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# PHYTOCHEMICAL SCREENING AND *IN-VITRO* STUDIES OF ANTIOXIDANT AND ANTIMICROBIAL ACTIVITY OF EXTRACTS OF DRIED *STEVIA REBAUDIANA* LEAVES

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**ABSTRACT:** The present study is based on the comparative evaluation of preliminary phytochemical screening and *in vitro* antimicrobial activity of methanolic and ethanolic extracts of *Stevia rebaudiana* leaves. The crude extracts of *S. rebaudiana* leaves were subjected to phytochemical screening tests by using standard procedures for the detection of the presence or absence of eight major secondary metabolites. The free radical scavenging activity of both the extracts was measured by using the 1,1- diphenyl-2-picrylhydrazyl (DPPH) assay. The dose dependent increase was observed in DPPH activity of methanolic and ethanolic extracts (10, 20, 40, 50, 100 µg/ml), and the range was 28.65 - 64.35 % and 28.15 – 61.57 % as compared to ascorbic acid 30.15 – 85.99 %. The antibacterial activity of *S. rebaudiana* leaves was tested by agar-well diffusion assay. The plant extracts of four different concentrations (25, 50, 75 and 100 mg/mL) were prepared in DMSO and tested against eight bacterial strains and was compared with that of standard drug tetracycline (10 µg/mL). The results of preliminary phytochemical screenings showed the presence of different kinds of phyto-constituents in methanolic and ethanolic extracts of *S. rebaudiana* leaves and the most abundant compounds were phenols followed by phytosterols, tannins, saponins, glycosides and flavonoids. Out of the two extracts, methanolic extract showed higher antibacterial activity at 100 mg/mL with inhibition zone of 25.93 ± 0.16 mm on *Zymomonas spp*. The results signified the effective therapeutic potential of *S. rebaudiana*.

**INTRODUCTION:** Plants have the ability to execute important biological functions by synthesizing a broad variety of naturally occurring chemical compounds for their normal metabolic activity commonly known as phytochemicals. They are basically non-nutritive plant derived chemicals having disease preventive properties; basically categorized into primary and secondary phytochemicals.

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Common sugars, proteins, fats and chlorophyll which are found in all plants are included in primary constituents while the smaller range of plants constitutes secondary compounds. Synthesis of aromatic compounds in plants like phenols or their oxygen substituted derivatives protects the plant from microbial infection and deterioration [1]. *Stevia rebaudiana* (Bert.) is a plant of *Asteraceae* family commonly used as low calorie natural sweetener and an alternative to artificial sugar and other nutritive sweeteners. It contains over 250 phytochemicals along with major eight sweet diterpene glycosides *viz*, stevioside, rebaudioside A-F, dulcoside and steviolbioside. Besides, it is well known for some notable health promoting properties due to presence of some bioactive

constituents or phytochemicals like alkaloids, sterols, phenols, diterpenes, triterpenes, austroinullin, labdane, flavonoids, chlorophylls, xanthophylls, lipids, aminoacids, β- carotenes, ascorbic acids, volatile oils, inorganic matters, essential oils and trace elements which creates a definite physiological action on the human body by treating different diseases such as hypertension, diabetes, cancer, neural disorders, cardiovascular disease, renal function and arthritis [2]. The presence of these compounds in the plant provides antioxidant, antimicrobial and antifungal property[3].

Analysis of phytochemicals and antioxidant activity is of paramount importance in identifying the new source of therapeutically and industrially valuable plant derived compounds having medicinal property. Therefore, the major objective of the present study was to carry out preliminary phytochemical screening and evaluating antioxidant activity along with the determination of antimicrobial potential of methanolic and ethanolic extracts of *S. rebaudiana* leaves. This study was performed to investigate the future prospects and to explore novel, potent and harmless antimicrobial compounds.

## MATERIALS AND METHODS:

**Collection of Plant material:** Fresh leaves of *S. rebaudiana* were collected in the month of June-August 2013 from Bioved Research Institute of Agriculture and Technology, Allahabad. The collected leaves were identified at the Department of Botany, University of Allahabad, India. The stems and other unwanted parts were removed and washed with slightly warm distilled water to remove the dirt particles and leaves were dried in shade after draining out excess water at temperature ranging from 25 ºC to 30 ºC for 24 to 48 hours. The dried leaves were blended to powder with a high speed blender for 30 s and were vacuum packed to avoid decomposition of various bioactive compounds.

