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SCREENING OF DIFFERENT ECO-TYPES OF *MUNRONIA PINNATA* (WALL) THEOB. FOR IMPORTANT PHYTOCHEMICAL PROPERTIES

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ABSTRACT: *Munronia pinnata* is a rare medicinal plant, naturally distributed in China, India, Vietnam, Indonesia, Malaysia, Philippines, and Sri Lanka. The entire plant is extensively used for medicinal purposes, especially for curing skin diseases and treating fever, including malaria. Four major ecotypes were identified in Sri Lanka that consist of 3, 5, 7, and 9 leaflets from various regions. This study was performed to investigate the phytochemical activity of four different ecotypes of Munronia. Leaves of ecotypes were collected and dried at 45 ºC for 15 h. The methanol extracts were prepared and used for screening of antioxidant activity, total polyphenol content, antifungal, and anti-diabetic activity. The findings were indicated that all ecotypes attest to significant differences in its activity but to varying degrees. The strongest antioxidant activity, highest polyphenol content, and mild inhibitory activity for Cladosporium cladosporioides were observed in ecotype with 3 leaflets. Ecotype with 7 and 9 leaflets showed moderate activity, and ecotype with 5 leaflets was the lowest for all tested properties. None of the ecotypes were given significant positive activity for alpha-amylase and alpha glycosidase enzymes. This study suggests that consideration of ecotypes will be important in the effective usage of Munronia in different medicinal applications.

INTRODUCTION: *Munronia pinnata* (Wall) is a rare medicinal plant species that belongs to family Meliaceae. It has a short stem and compound leaves arranged spirally around the hard and rigid stem. The leaflets found in one leaf varies between 3, 5, 7, 9 or 11 in number. *Munronia pinnata*is naturally distributed in China, India, Vietnam, Indonesia, Malaysia, Philippines, and Sri Lanka [1].

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This plant is growing in diverse ecosystems including rocky sites in the dry, intermediate and wet zone in Sri Lanka [1] and recorded in natural vegetation in Ritigala, Sigiriya, Lunugala, Botale, Haputale, Doluwa, Haldunmulla, Naula, Pollonnaruwa, Batticaloa, Wellawaya, Balangoda and Sinharaja rain forest area of Sri Lanka [2].

The entire plant is used for medicinal purposes in Ayurvedic medicine, especially for treating malarial, fever, dysentery, and purification of blood [2, 3]. However, the wild stock of Munronia has been depleted day by day due to increasing demand. It was included in the list of most important fifty, medicinal plants in Sri Lanka since it is categorized as a most valued and rare plant species under threat

of extinction [4]. This medicinal plant has recorded comparatively highest price per unit weight, and one kilogram is around US$ 50-1104. As less or no commercial-scale productions were reported in many countries, all materials have been collected from natural habitats. If this continues with high demand, this plant will be extinct from its natural habitats within a short period of time. Medicinal herbs produce a variety of phytochemicals or secondary metabolites and have been used for several purposes such as food preservatives, flavours, and for curing numerous diseases since thousands of years.

Today, appreciated lines of studies are conducting to determine the biological activities with potential benefits to human health. Research studies have been shown that medicinal plants need to be provided natural environmental conditions if introduce as cultivations [5]. Stress conditions that plants undergo in natural conditions lead to express genes responsible for the synthesis of secondary metabolites, but this would not be expressed under the monoculture or intensive management conditions that provide optimum conditions for plants [6]. Therefore, active ingredients could be much lower in commercial cultivations, where wild populations could have a high level of active ingredients, although the growth performances are poor.

