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ENZYME ASSISTED BIOACTIVE EXTRACTION FROM *FLACOURTIA MONTANA* AND INVESTIGATION OF ITS *IN-VITRO* ANTIOXIDANT AND ANTI-DIABETIC ACTIVITY

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ABSTRACT

Flacourtia species are known for medicinal properties since ancient times. Here in we report the efficacy of the enzyme assisted extraction of bioactive compounds from *Flacourtia montana* leaf using three different enzymes and in combination of same enzymes. The extraction was carried out by enzyme formulations which contained cellulase, pectinase and amylase in water bath at a temperature of 50^{0}C for 3 hours. Further the extract were used to determine the phenolic content, flavonoid content, antioxidant and anti-diabetic properties. The combination of enzyme used for extraction showed maximum total phenolic and total flavonoid content. The extract also showed strongest antioxidant activities and antidiabetic activity compared to other methods. The TPC ranged from 54.22± 1.25 to 31.25 ± 1.02 mg GAE/g of DW, TFC ranged from 21.77 ± 0.54 to 8.72 ± 0.2 mg QE/g of DW and TAC was found to be 149.83± 4.4 to 88.16 ± 6.0 mg EAA/g of DW. The IC50 values for anti-diabetic properties varied from 300 ± 0.01 µg/ml to 910 ± 0.02 µg/ml.

KEY WORDS

Cellulase, Amylase, Antioxidant activity, Antidiabetic activity, Enzyme extraction, Flacourtia montana, Pectinase.

INTRODUCTION

Plant based medicines are used for combating diseases since ancient times due to the presence of a large number of bioactive compounds [1], hence there is a continuous search for medicinal plants that are of rich in these compounds. It has been reported that among more than 25,000 secondary metabolites that have been identified in plants [2], phenolic compounds found to distributed in all parts of higher plants shown to exhibits high degree of free radical scavenging property which may be the prime reason behind antioxidant activity, anti-tumor, antibacterial, anti-aging, anti-allergic, anti-inflammatory and antidiabetic properties [3,4]. The increased demand for the antioxidants and antidiabetic activities from natural compounds have encouraged the research studies about enhanced extraction process. The conventional techniques of plant materials extraction are usually based on the choice of solvents and the use of heat to increase the solubility of the desired compounds. Usually, conventional techniques require longer extraction time, thus running a risk of thermal degradation of some of the bioactive compounds [5]. The solvents used in the extraction also increase the risk of environmental pollution. In last few years many new alternative methods have been developed for the extraction of phytochemicals from plants such as ultrasound-assisted extraction(UAE), enzyme assisted extraction(EAE),

microwave-assisted extraction (MAE), supercritical CO_2(SC-CO_2), pressurized fluid extraction (PFE). EAE has gained importance since it offers an eco-friendly approach, an improvement in quantity of target compound and enhancement in bioactivity. The extraction by EAE method is based on the ability of enzymes to degrade cell wall components, leaving the intracellular materials more exposed for extractioninto solvents [4]. Many studies have been extensively conducted in recent years to identify and quantify phenolic compounds and the relevant antioxidant activity of plants [6], *Flacourtia montana* belonging to family *Flacourtiaceae* is medicinal tree of Indian origin. The species of the family are well known fortheir pharmacological activities and has been used as a traditional medicine in many countries for centuries [*7*]. From the findings of above the objective of ourstudy was set to evaluate the efficiency of extraction of bioactive compounds by EAE in comparison to the traditional method. Later the extract were analyzed for invitro antioxidant and invitro antidiabetic studies.

MATERIALS AND METHODS

A. Processing of plant material

Barks, leaves, flowers, and nuts from *Flacourtia montana tree* were collected from Hassan district, Karnataka. The plant was authenticated by Prof. Dr.Sharanappa P (Department of Bioscience, Hemagangotri University, Hassan, Karnataka) and voucher specimen (PS27551016) was deposited at the Department. The fresh leaves of *Flacourtia montana* was separated from the stem and washed with running tap water to remove the surface impurities. The leaves were shade dried for a week and ground to fine powder and stored in refrigerator.