**Preparation of crude extracts:** The dried leaves of *S. rebaudiana* (500 g) were powdered and then extracted with 1000 mL of organic solvents (methanol and ethanol) separated in a conical flask. At room temperature it was incubated at 150 rpm in an orbital shaker for three days. The final extract was filtered with Whatman No. 1 filter paper (W and R Balson Ltd., England) and concentrated to dryness at 40 – 60 °C on hot water bath to get the semi solid crude extracts which were stored at 4 °C in airtight bottles till further use.

**Phytochemical Screening of extracts:** The crude methanolic and ethanolic extracts of *S. rebaudiana* leaves were subjected to phytochemical screening tests for the detection of major secondary metabolites by using standard procedures [4, 5, 6, 7, 8]. The qualitative results are expressed as (+) for the presence and (-) for the absence of photochemical.

**Free radical Diphenylpicrylhydrazyl (DPPH) Scavenging Assay:** The free radical scavenging activity was measured by using the 1,1- diphenyl-2-picrylhydrazyl (DPPH) assay [9] with some modifications. The preparation of stock solution was done by dissolving 33 mg of DPPH in 1L of methanol. The test tube was covered with aluminum foil to protect it from light and stored at 20 ºC until required. For control reading 5 ml DPPH was added to 1000µl methanol and immediately absorbance was taken at 517 nm. Different concentrations (10 – 100 µg $ml^{-1}$) of the extract as well as standard compound (ascorbic acid) were taken and the volume was made uniformly to 1000 µl with methanol and to each sample 5 ml DPPH was added. The reaction mixture was allowed to stand in dark for 10 -15 min at room temperature (37 °C), methanol was used as blank and the absorbance was recorded at 517 nm spectrophotometrically.

The percentage of scavenging activity was calculated using the following formula:

% scavenging = [(Absorbance control – Absorbance sample) /Absorbance control] × 100

**Test Microorganisms:**

**Bacterial strains:** The antibacterial activity of *S. rebaudiana* leaf extracts were tested individually against different gram positive (*Bacillus subtilis, Cellulomonas spp*.) and gram negative (*Zymomonas mobilis, Salmonella typhi, Pseudomonas aeruginosa, Pseudomonas fluorescens, Klebsiella pneumonia, Escherichia coli*) bacterial strains. All bacterial strains were procured in lyophilized form from the institute of microbial technology (IMTECH), Chandigarh.

**Growth and maintenance of bacterial strains:** Bacterial cultures were grown on nutrient broth (Hi-Media, India) at 37 °C for 24 hrs. All the bacterial strains were maintained at 4 °C in nutrient agar medium as bacterial slants. The stock cultures were sub cultured at regular intervals prior to each antimicrobial testing. For experiments the active cultures were prepared by transferring a loopful of culture to 10 mL of nutrient broth and incubated for bacterial proliferation at 37 °C for 24 hrs.

**Culture medium:** The culture medium was prepared by dissolving 13 gm of nutrient broth [3.0 gm Beef extract, 5 gm Peptone, 5 gm sodium chloride (NaCl) in 1000mL of distilled water and pH adjusted to (6.8 - 7.2 ± 0.2)], for nutrient agar add 15.0 gm agar to the medium and subjected to sterilization in an autoclave at 121 °C for 15 min at 15 lbs pressure for sterilization.

**Antibacterial assay:** The agar cup-plate diffusion method was used to analyze the antibacterial activity of the prepared methanolic and ethanolic extracts of *S. rebaudiana* leaves. The crude extracts were diluted in 100 % (DMSO) at a concentration of 25, 50, 75 and 100 mg/mL respectively [10]. 20mL of sterile nutrient agar medium was poured into sterile petri-dishes and was allowed to solidify.

The prepared medium was seeded by pour plate method with the micro-organisms using 4mL of sterile top agar containing 1 mL culture. On the agar plate, wells (6 mm) were made by using sterile cork borer No. 4. The different concentrations of (25 mg/mL, 50 mg/mL, 75 mg/mL, 100 mg/mL) leaf extracts were loaded into the wells and all the plates were incubated overnight at a temperature of 37 $^{o}$C for 24 hours with appropriate positive and negative controls. The drug tetracycline was used as a positive control in a concentration of 10 µg/mL and 100 % (DMSO) was used as negative control. The antibacterial activity of the plant extract was analyzed by measuring the diameter of the inhibition zone in mm with a transparent scale. The antibacterial assay for each of the extracts against all microorganisms tested was performed in triplicates.

