Synergistic Interaction between Hydroalcoholic Extracts of Aerial Parts of *Tridex Procumbens* L. and *Bryophyllum Pinnatum* L. Leaves to Promote In Vitro Hypoglycemic Activity

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Abstract: Current research around the world is focused on finding an alternative source of treatment from natural resources for diabetic management, apart from the available synthetic medicines. The present study is a preliminary study of a polyherbal formulation of two herbs viz. *Tridex procumbens* (Tp) and *Bryophyllum pinnatum* (Bp) and an assessment of its antidiabetic activity. The formulation was screened for its phytochemical constituents, total phenols, flavonoids, and antioxidant content. It was also analyzed for its inhibitory effect against the digestive enzymes α-amylase and α-glucosidase and to compare their individual hypoglycemic potential with their combination (Bt) in a specific proportion using the standard drug Acarbose. The study reports for the first time the synergistic effect of the extracts in the inhibition of alpha amylase activity. It can be concluded that the hydroalcoholic plant extracts interacted synergistically exhibiting the best inhibitory activity on the enzyme studied and the presence of phytochemicals like flavonoids, saponins, and tannins may have contributed greatly to the inhibitory activity of the extracts.

Keyword: *Tridex procumbens*, *Bryophyllum pinnatum*, hypoglycemic activity, α-amylase inhibition, antioxidant activity

I. INTRODUCTION

Recent advancements in the diagnosis and treatment of metabolic disorders, especially diabetes are focused on finding an alternative source of treatment from natural resources. The management of blood glucose level is the hallmark in the treatment of this disease. This may be achieved through the use of oral hypoglycemic drugs such as biguanides, insulin secretagogues, and α-glucosidase inhibitors. Considering the doubts about the efficacy of oral hypoglycemic agents known to medicine researchers have been making efforts to find better substitutes from plant sources. The purpose of the present study was to investigate the inhibitory effect of whole plant of *Tridex procumbens* and *Bryophyllum pinnatum* leaf extracts on the activities of α-amylase.

*Tridex procumbens* Linn. (Compositae family) is a native of tropical America and naturalized in tropical Africa, Asia, Australia and India. The plant is a small herb having short, hairy blade like leaves and is popularly called ‘coat buttons’ in English because of the appearance of flowers (yellow corolla). It has been extensively used in Ayurvedic system of medicine in India for various ailments (16-18). Different pharmacological activities of *Tridex procumbens* have been reported in the last few years such as radical scavenging (19), wound healing activity (20, 21), anti-diabetic activity (22) and blood pressure lowering effect.

*Bryophyllum pinnatum* (Lam.) (synonym: *Kalanchoe pinnata*, Lam.; common names: Life plant, air plant (Mexican), love plant, Canterbury bells, Cathedral bells, etc) is a perennial herb found perennially in various tropical and subtropical regions as well as mildly temperate regions worldwide that grows in the wild and is used as a traditional medicinal plant in tropical Africa, China, Australia, tropical America and Indian system of medicine- Ayurved(2). It habitats waste places, road sides and hedges throughout India (3). The leaf extracts of this plant have been routinely used for ailments like asthma, kidney stones and ulcers and have been reported to possess antimicrobial (5), antifungal(6), anti-ulcer(7), anti-inflammatory and analgesic(7), antihypertensive(8) and antimutagenic activities(10).

In the present work, the plant parts are combined based on previously available literature, indigenous knowledge, and various preliminary studies in a fixed proportion. Thus, the polyherbal formulation has been named “Bt”. Our aim was to investigate the antidiabetic potential of the polyherbal formulation (i.e., Bt) and prove its synergistic effect against various aspects involved in diabetes management.

II. MATERIAL AND METHODS

2. A Preparation of extracts of Tp, Bp and Bt

The plant parts were identified, collected and authenticated at Jawaharlal Nehru Cancer Hospital and Research Centre, Bhopal, Madhya Pradesh, India during the months of...
December 2015 to February 2016. For the study, aerial parts of *Tridex procumbens* (Tp) from the institute premises and fresh leaves of *Bryophyllum pinnatum* (Bp) were collected from the herbal garden of the institute. For extract preparation, the collected raw herb parts were allowed to shade dry for 2-3 weeks. The shade dried parts were pulverized, weighed and were divided into three portions. The portions were macerated in different separating funnels with 50% methanol, hexane and water. The mixtures were vigorously shaken intermittently for 72 hours. The extracts were collected in separate beakers and concentrated in water bath at 45°C. This process was repeated 3 times at least till colorless marc was obtained for each solvent. It was dried at 45°C in oven, powder of crude extract collected and weighed. After defatting with petroleum ether, the crude extracts were used for the study of phytochemicals and enzyme inhibition assay.

