BIOLICAL ACTIVITIES OF NOVEL IN VITRO RAISED STEVIA PLANT

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ABSTRACT

Objective: This communication explores a lead fraction from methanolic extract of novel Stevia species raised under in vitro conditions for its various biological activities.

Methods: The dried Stevia leaves were crushed in methanol to get the polar extract. This methanol extract was tested for pancreatic lipase and alpha-amylase inhibitory activity using quantitative plate assays. Antibacterial property of the extract was also evaluated against Staphylococcus epidermidis, Escherichia coli, Bacillus subtilis, Staphylococcus aureus, and Pseudomonas aeruginosa. Further, the antioxidant potential was evaluated using 1,1-diphenyl-2-picrylhydrazyl.

Results: The methanolic extract inhibited pancreatic lipase with IC₅₀ of 5.74 µg/ml in a similar manner to a well-known anti-obesity drug in the market orlistat. The methanolic extract also showed a better pancreatic α-amylase inhibitory activity (IC₅₀ = 88 µg/ml) than acarbose. Further, the lead fraction exhibited 88.48% antioxidant activity. It also exhibited broad spectrum antimicrobial activity against the spectrum of Gram-positive and Gram-negative bacteria tested under laboratory conditions with a minimal inhibitory concentration ranging from 1.95 to 31.25 µg/ml.

Conclusion: Thus, this study signifies the vast potential of the lead fraction from a novel Stevia species for further development into a herbal formulation for prevention of various infectious and non-infectious diseases.

Keywords: Antioxidants, Enzyme inhibitor, Minimal inhibitory concentration, Stevia.
of Stevia formulation ranging from 20 to 120 µg/ml were pre-incubated with 20 µl of porcine pancreatic lipase (40 U) at 37°C for 1 hr. Then, 100 µl of p-nitrophenyl laurate (PNPL) (2 mM) was added to start the reaction. The volume was made up to 250 µl using Tris buffer (pH 7.4). The plate was then subsequently incubated at 37°C for 3 hrs. The reading of the 96-well plate was measured at 410 nm using a 96-well plate reader. Orlistat was used as a positive control whereas negative control only comprised enzyme and PNPL.

**In vitro pancreatic amylase inhibition assay**

Inhibition of pancreatic amylase by Stevia formulation was done according to modified protocol [15]. All the concentrations were reduced to 300 µl, and the test was performed in 96-well plate. In this assay, acarbose served as positive control.

**In vitro assay for anti-oxidant activity**

The free radical scavenging property of methanolic extract of Stevia was determined by recording the change in the optical density of the 1,1-diphenyl-2-picrylhydrazyl (DPPH) radicals (DPPH, Sigma-Aldrich, USA). Briefly, to 1 ml of DPPH (100 µM), 50 µl of extract (1 mg/ml) was added and incubated at 37°C for half an hour. Further, the absorbance was recorded at wavelength 517 nm using a ultraviolet-visible spectrometer (Hitachi U-2900, Japan). Both positive (gallic acid) and negative (DMSO) controls were used in assay [16].

Free radical scavenging activity was calculated as:

\[
\text{% Free radical scavenging} = \left[ \frac{\text{OD control} - \text{OD test}}{\text{OD control}} \right] \times 100
\]

**Total phenolic content of methanol extract**

Total phenolic content of the mixture was estimated using Folin–Ciocalteu reagent based assay using gallic acid as standard. The methanolic extract (1 mg/ml) was mixed with 500 µl (HiMedia, Mumbai, India) of Folin–Ciocalteu reagent followed by addition of 1.5 ml of 2% sodium carbonate (HiMedia, Mumbai, India). The final volume was made to 5 ml using double distilled water. This mixture was then subsequently incubated at 37°C for half an hour. Using a UV–visible spectrophotometer, the absorbance was measured at 765 nm. The total phenolic content was estimated from the regression equation: \( y = 0.026x - 0.008 \) with \( R^2 = 0.99 \) [16].