**Analysis of relative percentage (%) zone of inhibition:** The relative percentage zone of inhibition of two extracts of *S. rebaudiana* leaves against eight bacterial strains was calculated by formula:

Percentage relative zone of inhibition in mm =

$$\frac{\text{Zone of inhibition of sample (Plant extract) - Zone of inhibition of negative control}}{\text{Zone of inhibition of positive control (antibiotic standard drug)}} \times 100$$

**Statistical Analysis:** The data obtained were expressed as mean ± standard deviation and the antibacterial activity of the samples was compared with standard antibiotic (tetracycline) by applying ANOVA (two way analysis of variance).

**RESULTS:** The results of qualitative analysis of phytochemicals in methanolic and ethanolic crude extracts of *S. rebaudiana* leaves revealed the presence of different phyto-constituents such as saponins, flavonoids, tannins, alkaloids, terpenoids, cardiac glycosides, phlobatannins and reducing sugars while *o-anthraquinone glycosides, proteins* and aminoacids were absent. The results support the cause for its broad range of biological activities furnished in **Table 1**.

**TABLE 1: PHYTOCHEMICAL ANALYSIS OF METHANOLIC AND ETHANOLIC EXTRACTS OF *S. REBAUDIANA* LEAVES**

| Phytoconstituents | Test name | Response | |
|---|---|---|---|
| | | Methanolic Extract | Ethanolic Extract |
| Alkaloids | Mayer's Test | ++ | ++ |
| | Dragondroff's Test | +++ | ++ |
| | Wagner's Test | + | + |
| Glycosides | Modified Borntrager's Test | - | - |
| | Keller-Killiani Test | ++ | ++ |
| Phytosterols | Salkowaski's Test | ++ | ++ |
| | Libermann Burchard's Test | + | + |
| Phenols | Ferric Chloride Test | +++ | +++ |
| Flavonoids | Alkaline Reagent Test | +++ | ++ |
| | Lead Acetate Test | +++ | ++ |
| Tannins | Ferric Chloride Test | ++ | ++ |
| | Formaldehyde Test | + | + |

| | | | |
|---|---|---|---|
| Saponins | Froth Test | + | + |
| | Foam Test | + | + |
| Carbohydrates | Molisch's Test | + | + |
| | Benedict's Test | + | + |
| Proteins and Amino acids | Ninhydrin Test | - | - |
| | Biuret Test | - | - |

+++ = shows high concentration.
++ = shows moderate concentration.
+ = indicates presence of phytochemicals and
- = indicates absence of phytochemicals

DPPH assay was used for measuring the scavenging activity of different extracts of *S. rebaudiana* leaves. Radical scavenging activity (%) was shown in **Fig 1**. A 100µg/ml of methanolic extract, ethanolic extract and ascorbic acid exhibited 62.76, 64.35 and 85.99 % inhibition respectively. The biochemical or physiological action of the plant cannot be ascertained only by the preliminary phytochemical analysis. Therefore, the *in-vitro* antibacterial activity against pathogenic bacterial strains was also evaluated. The present study has revealed the efficiency of methanol and ethanol extracts against the selected gram negative or gram positive bacteria employed quantitatively to analyze the presence or absence of inhibition (**Table 2**).