For the combination (Bt), the pulverizations of Tp and Bp were mixed in 2:3 ratio by weight and macerated in 50% methanol, hexane and water, following the same protocol as discussed above.

2. B Chemicals

Acarbose was purchased from Glucobay, Bayer Pharma, India. Sodium dihydrogen orthophosphate, disodium hydrogen phosphate (Himedia, India), Alloxan was purchased from Fluka BioChemika, India. Metformin was purchased from Sun Pharmaceuticals Ltd, India. Alpha Amylase; Thiobarbituric acid, 5-5’ dithio-bis-2-nitrobenzoic acid and starch were purchased from Sigma-Aldrich, USA. All other chemicals used were of analytical grade.

2. C Alpha Amylase Inhibitory Assay

This assay was carried out according to modified procedure of McCue and Shetty(18). Series of each extract solutions were prepared in varying concentrations (1.25-10 mg/mL). To the tubes, 250μL of 0.02M sodium phosphate buffer (pH 6.9) containing 0.5mg/mL of α -amylase was added. The solution was incubated at 25°C for 10 mins, after which 250μL of 1% starch solution in 0.02M sodium phosphate buffer was added at timed intervals and then further incubated in boiling water bath for 5 min and cooled to room temperature. The reaction was terminated by adding 500μL of dinitrosaliclyc acid (DNS). The tubes are again incubated in boiling water for 5 mins and cooled to room temperature. The reaction mixture was diluted with 5mL distilled water and the absorbance was measured at 540nm. A control was prepared by replacing the extracts with distilled water. Each experiment was performed in triplicates, along with appropriate blanks. Acarbose at various concentrations (10-100 μg/ml) was included as a standard. The result is expressed as percentage inhibition, which was calculated as,

\[
\text{Inhibition (\%) = } \left( \frac{A_{\text{control}} - A_{\text{sample}}}{A_{\text{control}}} \right) \times 100
\]

2. D Phytochemical Screening of Extracts

The crude extracts and their combination were subjected to qualitative chemical tests to identify various classes of bioactive chemical constituents present in the plants like amino acids, steroids, cardiac glycosides, phenols, tannins, terpenoids, alkaloids, flavonoids, saponins, carbohydrates, reducing sugar etc using standard procedures (2).

2. E Evaluation of Bioactive Constituents

2. E.1 Determination of Total Phenolic Content (TPC): The concentration of phenolics in the extract was determined using Folin-Ciocalteu assay (23). In brief, 1ml of extract (10mg/mL in DDW) or a series of standard solution of Gallic acid(1mg/mL) were taken in test tubes and volumes were made upto 10mL using DDW. 1.0mL of Folin-Ciocalteu reagent was added to the tubes and shaken. After 5 min, 1mL of saturated Na2CO3 was added to the tubes and the volume made upto 10mL using DDW. The reaction was kept in the dark for 90 C at room temperature and the absorbances noted against blank at 760nm using UV-1660, Shimazdu Spectrophotometer. All samples were analysed in triplicates.

2. E.2 Determination of Total Flavonoid Content (TFC): The determination of flavonoids was carried out according to Aluminiun Chloride colorimetric method(24) In brief, to 1ml of different concentrations of the extracts (1 mg/mL methanol) or standard solution of Rutin (20, 40, 60, 80 and 100 mg/L), 5ml of 2% AlCl3 in methanol was added. After a 60 minute incubation at room temperature (23 ± 2 C) the absorbance against blank consisting of water instead of extract was determined at 510nm using UV-1660, Shimazdu Spectrophotometer and the flavonoids content was calculated with (±) Rutin and the concentration was expressed as (±) Rutin equivalents. All samples were analysed in triplicates.

2 F Quantification of antioxidant activity

2.F.1 Hydroxyl radical scavenging activity: The assay was performed as described by Halliwell *et. al*. (15) with slight modification. The solutions were prepared freshly before the start of the experiment. The reaction mixture contained 100μl of 30mM 2-deoxy-D-ribose dissolved in NaH2PO4 - Na2HPO4 phosphate buffer (pH 7.4), 100μl of 100μM FeCl3 and 100μM EDTA (1:1 v/v), 100μl of H2O2 (1.0 mM) and 100μl ascorbic acid (1.0mM). The reaction mixture was added to 50μl solution of various concentrations of the extracts (50μg-500μg/ml) and DMSO standard series. After an incubation period of 1hr. at 37 C the extent of deoxy ribose degradation was measured by the TBA reaction. 1ml of TBA (1% in 50mM NaOH) and 1ml of TCA were added to the reaction mixture, tubes were heated at 100 C for 20 min. After cooling the absorbance was read at 532 nm against a blank (containing only buffer and deoxy ribose). The % inhibition was calculated by the formula

\[
\text{Inhibition (\%) = } \left( \frac{A_{0} - A_{1}}{A_{0}} \right) \times 100
\]
Where, \( A_0 \) is the absorbance of control and \( A_1 \) is the absorbance of sample. All the values expressed are the mean values carried out in triplicates. BHT was used as a positive control.