However, with increasing demand, bringing those important plants under commercial cultivations is a timely requirement, and the majority of pharmaceutical and other herbal based production industries prefer to have cultivated materials due to many advantages. The wild collection often provides adulterated materials as well as in irregular supply. But commercial cultivations guarantee a steady supply of raw material, facilitate correct post-harvest handling, quality control could be assured as well as product standards can be adjusted according to regulations and consumer preferences [7]. If growers can deliberately provide stress conditions as similar to the natural system, high-quality production could be obtained under commercial cultivations as well. Therefore, focus on commercially important phytochemicals and its compositions as well as the measures required to enhance phytochemical properties under cultivated conditions of important medicinal plants such as *Munronia pinnata* is vital. A large number of medicinal plant species have been screened as a viable source of antioxidants, polyphenols, and different properties such as antimicrobial and anti-diabetics [8-10], but there was no evidence under Indian or other Asian conditions on such studies for Munronia. The literature of *Munronia pinnata* under Sri Lankan conditions is limited, and few studies particularly on micropropagation [11-14] and morphology and agronomic practices [13, 15]. No records found on complete phytochemical analysis of different ecotypes under either local or global levels. This basic information is very much essential to make initiatives in conservation and commercialization of best ecotypes of Munronia.

Conservation of existing plants in natural habitats while introducing proper domestication and commercial cultivation programs will provide a better platform to save different ecotypes for future use without extinction. As different ecotypes growing in different climatic conditions, their chemical compositions might vary as it possesses different morphological characteristics, especially in leaf and floral biology. The selection and development of genotypes with commercially desirable traits facilitate the economic development of medicinal plant species as crops. This would lead to fulfilling the global demand through enhance economic stability in potential growing countries, further widening the industrial applications of Munronia.

The objective of the present study was to determine the presence and the activity of different phytochemicals to attest to the value of medicinally important and rare species of *Munronia pinnata*. Mainly, antioxidant activity, total phenolic content, antifungal activity, alpha-amylasee, and alpha-glucosidasee inhibitory activity of different ecotypes were focused.

MATERIALS AND METHODS:

Plant Material: Different ecotypes of *Munronia pinnata* were collected locally from IU3 ago-ecological zone covering different areas and raised at the net house of the Uva Wellassa University of Sri Lanka. Munronia ecotypes were authenticated and deposited as voucher specimens [PEK-MP-26022020(UWU)] in the National Herbarium, Department of National Botanic Gardens at

Peradeniya in Sri Lanka. Four ecotypes, including 3, 5, 7, and 9 leaflets, were raised in pots with the mixture of topsoil, sand in 3: 1 ratio.

Preparation of Extracts: The fresh leaves of mature plants were collected in January 2018 and air-dried under oven at 45 ºC for 15 h, and the leaves were crushed, and extracts were prepared by using methanol (Sigma-Aldrich, Germany). 10g of the dried leaf sample was taken in a conical flask, and 10 ml of methanol (Sigma-Aldrich, USA) was added. The crude methanol extract was obtained by extracting on a sonicator for 20 min. After that, the extract was filtered through Whatman filter paper 1, and the pellet was subjected to the same extraction procedure for another two times. The solvent extract was followed by distillation under reduced pressure using a rotary evaporator (45-50 ºC) until the solvent was completely dry. Finally, the extract was preserved in a sealed vial at 40 ºC until tested and analyzed.

Determination of Antioxidant Activity: The scavenging ability of methanol extract on 1, 1-diphenyl-2-picrylhydrazyl (DPPH) free radicals was estimated according to the method of Aqil in 2006 [16]. This method depends on the reduction of purple DPPH to yellow colored diphenyl picryl hydrazine. Sample stock solutions (1.0 mg/mL) were diluted to final concentrations of 500, 250, 125, 62.5 µg/mL in methanol. 1.5 ml of test samples in the half dilution concentration series (62.5 - 1000 µg/ml) was mixed with 0.6 ml of 0.005M DPPH (Sigma-Aldrich, USA) in methanol. Blank was conducted with 1.5 ml of various concentrations of sample and 0.6 ml of methanol. An equal amount of methanol and DPPH served as a control. The mixture was shaken and left to stand at room temperature in the dark. After 30 min, absorbance was measured at 518 nm against the blank using UV-Visible spectrophotometer (Infinite M200; Tecan, Mannedorf, Switzerland). Ascorbic acid (Himedia, India) was used as the positive control. Assays were carried out in triplicates. The percentage of inhibition of DPPH (IC_{50}%) was calculated using the following equation:

Percentage of DPPH radical scavenging activity (%) = $(A_o - A_1)/A_o \times 100\%$

Where A_o is the absorbance of the control reaction and A_1 is the absorbance of the different samples of the tested extracts. The percentage of free radical activity was plotted against the corresponding anti-oxidant substance concentration to obtain the IC_{50}% value, which is defined as the amount of antioxidant substance required to scavenge the 50% of free radicals present in the assay solution. IC_{50}% values are inversely proportional to the antioxidant potential. Finally, the unit of IC_{50}% was converted into Ascorbic acid equivalent capacity.

