



## Comparative Study of *In-Vitro* Antioxidant Potential of Crude Extracts of *Bryophyllum pinnatum* [(Lam) Oken] Leaves in Different Solvents and the *In-Vitro* Hypoglycemic Potential of its Hydroalcoholic Extract

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### ABSTRACT

The therapeutic potential of leaf extract of *Bryophyllum pinnatum* was screened for *in vitro* antioxidant potential and alpha amylase inhibitory action. Antioxidant activity of the extract was evaluated for hydroxyl radical scavenging activity by Fenton reaction, free radical scavenging by hydrogen peroxide scavenging and superoxide scavenging potential. The *in vitro* alpha amylase inhibition action of the plant was evaluated for hypoglycemic properties using starch as substrate. The phytochemical screening of the crude hydro-methanolic, aqueous and hexane extracts revealed the presence of flavonoids, alkaloids, tannins, glycosides, saponins, phenols, steroids and carbohydrates. The results indicated that 50%-methanol extract showed significant antioxidant potency at concentration of 250-500µg as compared to other solvent extracts and also possess alpha amylase inhibitory property. Hence it can be suggested that the leaf extract of *Bryophyllum pinnatum* has potential as an antioxidant and probably in biological systems as a nutraceutical for hypoglycemia.

**Keywords:** *Bryophyllum pinnatum*; Antioxidant activity; Phenolic content; α-Amylase inhibition

### INTRODUCTION

Diabetes mellitus (DM) including Type 2 DM (T2DM) is mainly a lifestyle disorder with increasing worldwide prevalence. It is a chronic metabolic disorder that affects the quality of life in all dimensions- physical health, social as well as psychological well-being with. The primary clinical manifestation of diabetes is hyperglycemia which contributes to diabetic complications by altering cellular metabolism, vascular matrix molecules and circulating lipoproteins [1]. The conventional therapies, as of yet, have been unable to achieve an effective cure for it. Hence, systematic and intensive search in medicinal plants for new drugs to treat T2DM seem to be of great utility. The present study aims to open new avenues for the utilization of hydro-alcoholic extract of *Bryophyllum pinnatum* for diabetes. *Bryophyllum pinnatum* (Lam.) (Crassulaceae) (synonym: *Kalanchoe pinnata*, Lam.; common names: Life plant, air plant, love plant, Canterbury bells, etc) is a perennial herb found in various tropical and subtropical regions as well as mildly temperate regions worldwide. It grows in the wild and habitats waste places, road sides and hedges throughout India [3]. It is used as a traditional medicinal plant in tropical Africa, China, Australia, tropical America and Indian system of medicine- Ayurveda [2]. The leaf extracts of this plant have been used as folkloric medicine for ailments like asthma, kidney stones and ulcers [4]. It has been reported to possess antimicrobial [4], antifungal [5], anti-inflammatory, analgesic [5,6], antihypertensive [7] and antimutagenic properties [9]. A number of active compounds, including alkaloids, glycosides, flavonoids, steroids, bufadienolides etc have been identified in *B. pinnatum* [11-13] that have been shown to possess variety of therapeutic activities such as antioxidant properties, antibacterial, insecticidal, antitumor and cancer preventive actions [14-18]. The role of oxidative stress in free radical

generation and associated diabetic complications has already been reported [8] and antioxidants can scavenge free radicals and play important role in prevention of diabetes. Hence in present study, antioxidant effects of *Bryophyllum pinnatum* leaves extracts, total phenolics present in various solvents along with the alpha amylase inhibition activity of its hydro-methanolic extract were evaluated.

## MATERIALS AND METHODS

### Collection of the material

Fresh leaves of *Bryophyllum pinnatum* was collected from the herbal garden of Jawaharlal Nehru Cancer Hospital and Research Centre (JNCHRC), Bhopal, Madhya Pradesh, India. The material was identified and authenticated by Dr Madhuri Modak, Professor, Dept of Botany, Shaheed Bhagat Singh Govt. Degree College, Ashta, Sehore, Madhya Pradesh with voucher specimen number -1131-60.01-263.