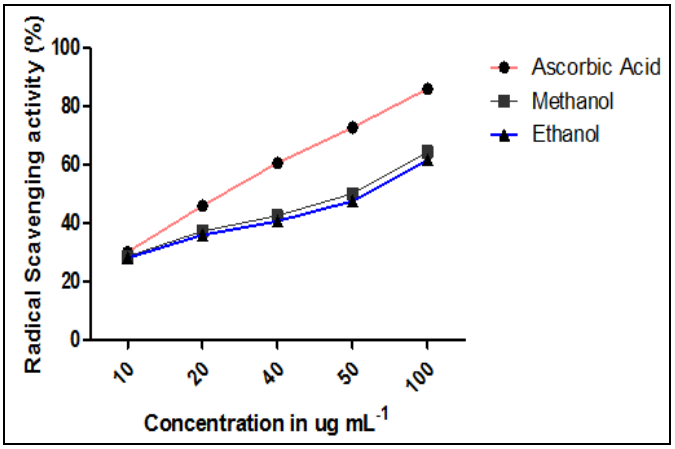


**FIG. 1: DPPH RADICAL SCAVENGING ACTIVITY IN DIFFERENT EXTRACTS OF DRIED *STEVIA REBAUDIANA* LEAVES**

**TABLE 2: ANTIBACTERIAL ACTIVITY OF METHANOLIC AND ETHANOLIC EXTRACTS OF *S.REBAUDIANA* LEAVES**

| Extracts/ Standard | Conc. (mg/ml) | Zone of inhibition (mm) | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | | *Zymomonas mobilis* | *Salmonella typhi* | *Bacillus subtilis* | *Pseudomonas aeruginosa* | *Cellulomonas spp* | *Pseudomonas fluorescens* | *Klebsiella pneumoniae* | *Escherichia coli* |
| Methanol extract | 25 | 11.83±0.23 | 11.06±0.16 | 12.03±0.12 | 11.83±0.12 | 12.95±0.04 | 15.13±0.20 | 13.00±0.21 | 13.15±0.12 |
| | 50 | 13.76±0.20 | 18.8±0.08 | 15.02±0.05 | 16.19±0.34 | 15.14±0.11 | 16.91±0.26 | 15.93±0.18 | 20.03±0.06 |
| | 75 | 21.76±0.20 | 20.43±0.75 | 17.9±0.29 | 17.03±0.13 | 18.13±0.11 | 18.27±0.22 | 18.05±0.16 | 21.01±0.08 |
| | 100 | 25.93±0.16 | 21.12±0.08 | 20.00±0.09 | 19.07±0.09 | 20.04±0.07 | 20.72±0.52 | 20.03±0.12 | 22.20±0.18 |
| Ethanol extract | 25 | 9.95±0.05 | 8.97±0.20 | 9.95±0.05 | 9.82±0.23 | 9.87±0.11 | 11.95±0.05 | 9.91±0.05 | 11.00±0.28 |
| | 50 | 12.84±0.20 | 14.93±0.07 | 12.91±0.10 | 14.76±0.20 | 12.87±0.13 | 15.95±0.05 | 13.87±0.09 | 13.90±0.04 |
| | 75 | 16.9±0.12 | 16.94±0.05 | 14.88±0.14 | 15.91±0.10 | 14.92±0.09 | 16.92±0.09 | 14.81±0.13 | 14.83±0.15 |
| | 100 | 19.92±0.09 | 18.91±0.10 | 16.88±0.09 | 16.93±0.05 | 15.85±0.13 | 17.94±0.06 | 16.92±0.05 | 16.11±0.08 |
| Tetracycline (Standard) | 10 (µg/ml) | 25.98±0.23 | 22.00±0.20 | 30.01±0.28 | 16.02±0.21 | 21.34±0.27 | 20.89±0.74 | 22.80±0.18 | 18.00±0.47 |
| Negative control (DMSO) | 100 % | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |

All values are expressed as mean ± standard deviation of mean (SD) of the three replicate

Out of the two tested extracts, methanol extract of *S. rebaudiana* leaves showed maximum zone of inhibition (in mm) at a concentration of 100 mg/mL as compared to ethanol extract against *Z. mobilis* (25.93 mm) followed by *E. coli* (22.20 mm), *S. typhi* (21.12 mm), *P. fluorescens* (20.72mm), *Cellulomonas spp.* (20.04 mm), *K. pneumoniae* (20.03 mm), *B. subtilis* (20.00 mm) and *P. aeruginosa* (19.07 mm) respectively. It was observed that on increasing the concentration of extracts, the anti-bacterial activity against tested pathogenic bacterial strains will also increase which indicates that antibacterial activity is concentration dependent. The phyto-constituents (alkaloids, glycosides, steroids, flavonoids and phenolics) might be responsible for antibacterial activity of the leaf extracts. In contrast, the other reason might be due to greater solubility of the extracts in these organic solvents [11]. The standard drug tetracycline (positive control) exhibited strong

antibacterial activity at concentration 10 µg/mL from 16.02 to 30.01 mm against all tested microorganisms. Negative antimicrobial activity was shown by control (DMSO). The analyzed results of the antibacterial activity of methanolic and ethanolic extracts of *S. rebaudiana* leaves were compared with antibiotic standard (tetracycline) for evaluating their relative percentage of inhibition zone (**Table 3**).