Estimation of Total Phenol Content: The amount of total phenol was determined using the Folin-Ciocalteu (Sigma-Aldrich, Germany) reagent method as described by Zahinin 2009 [17]. The 500 µl of Folin-Ciocalteu reagent (1/10 dilution) was added to 100µl of each sample, mixed well-using vortex and incubated at room temperature for 5 minutes. After that, 800 µl of 7.5% sodium carbonate (Daejung, Korea) solution was added. The final content was shaken well and incubated for 1h in the dark at room temperature. The absorbance of all samples was measured at 765 nm using a UV Visual spectrophotometer (Infinite M200; Tecan, Mannedorf, Switzerland). A standard curve was plotted by using Gallic acid (Research Lab Fine Chem Industries, India). A standard concentration curve from 0.1 to 0.01 mg/mL at 0.02 mg/mL interval was plotted. Triplicate measurements were carried out. The results were expressed in mg of Gallic acid equivalents (GAE) per milligram of crude extract of the plant.

Antifungal Activity: Extracts were screened against a fungus species *Cladosporium cladosporides*. Fungal colonies were obtained from the National Institute of Fundamental Studies, Hanthana, Sri Lanka. The test organisms were maintained on a nutrient agar plate and incubated at room temperature. Antifungal activity of methanol extracts of different ecotypes of *Munronia pinnata* was carried out using thin-layer chromatography referencing to a modified protocol of the Homans and Fuchs, 1970 [18]. The methanol extracts were submitted to thin-layer chromatography (TLC). Extracts of different ecotypes were spotted (500 µg in each spot) in TLC plates (GF 254 60; Merck) 250 µm thick were developed with $CHCl_3$: CH_3OH (90: 10) for 20-60 min, which separated components into a wide range of Retention Factor (R_f) values. The components were visualized under visible and UV light (254 and 366 nm) and clearly

marked the component separations. Spore suspension of *Cladosporium cladosporioides* in a glucose-mineral salts medium was directly sprayed to TLC plates. After spraying, the TLC plates were incubated in a moist atmosphere for 2-3 days at 25 °C. Inhibition zones were identified as the indicator for the presence of original fungi toxic compounds.

Alpha-Amylase Inhibitory Activity: Alpha-amylase inhibitory activity of extracts of different ecotypes of *Munronia pinnata* were tested with the colorimetric assay condition previously described by Bernfeld, 1955 [19]. Concentration series (31.25-1000 µg/ml) of different plant extract solutions were prepared by dissolving the crude extract in 1% Dimethyl sulfoxide (DMSO) (Sigma-Aldrich, Germany) solution. The 50 µL of the extract was mixed with 50 µL of 13U/mL alpha-amylase enzyme (Sigma-Aldrich, Germany), which was prepared by dissolving 6.5 mg in 5 mL of buffer solution and the tubes were incubated at 25 ºC for 30 min.

Sequentially, 100 µL potato starch (1% w/v) that was dissolved in 20 mM phosphate-buffered saline (pH 6.9) was added and incubated for another 3 minutes at room temperature. 100 µL of 3, 5-dinitrosalicylic acid (DNSA) (Sigma-Aldrich, Germany) color reagent, 12 g Potassium sodium tartrate (Daejung, Korea) dissolved in 8 mL of 2 M NaOH (Research Lab Fine Chem Industries, India) with 96 mM of 3, 5- dinitrosalicylic acid] was added into the previously incubated tubes and placed in a water bath maintained at 85- 95 ºC for 15 min.