### Preparation of extracts

Fresh and healthy leaves were collected and allowed to shade dry for 2-3 weeks. The shade dried parts were pulverized and weighed and the powdered leaves were divided into three portions. Each portion was macerated in a separating funnel with 50% methanol, hexane or 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 was used for the study of phytochemicals, antioxidant study and enzyme inhibition assay.

### Phytochemical screening of extract

The crude extracts were subjected to qualitative phytochemical tests to identify various classes of bioactive chemical constituents present in the plant using standard procedures. [16].

### Antioxidant assay

#### Hydroxyl radical scavenging activity:

**Non-site specific Hydroxyl Radical (OH<sup>•</sup>) mediated Deoxy-D-ribose degradation:** The experiment was performed with slight modifications as described by Halliwell *et al* [18]. The solutions were prepared fresh before the commencement of the experiment. The reaction mixture contained 100µl of 30mM 2-deoxy-D-ribose dissolved in NaH<sub>2</sub>PO<sub>4</sub>- Na<sub>2</sub>HPO<sub>4</sub> phosphate buffer (pH 7.4), 100µl of 100µM FeCl<sub>3</sub> and 100µM EDTA (1:1 v/v), 100µl of ascorbic acid (1.0mM) and 100µl H<sub>2</sub>O<sub>2</sub> (1.0 mM). 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 as the extent of inhibition of formation of thio barbituric acid reactive substance (TBARS) by adding 1ml of TBA (1% in 50mM NaOH) and 1ml of 5% TCA in distilled water to the reaction mixture; tubes were heated at 100° C for 20 min. After cooling the absorbance of the solutions were noted at 532 nm against blank solution (containing buffer solution and 2-deoxy ribose). The % TBARS inhibition was calculated by the formula

$$\% \text{ TBARS Inhibition} = [(A_{b_0} - A_{b_1}) / A_{b_0}] \times 100$$

Where, A<sub>b0</sub> is the absorbance of control and A<sub>b1</sub> is the absorbance of sample. All the values expressed are the mean values of experiments performed in triplicates. BHT was used as a positive control.

**Hydrogen Peroxide (H<sub>2</sub>O<sub>2</sub>) Scavenging Activity:** The estimation of H<sub>2</sub>O<sub>2</sub> scavenging activity was done by according to the reported method of Nabavi *et al* [12] The concentration of H<sub>2</sub>O<sub>2</sub> solution (40mM/L) prepared in 50mmol/L phosphate buffer (pH 7.4) was calculated by measuring the absorption of standard and extract solutions at 230 nm. Absorbance of extract at different concentrations (200µg, 400 µg, 600µg, 800µg and 1000 µg/mL) or standard Butylated Hydroxy Toluene (BHT) solution with 2mL of H<sub>2</sub>O<sub>2</sub> was determined after 10 mins against a blank solution of phosphate buffer. The percentage of H<sub>2</sub>O<sub>2</sub> scavenged was determined as-% H<sub>2</sub>O<sub>2</sub> Scavenging activity = [(A<sub>0</sub> - A<sub>1</sub>) / A<sub>0</sub>] x 100]

Where A<sub>0</sub> is the absorbance of BHT and A<sub>1</sub> the absorbance of *B.pinnatum* extracts or standards

**Superoxide radical (O<sub>2</sub><sup>•-</sup>) scavenging activity:** The estimation of superoxide radical scavenging activity of the extracts was performed as modified method described by Liu *et al* [14]. The superoxide is generated by reacting 0.1ml of Nitro blue tetrazolium (NBT) (50µm) solution with 0.3ml of different extract solutions in a concentration

of 1-10 $\mu$ g/ml. The reaction was initiated by adding 1ml of alkaline DMSO solution (10 $\mu$ m) to the reacting solutions. The solutions were then incubated at room temperature (or at 25°C) for 5 minutes and the absorbance was measured against the blank samples at 560nm. Positive control used was Butylated Hydroxy Toluene (BHT).

$$\% \text{ Superoxide radical scavenging} = [(A_0 - A_1) / A_0] \times 100]$$

Where  $A_0$  is the absorbance of the BHT and  $A_1$  the absorbance of *B. pinnatum* extracts or standards. The data at each point represent mean  $\pm$  standard deviation for triplicate values.