2.F.2 Superoxide Radical (\( O_2^- \)) Scavenging Activity: The estimation of superoxide radical scavenging activity of the extracts was done based on the modified method described by Liu et al (12). Alkaline DMSO was used as a super oxide generating system. The superoxide is generated by reacting 0.1ml of Nitro blue tetrazolium (NBT) (50μm) solution with 0.3ml sample solution of different extracts in a concentration of 1-10μg/ml .The reaction was started by adding 1ml of alkaline DMSO solution (10um) to the mixture. The reaction mixture was incubated at 25°C for 5 min. and the absorbance at 560nm was measured against the blank samples. Butylated Hydroxy Toluene (BHT) was used as positive control.

\[
\% \text{ Inhibition} = \left( \frac{A_{\text{control}} - A_{\text{sample}}}{A_{\text{control}}} \right) \times 100
\]

Table 1. Phytochemical screening of Tp, Bp and Bt in different solvents

<table>
<thead>
<tr>
<th>S.N</th>
<th>PHYTOCHEMICAL</th>
<th>Aqueous Extract</th>
<th>Methanolic Extract</th>
<th>Petroleum Extract</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Tri</td>
<td>Bry</td>
<td>Bt</td>
</tr>
<tr>
<td>1</td>
<td>Flavonoids</td>
<td>+</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>2</td>
<td>Tannins</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>3</td>
<td>Alkaloids</td>
<td>+</td>
<td>+</td>
<td>+</td>
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<tr>
<td>4</td>
<td>Glycosides</td>
<td>-</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>5</td>
<td>Saponins</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>6</td>
<td>Phenols</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>7</td>
<td>Terpenoids</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>8</td>
<td>Steroids</td>
<td>-</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>9</td>
<td>Carbohydrates</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>10</td>
<td>Proteins</td>
<td>+</td>
<td>-</td>
<td>+</td>
</tr>
</tbody>
</table>

+ tests positive; -- tests negative

3. B Total Phenolics and Flavonoids

The total phenolic content was calculated using calibration curve for Gallic acid standard curve (\( y = 0.002x + 0.004 \); \( R^2 = 0.956 \)). The results were expressed as Gallic acid equivalent per gram dry weight of extract (mg of GAE/g of extract) (Table 2). The total phenolic content of methanolic extract of \( T.\) procumbens was 24.99 ± 3.1 mg gallic acid equivalent/g of extract which was highest in comparison to other extracts. Similarly, the total phenolic content of methanolic extract of \( B.\) pinnatum was 53.18 ± 4.2 mg of GAE/g of extract which was highest in comparison to other extracts as well as to that of \( T.\) procumbens. The total flavonoid content was calculated using calibration curve for Rutin standard curve (\( y = 0.006x + 0.014 \); \( R^2 = 0.985 \)) and was found to be 28.331 ± 2.6 mg Rutin equivalent/g for methanolic extract of \( T.\) procumbens and for \( B.\) pinnatum was found to be 21.27 ± 2.6 mg Rutin equivalent/g of methanolic extract.

3.C Antioxidant Assay

Statistical analysis: All the determinations were done in triplicates. The statistical processing of the data obtained from all studies is expressed as means ± standard deviation (SD) of three separate experiments. IC\(_{50}\) values were calculated from linear regression analysis.

III. RESULTS

3. A Phytochemical screening

The phytochemical screening of \( Tp, Bp \) and \( Bt \) extracted in different solvents is shown in table 1. Aqueous and Methanolic extracts seem to possess a variety of active components that include alkaloids, flavonoids, glycosides, saponins, polyphenols, tannins etc which may relate to its therapeutic properties.
3.C.1 Hydroxyl radical scavenging activity: The Fenton reaction was used to generate OH radicals in the system and the free radical activity was determined by the degradation of deoxyribose, as standardized by Elizabeth and Rao. The OH radicals attack the deoxyribose and initiate a series of reactions that eventually result in the formation of Trichloroacetic acid reactive substance (TBARS). The radical scavenging by the protectors result in inhibition of TBARS as shown by the extracts in Fig 1. The alcoholic extracts of T.procumbens showed better scavenging as compared to aqueous and n-hexane extracts. The effect of alcoholic extract eventually got stabilized above concentration of 80 μg ml⁻¹ with IC50 value of 53.15. The IC50 value for BHT was found to be 44.54 μg/ml. The data of studies on B.pinnatum shows IC50 value of of methanolic extract to be highest at 62.08, followed by aqueous extract with IC50 74.58 and minimum in case of 2-hexane extract (IC50 165.37). The IC50 vale for Bt was found to be 49.11 which is significantly closer to the standard over concentration of 300 μg/ml. The scavenging effect of the T.procumbens, B.Pinnatum, their combination and standards with the superoxide radical was found to follow the order: BHT (84.68%) > Btm (66.4%) > Tpm (62%) > Tpa (51.54%) > Bpm (36.21%) > Tph (35.44%) > Bph (13.1%).