**In vitro broth dilution assay for minimal inhibitory concentration (MIC) estimation**

**In vitro broth dilution assay** to estimate MIC of the extract was performed as per described by Gomber and Saxena [17]. Briefly describing, 125 µl of Mueller-Hinton broth was dispensed into each well. Subsequently, 50 µl of 0.5 McFarland adjusted 18 hrs old test organisms were added into wells, and the plate was kept at 37°C for 2.5 hrs. Further, 25 µl of the test extract (i.e., two-fold serial dilutions of concentrations between 62.5 and 0.48 µg/ml) was dispensed into each well, and the plate was incubated at 37°C for 24 hrs. Further, 20 µl of 0.02% of 3-(4,5-Dimethylthiazol-2-Yl)-2,5-diphenyltetrazolium bromide (MTT) was added, and the plate was again incubated at 37°C for 60 minutes. The change in color in the wells due to difference in antimicrobial activities from pink (live) to yellow (dead) was observed and MIC was calculated. All the tests were performed in triplicates.

**RESULTS**

**Dose response activity of methanolic extract of Stevia against pancreatic lipase**

The quantitative plate assay ascertained the reduced pancreatic lipase activity by the methanolic extract of novel Stevia sp. and the only lipase inhibitor orlistat. Stevia extract inhibited pancreatic lipase with an IC\(_{50}\) of 2.7 µg/ml which was quite comparable to orlistat (IC\(_{50}\)=5.7 µg/ml). The inhibition pattern of Stevia extract was also similar to orlistat (Fig. 1).

**In vitro pancreatic α-amylase inhibitory activity of methanolic extract of Stevia**

This study showed the greater potential of Stevia species for inhibition of pancreatic amylase. The IC\(_{50}\) value of the methanolic extract was found to be 88 µg/ml as compared to that acarbose (IC\(_{50}\)=91.1 µg/ml) (Fig. 2).

**In vitro assay for antioxidant activity of methanolic extract of Stevia**

The methanolic extract of Stevia in the DPPH assay exhibited 87.48±2.08% antioxidant activity which was better than as compared to standard gallic acid which showed 84.16±1.49% antioxidant activity. There was a noticeable change in color from purple to yellow following scavenging reaction. Further, the total phenolic content of the lead fraction amounts to 21.42 µg/ml.

**In vitro broth dilution assay for MIC estimation**

The methanolic extract of in vitro raised Stevia exhibited better MIC value (1.95-3.91 µg/ml) for Staphylococcus epidermidis, Escherichia coli, and Bacillus subtilis whereas it showed higher MIC values (15.625-31.25 µg/ml) for Staphylococcus aureus and Pseudomonas aeruginosa when compared to streptomycin under in vitro conditions (Table 1).

**DISCUSSION**

Stevia is a safe, calorie-free, and natural sweetener with no side effects and it is also considered as a potential antidiabetic supplement also

![Fig 1: Dose-response curves for inhibition of pancreatic lipase by Stevia formulation. Orlistat served as positive control](image1)

![Fig 2: Dose-response curve for inhibition of α-amylase by Stevia extract. Acarbose served as positive control](image2)
Table 1: MIC of methanolic Stevia extract and streptomycin against Gram-positive and Gram-negative bacteria

| Test organism                       | MIC in µg/ml |
|-------------------------------------|--------------|
|                                     | Stevia extract | Streptomycin |
| Staphylococcus aureus               | 15.62*        | 7.18         |
| Staphylococcus epidermidis          | 3.91*         | 7.81         |
| Escherichia coli                    | 1.95          | 3.91         |
| Bacillus subtilis                   | 1.95          | 3.91         |
| Pseudomonas aeruginosa              | 31.25*        | 15.625       |