#### Determination of Total Phenolic Content (TPC)

The total phenolic content 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  $\text{Na}_2\text{CO}_3$  was added to the tubes and the volume made upto 10mL using DDW. The reaction was kept for 90 minutes at room temperature in the dark and the absorbances noted against blank at 760nm using UV-1660, Shimadzu Spectrophotometer. All data are represented as mean  $\pm$  standard deviation for triplicate experiments.

#### Determination of Total Flavonoid Content (TFC)

The determination of flavonoids was carried out according to Aluminium 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%  $\text{AlCl}_3$  in methanol was added. After incubation at room temperature ( $23 \pm 2$  C) for 60 minutes, the absorbance against blank consisting of water instead of extract was determined at 510nm using UV-1660, Shimadzu Spectrophotometer and the flavonoids content was calculated with ( $\pm$ ) Rutin and the concentration was expressed as ( $\pm$ ) Rutin equivalents. All data are expressed as mean  $\pm$  standard deviation for triplicate values.

#### Alpha amylase inhibitory assay

This assay was carried out according to modified procedure of McCue and Shetty [8]. Series of each extract solutions were prepared in varying concentrations (1.25-10 mg/mL). To the tubes, 250 $\mu$ L of 0.02M sodium phosphate buffer (pH 6.9) containing 0.5mg/mL of  $\alpha$ -amylase was added. The solution was incubated at 25°C for 10 mins, after which 250 $\mu$ L of 1% starch solution in 0.02M sodium phosphate buffer was added at regular intervals and then again incubated in boiling water bath for 5min and cooled to room temperature. The reaction was terminated by adding 500 $\mu$ L of dinitrosalicylic acid (DNS). The tubes are again incubated in boiling water for 5mins and cooled to room temperature. The absorbance was measured at 540nm. A control was prepared by replacing the extracts with distilled water ( $A_0$ ). Each experiment was performed in triplicates. Acarbose at various concentrations (10-100  $\mu$ g/ml) was included as a standard. Absorbances of extract and standard were noted as ( $A_1$ ). The result is expressed as percentage inhibition of enzyme activity, which was calculated as, % Inhibition =  $[(A_0 - A_1) / A_0] \times 100$ . The result is also expressed in terms of  $\text{IC}_{50}$  value.

#### Statistical analysis

The statistical analysis of the data obtained from all the experiments is expressed as mean  $\pm$  standard deviation of three different determinations.  $\text{IC}_{50}$  values were calculated from statistical linear regression analysis.

## RESULTS

The active components found in the extract include alkaloids, flavonoids, glycosides, saponins, polyphenols, tannins etc. the results are shown in table 1.

#### 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 total phenolic content of hydro-methanolic extract of *B.pinnatum* was  $53.18 \pm 4.2$  mg/g mg gallic acid equivalent/g of extract (mg of GAE/g of extract) which was higher in comparison to aqueous and hexane extracts. 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  $16.27 \pm 2.6$  mg Rutin equivalent/g of hydro-methanolic extract.