**TABLE 3: RELATIVE PERCENTAGE (%) ZONE OF INHIBITION (MM) OF METHANOLIC AND ETHANOLIC EXTRACTS OF *S.REBAUDIANA* LEAVES AGAINST TEST MICROORGANISMS**

| Extracts/ Standard | Conc. (mg/ml) | Relative percentage (%) zone of inhibition (mm) | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | | *Zymomonas mobilis* | *Salmonella typhi* | *Bacillus subtilis* | *Pseudomonas aeruginosa* | *Cellulomonas spp* | *Pseudomonas fluorescens* | *Klebsiella pneumoniae* | *Escherichia coli* |
| Methanol extract | 25 | 45.53 | 50.27 | 40.08 | 73.84 | 60.68 | 72.42 | 57.01 | 73.05 |
| | 50 | 52.96 | 85.45 | 50.04 | 101.06 | 70.94 | 80.94 | 69.86 | 111.27 |
| | 75 | 83.75 | 92.86 | 59.64 | 106.30 | 84.95 | 87.45 | 79.16 | 116.72 |
| | 100 | 99.80 | 96.00 | 66.64 | 119.03 | 93.90 | 99.18 | 87.85 | 123.33 |
| Ethanol extract | 25 | 38.29 | 40.77 | 33.15 | 73.84 | 46.25 | 57.20 | 43.46 | 61.11 |
| | 50 | 49.42 | 67.86 | 43.01 | 92.13 | 60.30 | 76.35 | 60.83 | 77.22 |
| | 75 | 65.05 | 77.00 | 49.58 | 99.31 | 69.91 | 80.99 | 64.95 | 82.38 |
| | 100 | 76.67 | 85.95 | 56.24 | 105.68 | 74.27 | 85.87 | 74.21 | 89.50 |

The methanol extract at 100 mg/ml exhibited maximum (123.33%) relative percentage inhibition against *E. coli*, followed by *P. aeruginosa* (119.03%), *Z. mobilis* (99.80%) *P. fluorescens* (99.18%), *S. typhi (96%), Cellulomonas spp* (93.90%), *K. pneumoniae* (87.85%) and *B. subtilis* (66.64%) while the ethanolic leaf extract at 100 mg/ml showed maximum relative percentage inhibition against *P. aeruginosa* (105.68%), and this percentage inhibition followed by *E. coli* (89.50%), *S. typhi* (85.95%), *P. fluorescens* (85.87%), *Z. mobilis* (76.67%), *Cellulomonas spp* (74.27%), *K. pneumoniae* (74.21%) and *B. subtilis* (56.24%).

The data analyzed by two-way (ANOVA) test on antimicrobial activity of methanol and ethanol extracts on eight bacterial species showed that the organic solvents used in extraction process had significant effect ($P<0.0001$) on the level of inhibition observed. Thus, the above results revealed that *S. rebaudiana* leaf extracts have great potential to work as antimicrobial agent and they can be used in the treatment of infectious diseases caused by resistant microorganisms.

**DISCUSSION:** The preliminary phytochemical screening tests are helpful in quantitative estimation and qualitative separation of various bioactive principles that are pharmacologically active chemical compounds which perform different activities, for example antimicrobial, antifungal, antioxidant, anti-inflammatory, anti-allergic, anti-angionic, antihypertensive, anti-obesity, antidiabetic and anticancer [12, 13, 14]. Different biological mechanisms facilitate in improved identification and isolation of major secondary metabolite components in the polar plant crude extracts which display antimicrobial and antioxidant properties [15]. The phytochemical screening conducted on methanol and ethanol extracts of *S. rebaudiana* revealed the presence of active chemical constituents such as alkaloids, flavonoids, saponins and tannins (**Table 1**).

Moreover, the presence of different phytoconstituents in the two different extracts may be responsible for the remedial properties of *S. rebaudiana*. Phenols, flavonoids and tannins are important bioactive components found to be present in methanol and ethanol crude extracts that act as excellent antioxidant and free radical scavengers which can cause inhibition of the cellular oxidative damage and lipid peroxidation.