B. Preparation of Extract

The enzyme assisted extraction was carried as described by [8] with slight modification, usingcommercial enzymes amylase, pectinase and cellulase. The plant extract and distilled water was taken at the ratio of 1:10 (W/V), to itenzyme at the concentration of 0.5% was added and then placed in water bath for 3 hours at 50^0C. After the period of incubation, the temperature was raised to 100^0C for 20 min to denature the enzymes. The extract was strained through double layered muslin cloth followed by centrifugation at 10,000 rpm for 10 min at 20^0 C to remove the undigested residue. The resultant supernatant was filtered using whattman filter paper and concentrated in the oven at 55°C until dryness. The same procedure was repeated for all the enzymes and its absence.Eachextract were re-suspended in water to make a 1mg/ml and1 mlof aliquot of leaf extract were used for all the assays.

C. Determination of Total Phenolic Content

The total phenolic content of the extracts was determined by using Folin–Ciocalteu's methoddescribe by [9] with slight modification. Briefly, 1mlof extractwas mixed with 5 ml of Folin–Ciocalteu's reagent (diluted tenfold with disittled water) and incubated in room temperature for 2 minute. After the period of incubation, 4 ml of 7.5 % sodium carbonate solution was added and mixed thoroughly andincubated for 120 min in dark at room temperature. The absorbance of sample was measured at765nm.The appearance of blue colour indicates the reduction of products. Blank was prepared without extract. The total phenolic content in the extract was calculated as Gallic acid equivalent (GAE) per gram of dry weight (DW)established from the calibration curve in mg/ml.

D. Total flavonoid content

The Total flavonoid content of the unrefined leaf extract was assessed utilizing the aluminum chloride colorimetric method as describe by [10] with a slight alteration. Briefly, a volume of 1 ml of the extract was blended with 2.8 ml of distil water and after that 0.1 ml of 1 mg/ml potassium acetic acid was added. To this, 0.1 ml of 10% aluminum chloride was added, the blend was allowed to stand for 30 min and absorbance was measured at 415nm. The appearances of yellow colour indicate the presences of flavonoids. The TFC was expressed as mg of quercetin equivalent (QE) per gram of a dry weight.

E. Total antioxidant captivity (TAC)

The TAC of the extracts were evaluated by the phosphomolybdenum method according to the procedure described previously [6] with some modification. Briefly1 ml extracts were mixed with 3.0 ml of complex reagent solution (including 0.6 M sulfuric acid, 28 mM sodium phosphate and 4 mM ammonium molybdate, 1:1:1). The reaction solution were incubated at 95^0C for 90 min and then cooled to room temperature. Finally, the absorbance of the solution was measured at 695 nm against a blank on a UV-visible spectrophotometer. A typical blank contained 1 ml of the reagent solution along with an appropriate volume of the solvent and incubated under similar conditions.

Antioxidant capacity of ascorbic acid has been used as a reference standard from which plant extracts with potential antioxidant activity are compared. The total antioxidant capacity (TAC) was expressed as mg equivalents of ascorbic acid per gram (EAA/g).

F. Antidiabetic activity

Inhibition of Amylase Enzyme

Antidiabetic activity was examined by amylase inhibition using dinitro salicylic acid(DNS) method. Briefly, a volume of 500 µl of plant extract having concentration of 200-1000 µg/ml were taken in 500 µl of sodium phosphate buffer containing α-amylase (0.5 mg/ml) and incubated at 37 ^{0}C for 10 min. After a time of incubation, 500µl of 1% starch solution in 0.02 M sodium phosphate buffer (pH 6.9) was added. This reaction blend was then incubated for 10 minutes at 25°C, followed by addition of 1.0 ml of DNS. The reaction was ceased by incubating in boiling water for 10 min than diluted with 10 ml distilled water, and the absorbance was measured at 540 nm. The mixture without plant extract was used as a control [11].

The α-amylase inhibitory activity was expressed as percentage inhibition (%) by using the formula

I % = (Ac-As)/Ac X 100

Where Ac is the absorbance of the control and as is the absorbance of the sample. The IC50 value was defined as the concentration of the sample extract to inhibit 50% of α-amylase activity under assay condition.

Statistical Analysis

All the assays of TPC, TFC, and TAC were carried out in triplicates and results were subjected to statistical analysis. All data were expressed as mean ±standard error (SE) and were subjected to variance anayalsis, $p < 0.05$ was considered statistically significant. For antidiabetic assays, IC50 value was used to determine.