Table1. Phytochemical screening of the crude extracts of *B pinnatum* leaves

| S No | Phytochemical | Aqueous Extract | Hydro-methanolic Extract | Hexane Extract |
|------|---------------|-----------------|--------------------------|----------------|
| 1    | Flavonoids    | -               | +                        | +              |
| 2    | Tannins       | +               | +                        | +              |
| 3    | Alkaloids     | +               | +                        | -              |
| 4    | Glycosides    | +               | -                        | -              |
| 5    | Saponins      | -               | +                        | -              |
| 6    | Phenols       | +               | +                        | +              |
| 7    | Tepenoids     | +               | +                        | +              |
| 8    | Steroids      | +               | +                        | -              |
| 9    | Carbohydrates | +               | +                        | +              |
| 10   | Proteins      | -               | +                        | +              |

+ Tests positive; -- Tests negative

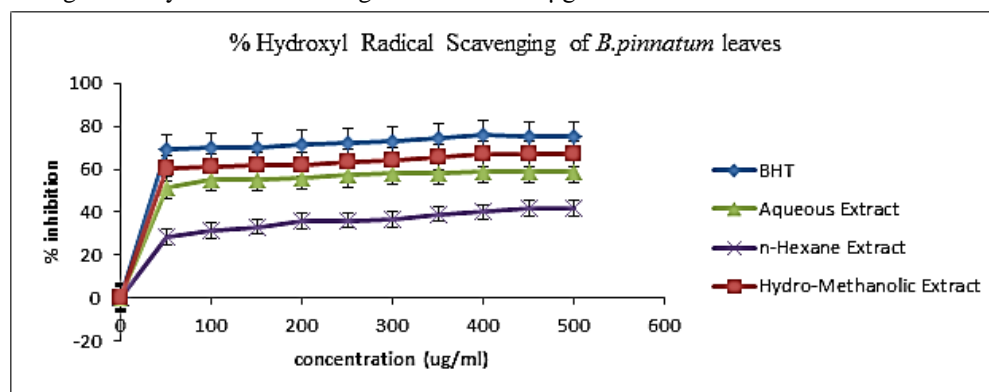
Table 2: Total Phenolic and Flavonoid content of *Bryophyllum pinnatum*

| Sample Extracts in different solvents | Total Polyphenols* (mg GAE/g extract) | Flavonoids* (mg Ru/g extract) |
|---------------------------------------|---------------------------------------|-------------------------------|
| Methanol                              | 53.18 ± 4.2                           | 21.27 ± 2.6                   |
| Water                                 | 22.19 ± 2.4                           | 15.12 ± 3.1                   |
| Petroleum ether                       | 12.07 ± 0.04                          | 07.11 ± 0.5                   |

\*Values showing Mean ± S.D of triplicates

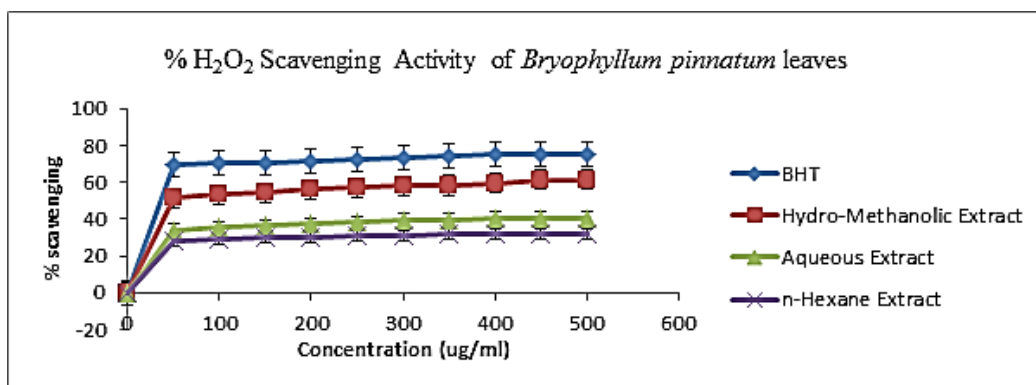
### Hydroxyl radical scavenging activity

The Fenton reaction was used to generate OH<sup>•</sup> radicals in the system and the free radical activity was determined by the degradation of deoxyribose, as standardized by Elizabeth and Rao [10]. The OH<sup>•</sup> Radicals attack the deoxyribose and initiate a series of reactions that eventually result in the formation of Thiobarbituric acid reactive substance (TBARS). The radical scavenging by the protectors result in inhibition of TBARS as shown by the extracts. The data shows maximum protection offered by hydro-methanolic extract with IC<sub>50</sub> 44.08, followed by aqueous extract with IC<sub>50</sub> 74.58 and minimum in case of n-hexane extract (IC<sub>50</sub> 165.37). The values of aqueous and hexane extracts varied significantly with BHT having IC<sub>50</sub> of 44.545 µg/ml.

