallo-beta lactamase producing uropathogens (Tariq et al., 2015). In previous report where extract of *Jatropha elliptica* was identified as resistance modifying agent which can help in reducing the drug resistance of *Staphylococcus aureus* and make it more sensitive towards a range of antibiotics (Marquez et al., 2005). The ajwain extract when used in combination with ampicillin against ESBL and MBL uropathogens significantly showed 99% reduction in the MBC of ampicillin, thereby indicating synergy between ajwain extract and ampicillin (Tariq et al., 2014). The synergy mechanism between *Stevia rebaudiana* extract and ampicillin is not yet reported to best of our knowledge, and this study proves to be significant for treatment of UTI.

Antioxidants from plant origin possess the ability to protect the body from oxidative damage (Ahmad et al, 2013). The antioxidant capacity of *Stevia rebaudiana* was measured in terms of IC_{50} and was found to be 32.765 µg/ml. It means that leaf extract of *Stevia rebaudiana* is capable of detoxifying DPPH free radicals. Similar studies were done by using methanolic extract of *Stevia rebaudiana* which showed 30.33% inhibition of DPPH (Rao et al., 2014). Ascorbic acid was used as standard control in the current study and the IC_{50} value was found to be 6.474µg/ml which was in accordance with Bendoud et al. (2009).

The bioactive components present in *Stevia rebaudiana* was studied using HR-LCMS and eight components were reported in our studies. The presence of flavanoids such as rutin and quercitrin in *Stevia rebaudiana* extact have been reported to prevent the neurotoxic effect (Azevedo et al., 2013). In the present study, Dihydrodeoxystreptomycin was found and it might have attributed to antibacterial activity of *S.rebaudiana*. Similar studies done on seeds of *Pinda concanensis* extract showed the presence of antibacterial Dihydrodeoxystreptomycin (Patil et al., 2016). Our study also reported few antioxidants such as delphinidin, rosamarinic acid and khayasin C that inhibit the oxidative stress. The antioxidant effect of Delphinidin was reported in the study done on extract of *Aristotelia chilensis* (Watson and Schonlau, 2015). Previous studies have shown that rosamarinic acid present in herb *Salvia officinalis* extract inhibit the reactive oxygen species formation (Luvone et al., 2006). The studies done on bark extract of *Neobegueae mahafalensis* revealed Khayasin as major antioxidant component (Naidoo et al., 2003).

In conclusion, the methanolic extract of *Stevia rebaudiana* proved to be very effective against antibiotic resistant ESBL producing uropathogens. Morever, it has been proved to be safe and does not cause any DNA damage as proved by genotoxic studies done by Sekhihashi et al. (2002). Thus, it has potential to be used as an alternative therapy for UTI patients.

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