The presence of tannins in the extracts have several physiological effects, such as to reduce blood pressure, cancer prevention, accelerate blood clotting, decrease the serum lipid level and increase spasmolytic activity in smooth muscle cells; it also inhibits several hydrolytic enzymes used by plant pathogens like pectolytic macerating enzymes [16, 17]. The plant extracts showed positive results for alkaloids which serve as plant regulators in actions like growth, metabolism and reproduction along with protein synthesis. It prevents damage to plant by functioning as detoxicating agents by methylating, condensing, and cyclizing the compounds.

Many pharmacological activities is also shown by alkaloids in protecting the body against chronic diseases for example antiasthma, antimalarial, anticancer [18] cholinomimetic [19] vasodilatory and antibacterial activities [20].

Glycosides are also present in the extracts which being non-toxic and its hydrolysis release phenolics which are toxic to microbial pathogens. It also functions as diuretics and cardiotonics basically used in the treatment of atherosclerosis, cardiovascular disease, rheumatic heart disease, cardiac arrhythmia and hypertension [21].

Presence of saponins functions as potential antimicrobial agents and they are involved in plant defense system [22]. They also inhibit inflamed cells and protect against congestive heart failure, hypercholesterolemia and hypotension [23]. Steroids and triterpenoids play an essential role in traditional herbal remedies. Both are generally used as drugs and constitute analgesic, anti-inflammatory, contraceptive and anticancer agents. Thus, these results show a clear picture that leaves of *S. rebaudiana* are useful in folklore remedies and responsible for numerous pharmacological actions and medicinal applications.

The single or combined effect of plant derived phytochemicals or secondary metabolites are responsible for antioxidant and antimicrobial potency of the plant. From the obtained results, when the efficacy of methanol and ethanol extracts were compared, it was found that methanol extract of *S. rebaudiana* leaves had remarkable antioxidant and antibacterial activity against the tested bacterial strains and this plant can be used to discover new antibiotics and some natural bioactive products that can provide enormous therapeutic potential in developing new pharmaceutical drugs with lesser side effects.

The presence of bioactive components in these extracts generally hinders the growth and metabolism of microorganisms in a negative manner and is quantified by determining the minimum bactericidal activity and minimum inhibitory concentration. The values obtained are used as guide for the treatment of major infections [24]. The current study provides essential information that maximum antimicrobial activity was shown by methanol extract at 100 mg/mL with inhibition zone of 25.93 ± 0.16 mm on dreaded pathogen like *Zymomonas spp*. So, the leaves of *S. rebaudiana* can be used to discover naturally occurring bioactive products that may serve for the development of phytomedicine that act against microbes. Sensitivity of bacterial strains was compared among leaf extracts of *S. rebaudiana* and artificial antibiotic (tetracycline), it was found that zones of inhibition was comparable between two and plant extracts can substituted in place of antibiotics as these extracts have lesser side effects which may arise due to consumption of synthetic antibiotics [25].

Further investigations should be performed on *S. aureus* bacteria and food spoiling fungi like *A. niger*, so that *Stevia* can be used for value addition as natural food preservative and also as substitute to artificial sweeteners.

In another study the methanol extracts shows inhibitory activity against *S. aureus* and *E. coli* with 8.33 mm and 13.0 mm zones of inhibition [26]. The presence of phytoconstituents in these extracts generally interferes with the growth and metabolism of microorganisms and it can be analyzed by determining the minimum inhibitory concentration and the minimum bactericidal activity [24]. However, *in vitro* antimicrobial evaluation of *S. rebaudiana* leaves extracts forms a major platform for promoting phytochemical and pharmacological studies.

**CONCLUSION**: The obtained results revealed the presence of medicinally important phytochemical constituents and antioxidants in the studied plant. The antimicrobial activity of *S. rebaudiana* leaves extracts is might be due to presence of these phytochemicals and antioxidants. Both the extracts (methanol and ethanol) show antimicrobial growth inhibition in agar-well diffusion assay, but methanol extract shows greater resistance against the number of pathogenic microorganisms that may cause variety of disease conditions. The extracts from this plant could be a good source for useful drugs. Elucidation of mechanism of action of this plant extracts on human health should be encouraged to carry out in future.

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