Figure 1: % Hydroxyl radical scavenging of *b. pinnatum* leaves by fenton reaction

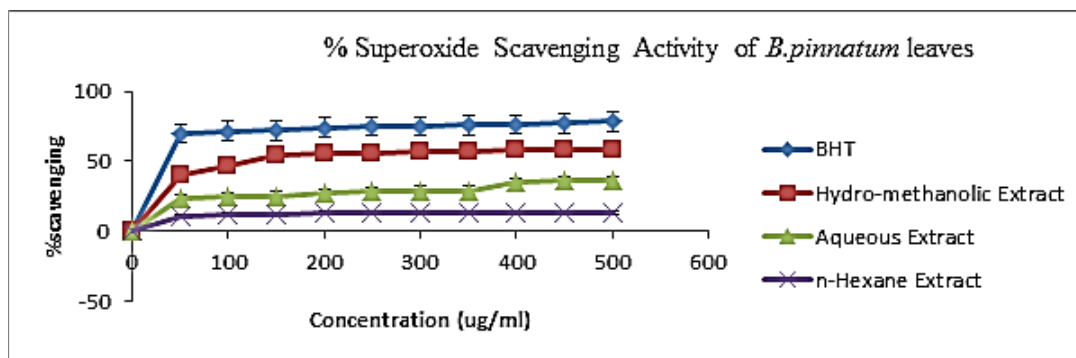
### Hydrogen peroxide scavenging

It was found that the radical scavenging activity of extract augmented with the increase in concentration. The hydro-alcoholic extract showed better scavenging as compared to aqueous and n-hexane extracts. The hydro-methanolic extract exhibited good reducing power at 100 - 500 µg ml<sup>-1</sup> but its activity was not comparable with that of BHT (P>0.05). All extracts were capable of scavenging hydrogen peroxide in a concentration dependent manner with maximum activity at 250-450 µg ml<sup>-1</sup> as shown in Figure 2. The effect of hydro-alcoholic extract eventually got stabilized above concentration of 300 µg ml<sup>-1</sup>. The scavenging effect was found to follow the order: BHT (IC<sub>50</sub> 42.72) > 50% hydro-methanolic (IC<sub>50</sub> 59.1176µg/ml) > aqueous (IC<sub>50</sub> 85 µg/ml) > n-hexane (IC<sub>50</sub> 142µg/ml).

Figure 2: % H<sub>2</sub>O<sub>2</sub> scavenging activity of *bryophyllum pinnatum* leaves

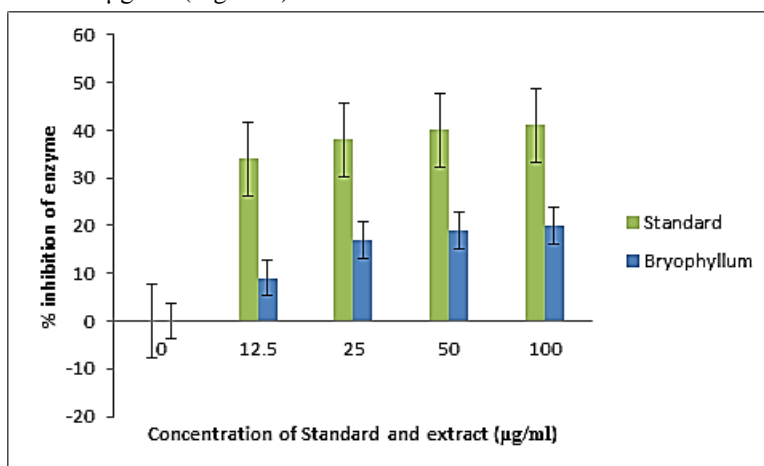
### Superoxide radical (O<sub>2</sub><sup>-</sup>) scavenging activity