RESULTS

Comparison of EAE with aqueous Extraction Method on TPC and TFC

In present study the efficiency of extraction with and without enzyme on TPC and TFC were compared and values are shown in figure. The TPC and TFC values of the extracts were validated by a linear correlation between the concentration and absorbance. Based on the calibration curve an equation (y = 0.003x - 0.0043, R^2 = 0.9957) and (y = 0.0091x + 0.0429 R^2 = 0.9752) were obtained respectively, where Y represents the absorbance and X represents equivalent (mg/g). The TPC and TFC values varied from 31.25 ± 1.02 to54.22 ± 1.25 (mg GAE/g DW) and 21.77 ± 0.54 to 8.72 ± 0.2 (mg QE/g DW). According to the statistical analysis (P≤0.05) the TPC and TFC were markedly higher in an enzyme extract than the aqueous extraction. As shown in (Fig. 1 and Fig. 2), the EAE method had the highest extraction yield of TPC and TFC compared to aqueous extraction. Among the EAE extraction mixture of enzymes showed maximum phenolic content 54.22± 1.25 mg GAE/g DW and flavonoid content 21.77 ± 0.54 mg QE/g DW followed by cellulase and pectinase. These results indicated the influence of the extraction by enzyme on the total content of phenolic compounds extracted. The order of yield of TPC and TFC extracted with different methods was similar to the results of former studies (*12*).

Evaluation of Antioxidant Capacity

The TAC was chosen to determine the antioxidant capacity of extracts obtained from different extraction method. It is based on the reduction of Mo (VI) to Mo (V) by the extract and subsequent formation of green phosphate/Mo (V) complex at acid pH. TAC of the phosphomolybdenum model evaluates both water-soluble and fat-soluble antioxidant capacity (total antioxidant capacity). The results are shown in Fig. 3, all the extract obtained from extraction has showed the antioxidant activity in different range. Among four types of enzyme extraction the combination of enzyme as showed highest antioxidant activities 149.83± 4.4EAA mg/g and lowest activity was shown by amylase. Conversely, extraction without enzyme yielded the lowest antioxidant activities of 88.16 ± 6.0 EAA mg/g. This results were supported by many studies in literature.Feng [4] reported the cellulase extraction of *Trapa quadrispinosa* as showed the TAC of 45.2 ± 2.2 mg GAE/gDW at concentration 2% cellulase at 50^0 C.