*p=0.05 (Tukey’s post hoc analysis). MIC: Minimal inhibitory concentration

possessing antioxidant and antimicrobial properties [18,19]. There are over 240 species of Stevia, and they have been largely focussed on their sweetness properties and in the development of anti-diabetic agent [20]. Apart from this, several studies have been carried which determined in vitro antioxidant, anti-cancer, anti-inflammatory and anti-angiogenic properties of the Stevia plant [6]. However, very few or almost no efforts have been made to explore the lipase inhibitory (anti-obesity) activity and correlate it with the anti-diabetic property of the plant. There exists only one report regarding pancreatic lipase inhibitory activity of Stevia rebaudiana [21]. Since obesity and diabetes are interrelated; this would be a novel avenue for management of two of the most threatening diseases of the modern world. In this context, we chose to evaluate both the anti-obesity and anti-diabetic property of the in vitro raised Stevia plant. The study was focussed on inhibition of two enzymes, viz., pancreatic lipase and α-amylase for anti-obesity and anti-diabetic property, respectively [1,4]. The lipase inhibitory property of the methanolic extract of Stevia plant (IC~50~ =5.74 µg/ml) in this study was found to be much better than that observed in Stevia (IC~50~ =530 µg/ml) [21]. Pancreatic lipase inhibitory potential of the lead fraction was better than hesperidin and carnosic acid isolated from citrus fruits and Salvia officinalis, respectively [22,23]. It is believed that further purification of the methanolic fraction will increase the inhibition response, which would further able to demonstrate exact IC~50~ of the purified compound [1]. Similar pattern was observed in several microbial lipase inhibitors such as vibralactone and Percy Quin whose phospholipids inhibitory efficiency increased on purification [24,25].

Alpha-amylase is a key enzyme which is accountable for degradation of starch into monosaccharides. Alpha-amylase hydrolyses complex sugar molecules into simpler sugars that are absorbed by the villi of small intestine, hence passing into the hepatic portal vein. These sugars are responsible for increase in postprandial glucose levels. The inhibitors of alpha-amylase have been given another name as starch blockers because they prevent absorption of dietary starch in the body ultimately lowering postprandial glucose levels [26]. The lead fraction in this study also exhibited comparable α-amylase inhibitory activity. In this study, the α-amylase inhibitory activity (IC~50~ =88 µg/ml) was found to be far better than aqueous extracts of S. rebaudiana reported by Ruiz-Ruiz et al. [15] and Patil et al. [27]. The extract might exhibit α-amylase inhibitory activity by binding at substrate binding site/active site of alpha-amylase, an enzyme responsible for the breakdown of α-1,4 glycosidic bonds in starch and other polysaccharides that increase the sugar level in body and subsequently leading to postprandial hyperglycaemia. Therefore, this report supports the theory that moieties from medicinal plants having a potential to inhibit α-amylase can be used as a pharmaphore for managing postprandial hyperglycaemia with minimal side effects [15].

Calculation of MIC using MTT is a widely known precise technique to estimate the response of a microorganism to a specific antibiotic [28]. This study reports the potential of methanolic fraction of Stevia plant to inhibit S. epidermidis, E. coli, and B. subtilis with a lower MIC value (1.95-3.91 µg/ml) whereas it displayed higher MIC value (15.62-31.25 µg/ml) for S. aureus and P. aeruginosa when compared to streptomycin. The antibacterial property reported in this study is far better than the earlier studies reporting MIC of S. rebaudiana against B. subtilis and E. coli to be 500 µg/ml [29]. Earlier studies have also reported that the aqueous and ethanolic extract of Stevia possesses broad spectrum antimicrobial activity against microorganisms such as S. aureus, S. epidermidis, B. subtilis, and P. aeruginosa. Stevia has been known to possess antibacterial, anti-fungal, and anti-viral properties [30]. Further, the lead fraction also exhibited strong antioxidant activity via free radical scavenging activity. The antioxidant activity of this methanolic extract (88.48%) was better than that of antioxidant activity reported from earlier Stevia extract (71.75%) and 86.4% [12,31]. Following scavenging reaction, there was a noticeable change in color from purple to yellow. The change in color was due to the reaction of the extract with the antioxidant molecule which ultimately resulted in the scavenging of the radical by hydrogen donation [12]. Stevia has been reported to have antioxidant and anti-diabetic properties in diabetic mice [32]. Thus, the Stevia plant offers to be a promising source of bioactive compounds with efficient medicinal properties. To date, there are no reports on determination of lipase inhibitory and α-amylase inhibitory activity from an extract of in vitro grown Stevia plant. This study is the pioneering work where the lipase and α-amylase inhibitory activities of the methanolic extract of Stevia plant along with potential antioxidant and antimicrobial activities were explored.

CONCLUSION

In vitro raised novel strain of Stevia plant contains a potential pharmacophore which can be used as an herbal formulation for a safer management of obesity and related diseases. Market today needs a natural drug for the two most prevailing and interrelating diseases. Purification, structural and biochemical characterization of the bioactive compound in the lead extract will lead to a natural drug for obesity and diabetes management.

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