The scavenging activity of *Bryophyllum pinnatum* on superoxide radicals enhanced with increase in concentration as given in Fig 3. The % scavenging effect of hydro-methanolic extract was the highest among the extracts and stabilized over the concentration of 150 µg/ml. The scavenging effect of the *B.pinnatum* and standards with the superoxide radical was found to follow the order: BHT (IC<sub>50</sub> 28.5) > hydro-methanolic (IC<sub>50</sub> 58.24µg/ml) > aqueous (IC<sub>50</sub> 79µg/ml) > hexane (IC<sub>50</sub> 132.5µg/ml).

Figure 3: % Superoxide Scavenging Activity of *B. pinnatum* leaves

### Alpha amylase inhibition

Henceforth, the antihyperglycemic effects of the hydro-methanolic extracts only were studied ( $y = 0.57x + 39.13$   $R^2 = 0.989$ ) and it was found that the hydro-alcoholic extract of *B.pinnatum* has IC<sub>50</sub> value of 94.15µg/ml. HMBP showed significant alpha amylase inhibition at concentration of 50µg/ml. Acarbose showed  $\alpha$ - amylase inhibitory activity with IC<sub>50</sub> value of 0.33 µg/ml. (Figure 4)

Figure 4: Amylase Inhibition of *B.pinnatum* leaves

The mode of inhibition of the aqueous extract of *HMBP* leaf on  $\alpha$ -amylase was determined using the Lineweaver-Burk plot which displayed a mixed noncompetitive inhibition of the enzyme (Figure 5 a and b). Michaelis-Menten plot shows lesser inhibition of the enzyme by *HMBP* as compared to the standard used.

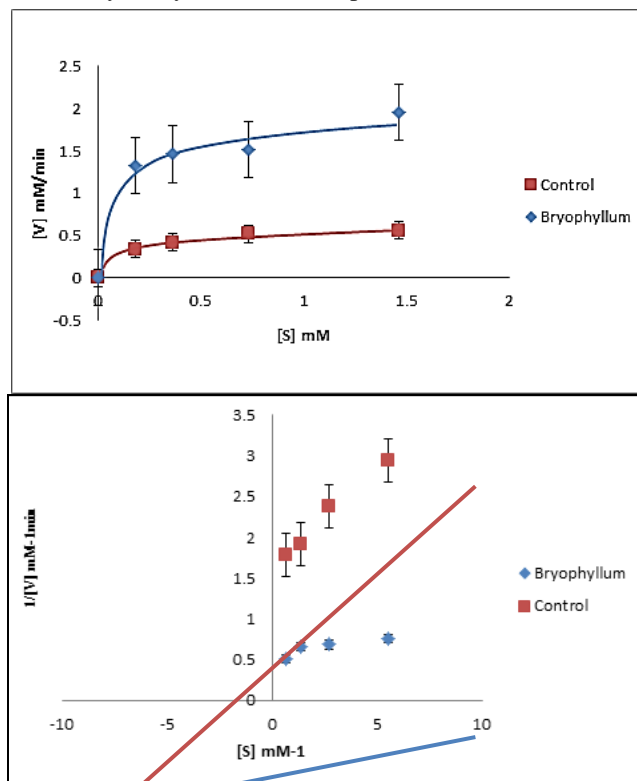


Figure 5: Mode of inhibition of  $\alpha$ -amylase by *HMBP* (a) Michaelis-Menten plot and (b) Lineweaver-Burk plot