The mixtures in each test tube were diluted with 900 µL deionized water, and the absorbance was measured at 540 nm using UV-Visible spectro-photometer (Infinite M200; Tecan, Mannedorf, Switzerland). For each concentration of extract used, blank incubation was prepared with interchanging the starch solution with DNSA color reagent. Control tests, representing 100% enzyme activity, were carried out in a similar manner by replacing plant extract with 50 µL of 1% DMSO. All the tests were run in triplicates, and the level of inhibition was calculated according to the formula;

Inhibition Percentage (%) = Abs 540 (control) – Abs 540 (extract) / Abs 540 (control) * 100

The IC_{50} values were determined from plots of percent inhibition versus inhibitor concentration. Acarbose (Sigma-Aldrich, Germany) was used as the reference for the test.

Alpha Glycosidase Inhibitory Activity: Analysis of alpha-glucosidase enzyme inhibitory activity was carried out according to Matsui in 2001 [20], and P-Nitrophenyl-α-D-glucopyranoside (4 mM) (Sigma, USA) was used as the substrate. The enzyme alpha-glucosidase (50 mU/Ml) (Sigma, USA) was dissolved in buffer (pH 5.8 ± 0.1) and was used as the enzyme extract.

Plant extracts used were in the concentration of 1 mg/mL, and different plant extracts were incubated for 30 min at 37 ± 2 ºC. Absorbance was read at 400 nm. The control samples were prepared without any plant extracts. The % inhibition was calculated according to the formula.

Inhibition (%) = Abs 400 (control) – Abs 400 (extract)/ Abs 400 (control) × 100

The IC_{50}% values were determined from plots of percent inhibition versus log inhibitor concentration and calculated by nonlinear regression analysis from the mean inhibitory values.

Acarbose was used as the reference for the alpha-glucosidase inhibitor. All tests were performed in triplicates.

Statistical Analysis: Data were statistically analyzed using One- way Analysis of Variance (ANOVA) with Minitab 17 statistical package and means were separated using Tukey pairwise comparison.

The experiments were laid out in a Complete Randomized Design (CRD), and values were considered significant at $p<0.05$.

RESULTS: The study was conducted to analyze phytochemicals of different ecotypes of *Munronia pinnata*, and the results indicated that the different ecotypes exhibit positive biochemical functions at varying degrees. All the crude extracts were obtained through a series of methanolic extraction.

Antioxidant Activity: The results of antioxidant activity of leaf extracts of different ecotypes of *Munronia pinnata* were shown in **Table 1**.

TABLE 1: MEAN ANTIOXIDANT ACTIVITY OF FOUR DIFFERENT ECOTYPES OF *MUNRONIA PINNATA*

	Different ecotypes	IC_{50}% (µg/mL)
Methanol extracts	3 leaflets	22.02 ± 3.03^{c}
	5 leaflets	179.6 ± 3.68^{a}
	7 leaflets	53.67 ± 16.07^{b}
	9 leaflets	75.08 ± 8.93^{b}
Standard	Ascorbic acid	7.013 ± 0.017^{c}

Data are means of triplicates with measurements ± standard deviation. Means not sharing a common single letter were significantly different at $p < 0.05$

A lower IC_{50}% indicates a stronger free radical inhibition as strong free radical inhibitors are active at low concentrations. In this study, lowest IC_{50}% value was observed in the 3 leaflets of *Munronia pinnata*, and it exhibited the highest scavenging activity with an IC_{50}% of 22.02 ± 3.03 µg/mL **Table 1**, which was significantly similar to the standard antioxidant of ascorbic acid (7.013 ± 0.017 mg/mL) at $p< 0.05$. The ecotype with 7 leaflets (53.67 ± 16.07 µg/mL) and 9 leaflets (75.08 ± 8.93 µg/mL) showed relatively lower scavenging ability that was significantly different to 3 leaflets of Munronia ($p< 0.05$). The ecotype of 5 leaflets showed the lowest IC_{50} value (179.6 ± 3.68 mg/mL), and it was significantly different from all other three ecotypes at $p< 0.05$.