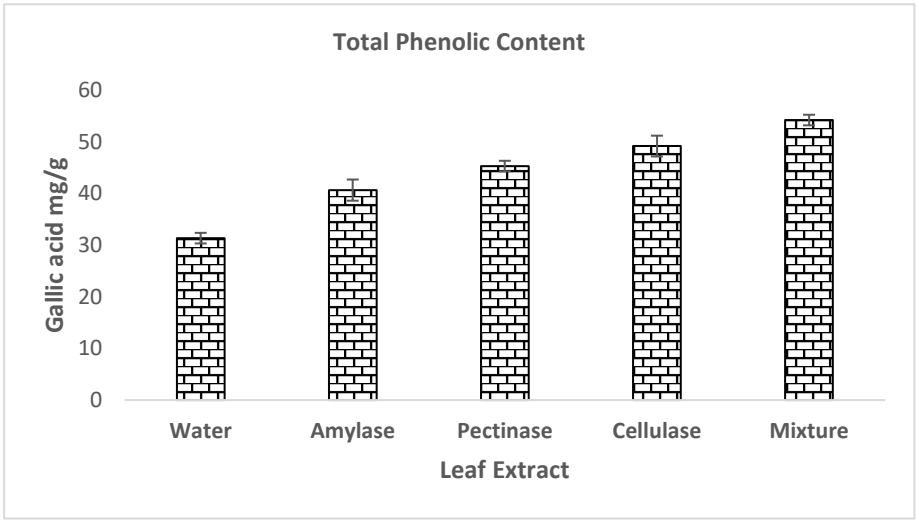

Figure 1. Total Phenolic content in various leaf extracts of *F. montana*

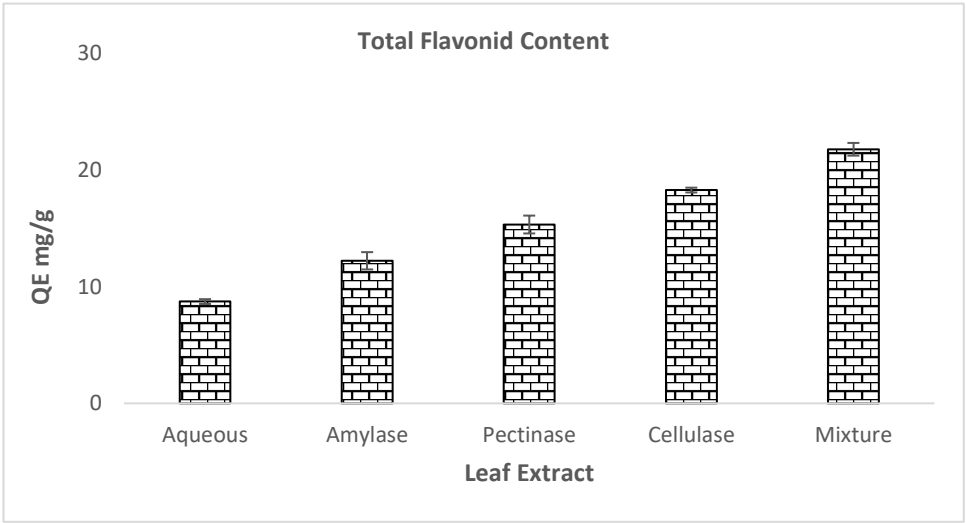

Figure 2. Total flavonoid content in various leaf extracts of *F. montana*

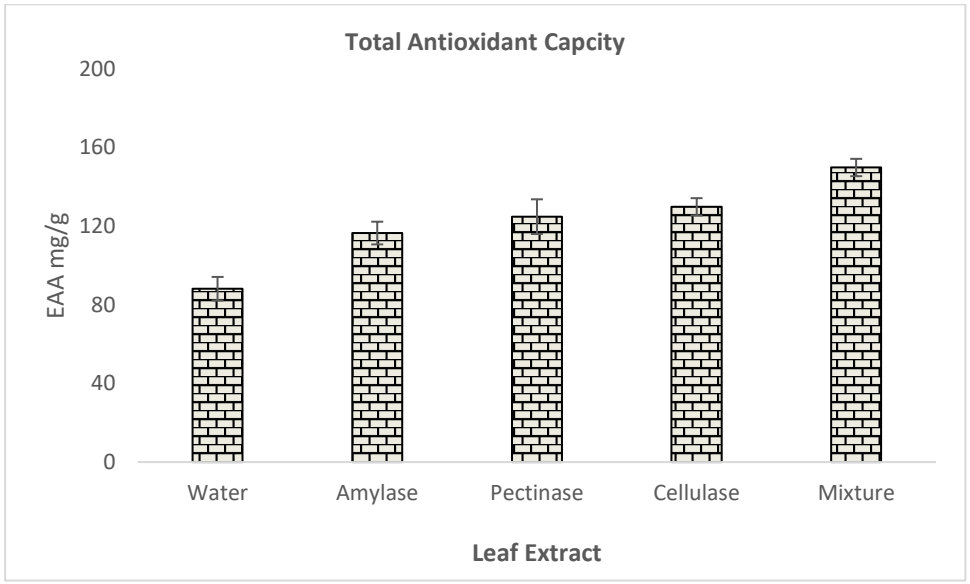

Figure 3. Total antioxidant content in various leaf extracts of *F. montana*

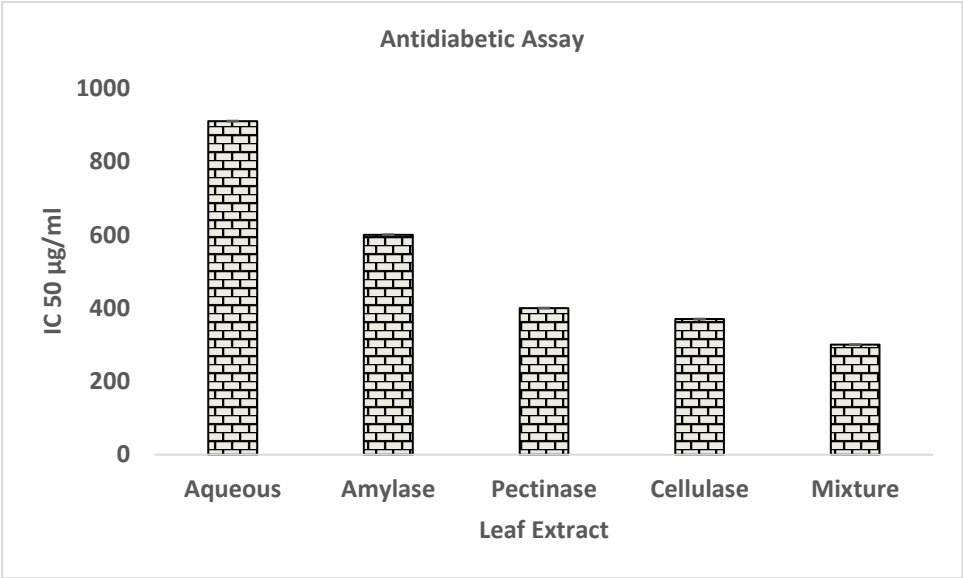

Figure 4. Invitro Antidiabetic Activity of various leaf extracts of *F. montana*

Antidiabetic activity

In the present research, the study has been carried out to screen the effect of EAE and aqueous extracts of *F. montana* plants in inhibiting α-amylase. The results of the inhibitory assay and with IC50 values are summarized in Fig. 4. All the above extraction showed varying inhibitory effect on enzyme which varied from the range 300 ± 0.01 µg/ml to 910 ± 0.02 µg/ml. This may be due to the presence of higher secondary metabolites. These observations produce a scientific basis for the use of this medicinal plant in traditional medicine for the treatment of diabetes. The dose dependent invitro antidiabetic activity of plant extract from *Nigella sativa, Eugenia jambolana, Andrographis paniculata and Gymnema sylvestre* [13], *C. swietenia* [14], *Ceropegia juncea*[15] was reported in literature.

DISCUSSION

Flacourtia sps has reported for valuable pharmaceutical properties, as it's contain abundant phenols, and exhibit

strong antioxidant activity [16,17]. These property is due to the phytochemicals, especially plant phenolics and flavonoids [18]. From the previous study the presence of bioactive compounds like alkaloids, Flavonoids, Tannins in aqueous extract of *F. montana* has been reported [7]. The bioactive compounds, such as terpenoids and flavonoids are the principal antioxidants that apply a scavenging effect on active oxygen free radicals by various mechanism including the inhibition of enzymes responsible for free radical generation, the chelation of metal ions, such as iron and copper and inhibit lipid oxidation at an early stage. Hence the extraction of secondary metabolites from the plant is a primary step to enhance the release of phenolic compounds, keep their stability and antioxidant activities [19]. Thus, the