## DISCUSSION

Recent researches have shown that plant phenolics are highly effective free radical scavengers and antioxidants due to their hydrogen donating ability [9],[10]. In general, antioxidant activities of plant are often explained with respect to their total phenols, flavonoids tannin content and antioxidants [25], [26]. Antioxidants not only protect lipids from oxidation, but also provide health benefits associated with diseases involved in biological degeneration [27]. Table 1 gives the total Polyphenols and flavonoids from different solvent extracts of *Bryophyllum pinnatum*, which followed the order: hydro-methanol > water > Hexane. Hence hydro-methanolic extract (*HMBP*) contained higher levels of Polyphenols and flavonoids. All the extracts of the plant leaf inhibited the degradation of deoxyribose *in vitro* in the  $\text{Fe}^{3+}$ -Ascorbate-EDTA- $\text{H}_2\text{O}_2$  system indicating  $\text{OH}^\cdot$  scavenging activity. Alcoholic extract showed significant  $\text{OH}^\cdot$  scavenging activity starting from  $150\mu\text{g/ml}$  whereas the effect of n-hexane extract was not credential.

Superoxide radical ( $\text{O}_2^{\cdot-}$ ) is much less reactive than  $\text{OH}^\cdot$  but a number of biological targets are sensitive to it. Moreover, it is known to be a precursor of more reactive oxygen species [18] in a variety of cellular components. The superoxide radical is known to be produced *in vivo* and the *in vivo* conversion of  $\text{O}_2^{\cdot-}$  to  $\text{H}_2\text{O}_2$  together with their conversion into more reactive species, e.g., the hydroxyl radical, also can have many metabolic consequences [8]. The hydro-methanolic extract was markedly a more potent scavenger of superoxide anion than other extracts. However, the reference compound, BHT exhibited higher superoxide scavenging activity than the extracts.

Scavenging of  $\text{H}_2\text{O}_2$  by *B. pinnatum* extracts may be attributed to its phenolics, which can donate electrons to  $\text{H}_2\text{O}_2$ , thus neutralizing it to water. Although hydrogen peroxide itself is not very reactive but removing  $\text{H}_2\text{O}_2$  is very important throughout food systems as it can sometimes cause cytotoxicity by giving rise to hydroxyl radicals in the cell [10], [27]. The experimental results show that the extracts were capable of scavenging hydrogen peroxide in a concentration dependent manner with *HMBP* showing good activity. Alpha Amylase enzyme is involved in the digestion of carbohydrate into glucose and the processing of the oligosaccharide moieties of glycoprotein. The search for amylase inhibitors from plants is ever expanding [18-20], not only because chemical inhibitors come with

an array of side effects but also because plant based inhibitors are important biochemical tools for studying the mechanism of enzymes [8], [19]. The drugs that have inhibitory effect on carbohydrate hydrolyzing enzymes have been shown to decrease postprandial hyperglycemia and improve glucose metabolism without promoting insulin secretion [24]. The mode of inhibition studies show that the extract *HMBP* acts as an allosteric inhibitor, binding to another site on the enzyme such that, when bound, it changes the entire shape of the enzyme so that its active site is also altered rendering it unable to bind to its natural substrate. As with increasing extract concentration, fewer active sites are available, so reaction velocity decreases. Thus the present work shows that hydro-methanolic extract of *Bryophyllum pinnatum* is beneficial against oxidative stress by activation of various antioxidants and effective in the control of Diabetes.

### CONCLUSION

The results clearly showed that hydro-methanolic extract of *Bryophyllum pinnatum* (HMBP) has potential active principles which are responsible for reducing postprandial glucose levels via  $\alpha$ -amylase inhibitory action justifying the traditional uses of the plant in the treatment of infectious diseases and free radical damages. These effects of *B. pinnatum* are especially promising in the light of preventing diseases linked to oxidative stress and cellular damage like cardiovascular diseases, cancer and diabetes. The present study can be used to further investigate the mechanism of action related to these effects.

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