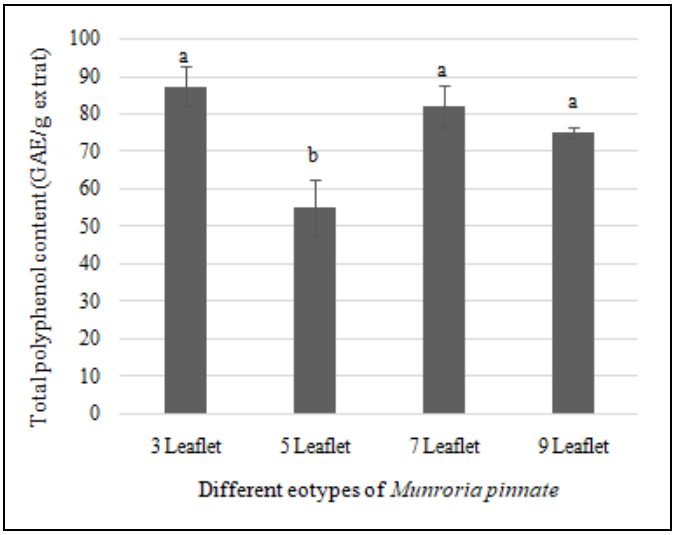

FIG. 1: POLYPHENOL CONTENT OF DIFFERENT ECOTYPES OF *MUNRONIA PINNATA* Data are means of three replicates. Means not sharing a common single letter were significantly different at $p < 0.05$. Error bars indicate the level of variation between replicates.

Polyphenol Content: Gallic acid is the most common polyphenol in natural products and it was used to determine the polyphenol content 17 of different ecotypes of *Munronia pinnata*. The polyphenol content in all four extracts was found to be in the range of 55.35 mg to 87.38 mg corresponds to gallic acid. The total concentrations of phenolic compounds in the extracts were shown in **Fig. 1**. The ecotype of 3 leaflets showed the highest total concentration (87.38 ± 5.25 mg Gallic acid equivalents (GAE)/g extract), followed by 7 leaflets and 9 leaflets (82.28 ± 5.66 and 75.192 ± 1.222 mg GAE /g extract, respectively) which were not significantly different at $p< 0.05$. The lowest concentration of polyphenols content was recorded in the ecotype 5 leaflets (55.35 ± 7.45 mg GAE/g extract) and it significantly different from the other three ecotypes ($p < 0.05$) used in the study.

Antifungal Activity: Study of the antifungal and antimicrobial activity of different plant extracts is an interesting area as it could be effective in treating of infectious diseases while mitigating many of the side effects that are often associated with conventional drugs. In this study, a simple bio autographic technique was followed to detect the presence of fungi toxic substances in the extracted plants. This antifungal screening technique was developed by Homans, and Fuchs in 1970 was proven to be elegant because of its high sensitivity and possibility of keeping records. It has been used by numerous studies due to its transparency in results [21-23]. However, in the present study, the plant extracts showed mild inhibition activity for the fungi as it showed small inhibition on the TLC plate after three days of the cultures. Ecotype with 7 leaflets is slightly active in suppressing the growth of fungi of *Cladosporium cladosporioides* compared to the other three ecotypes.

Alpha-Amylase and Alpha Glucosidase Activity: The inhibitory activity of methanolic extracts of *Munronia pinnata* on wheat alpha-amylase and yeast alpha-glucosidase was investigated in this study, and the results were shown in **Table 2**. The alpha-amylase test results showed that ecotype 3 and 5 leaflet extracts (1 mg/mL) were able to inhibit the enzyme at a low rate (6.947 and 4.771%, respectively). However, the alpha-amylase inhibition was not significant as compared with the standard compound acarbose that shows an IC_{50}% value as 3.241 µg/mL. It was observed that ecotype 7 and 9 leaflets showed negative results for alpha-amylase **Table 2**. Further, there was no inhibition of alpha-glucosidase enzyme for any ecotype extracts (1 mg/mL) and all the four ecotypes

showed negative values for alpha-glucosidase bioassay (IC_{50}% for acarbose, a standard reference compound, 5.68 µg/mL)