extraction parameters such as time, temperature, type of solvent, particle size of extract, type of enzyme, and concentration of the enzyme used influence the extraction efficiency. It has been reported that a long extraction time presented a favourable effect on the production of phenolic compounds. However, excessive extending of extraction time may induce the difference in phenolic compounds molecule structure and bioactivities due to oxidization or hydrolysis [4]. Recent advances in understanding the activity of intestinal enzymes helped in the development of newer pharmacological agents [20].α- amylase enzymes present in the intestinal human play main roles in the digestion of carbohydrates to degrade starch and oligosaccharides. The inhibition action of enzyme would slows the breakdown and absorption of glucose from the GI tract and leads to reduction of postprandial glucose level elevation. This delay digestion and breakdown may have beneficial effects on insulin resistance and glycemic index control in people with diabetes [21]. It has been also reported phenolic compounds are known to reduce the risk of metabolic syndromes, and the related complications of type 2 diabetes [22]. Gallic and protocatechuic acids have activities that inhibit α -amylase, which is key enzymes for the digestion of dietary carbohydrates [23].

CONCLUSIONS

There are several reports which supports the antioxidant activities and antidiabetic activities of *Flacourtia species* but, there are no previous reports, to the best of our knowledge, about *F. montana*. This paper provides a strong evidence for the antioxidant activities and antidiabetic activities of *F. montana*. The work herein indicates that enzyme assisted extraction has higher antioxidant activity and antidiabetic activities in vitro, in comparison with aqueous extract. The knowledge obtained from this study should be helpful for further exploitation and application of this resource. Hence, further, studies has to be focused on the optimization of extraction process in respective of time, temperature and enzyme concentration and bioactive compounds that are responsible for the inhibition of α-amylase activity.

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