Table 2. Quantitative Bioactive Constituents of the extracts

<table>
<thead>
<tr>
<th>Sample Extracts in different solvents</th>
<th>Total Polyphenols (mg GAE/g extract)</th>
<th>Flavonoids (mg Ru/g extract)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>T. procumbens</td>
<td>B. pinnatum</td>
</tr>
<tr>
<td>Methanol</td>
<td>24.99 ± 3.1</td>
<td>53.18 ± 4.2</td>
</tr>
<tr>
<td>Water</td>
<td>22.86 ± 2.4</td>
<td>22.19 ± 2.4</td>
</tr>
<tr>
<td>Hexane</td>
<td>16.12 ± 1.2</td>
<td>12.07 ± 0.04</td>
</tr>
</tbody>
</table>

3.C.2 Superoxide Radical (O₂⁻) Scavenging Activity: The data for Superoxide Radical (O₂⁻) Scavenging Activity show highest % inhibition by Bt methanolic extract among all other extracts. Its value gets stabilized above concentration of 250 μg/ml showing 66.47% inhibition. The alcoholic extracts of individual plants show better inhibition than the aqueous or n-hexane fractions. The scavenging effect of the T.procumbens, B.Pinnatum, their combination and standards with the superoxide radical was found to follow the order: BHT (84.68%) > Btm (66.4%) > Tpm (62%) > Tpa (51.54%) > Bpm (36.21%) > Tph (35.44%) > Bph (13.1%).

3. D Alpha amylase inhibition

Henceforth, the antihyperglycemic effects of the methanolic extracts only were studied (y = 0.57x + 39.13 R² = 0.989) and it was found that T.procumbens has IC50 value of 19.07 μg/ml and the alcoholic extract of B.pinnatum has IC₅₀ value of 94.15μg/ml. Acarbose showed α- amylase inhibitory activity with IC₅₀ value of 0.33 μg/ml.
IV. DISCUSSION

One of the anti-diabetic therapeutic strategies is inhibition of carbohydrate digesting enzymes such as alpha-amyrase and alpha-glucosidase (Narkhede et al., 2011). Alpha-amyrase hydrolyzes complex starches to oligosaccharides, while, alpha-glucosidase hydrolyzes oligosaccharides to glucose and other monosaccharides. Inhibition of these enzymes produces postprandial anti-hyperglycemic effect by reducing the rate and extent of glucose absorption from small intestine (Okoli et al., 2011). Many natural products have shown inhibitory activity against these enzymes (Tripathi et al., 2011; Kumar et al., 2012; Srivastava et al., 2012). In the present study, extracts of Tridax procumbens and Bryophyllum pinnatum and their combination Bt in 2:3 ratio were evaluated for their effect on alpha-amyrase enzyme using in vitro assays. Both the plants have shown reduction of blood glucose levels in diabetic rats and in hyperglycemic subjects (Taddai, et al., 2000; Kamat et al., 2012). Inhibition of carbohydrate digesting enzymes such as alpha-amy-lase could be one of the mechanisms. The phytochemical study reveals that both the plants are rich in a number of bioactive components.

The role of oxidative stress in diabetes and diabetic complications has been reported (Giacco and Brownlee, 2010). Antioxidants can scavenge free radicals and play important role in prevention of diabetes. The combination Bt showed maximum antioxidant activity in hydroxyl radical activity and superoxide radical scavenging activity. Natural antioxidants not only protect lipids from oxidation, but also provide health benefits associated with preventing damage due to biological degeneration and it is well-known that plant phenolics are highly effective free radical scavengers and antioxidants due to their hydrogen donating ability. Table 2 gives the total Polyphenols and flavonoids from different solvent extracts in both the plants, which followed the order: methanol > water > Hexane. Hence methanol extract contained higher levels of polyphenols, flavonoids and tannins. So further hypoglycemic studies were undertaken using methanolic extracts only. The results show that the combination Bt can be an effective natural antidote for the treatment of hyperglycemia.

REFERENCES

[11]. 4. Elmaštaj M, Guličić L, Išidžak O, Klfićević B, Bilbija AR, Bilia AR, Bilia MC, Bilia AR, 2012. Inhibition of carbohydrate digesting enzymes such as alpha-amyrase could be one of the mechanisms. The phytochemical study reveals that both the plants are rich in a number of bioactive components.

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