TABLE 2: THE PERCENTAGE INHIBITION OF ALPHA AMYLASE AND ALPHA GLUCOSIDASE BY METHANOLIC EXTRACTS OF *MUNRONIA PINNATA*

Different Ecotypes	Percentage inhibition in alpha-amylase bioassay	Percentage inhibition alpha-glucosidase bioassay
3 leaflets	6.947	-1.35
5 leaflets	4.771	-8.69
7 leaflets	-30.848	-14.82
9 leaflets	-34.703	-10.61

The present study indicated that *Munronia pinnata* could not be used in the control of postprandial hyperglycemia as it showed weak alpha-amylase and no alpha-glucosidase enzyme inhibition.

DISCUSSION: Phytochemical screenings are carried out with the purpose of detecting a diverse group of naturally occurring active phytochemicals. There, discovering the bioactive profile of plants with therapeutic value is prominent. In the present study, determining the antioxidant activity of Munronia, DPPH assay was employed, and DPPH is consists of stable free radical at room temperature and accepts an electron to become a stable molecule [24]. The reduction capability or the decrease in absorbance of DPPH radical is done by antioxidants and is determined by the decrease of its absorbance at 5l7 nm. Because of the reaction between antioxidant molecules and free radicals, change in color from purple to yellow shows the scavenging of the radical by hydrogen donation.

As it is visually noticeable and the ability to generate free radicals, DPPH is usually used as a substrate to evaluate the anti-oxidative activity [16, 25, 26]. When the IC_{50}% value gets decreased, it is clear that the less DPPH free radicals remain. Even the samples show a positive result in scavenging, the capacity of scavenging is inferior to ascorbic acid at 518 nm. All these data clearly indicated that all four ecotypes of *Munronia pinnata* are effective electrons or hydrogen atoms donor to DPPH, showing the antioxidant activity but at varying degrees. Many herbal plants contain antioxidant compounds that have the ability to protect cells against the degenerative effects of Reactive Oxygen Species (ROS) such as singlet oxygen, superoxide, peroxyl, radicals, hydroxy radicals [27, 28]. These biomolecules may contribute to the anticipation of many chronic diseases such as cancer, cardiovascular disease, atherosclerosis, diabetes, asthma, hepatitis, and arthritis [29]. A large number of plants have been screened as a viable source of antioxidant [30-32]. But, there was no previous evidence reported on the presence of antioxidants in different ecotypes of Munronia apart from the present study. Several researchers have found that there is a positive linear relationship existed between the antioxidant activity and total phenolic content in plants [33, 17] polyphenols are biological metabolites of plants, and generally involved in defense against ultraviolet radiation or aggression by pathogens. Moreover, there is an advantage of studying the biological compounds in such importance due to many beneficial effects on human health.

As the phenolic compounds have the ability to scavenge free radicals and active oxygen species, such as singlet oxygen, superoxide free radicals, and hydroxyl radicals, they are known as high-level antioxidants [29]. As stated before, there is a positive correlation between the antioxidant activity and polyphenol content, it is clearly evident in the present study as the highest antioxidant activity, and sequentially highest amount of polyphenol content was observed in the same ecotype with 3 leaflets of *Munronia pinnata*.

The antifungal activities of plants may vary in relation to the test organism, and *Cladosporium cladosporioides* is the pathogenic fungi used for the screening. It is the most common species of indoor contaminants occasionally linked to health problems [34]. Plant extracts that contained a high level of phenolics exhibited antifungal and antibacterial effect [35]. There were no previous studies related to *Munronia pinnata* on anti-microbial activity. Antifungal or antimicrobial activity refers to the process of killing or inhibiting the disease-causing microbes, and all of them have different modes of action by which they act to suppress the infections. When considering recent studies, some plants consist of potential antifungal activity, and they are good sources for natural compounds that could act as new anti-infection agents [36, 37]. This study supports the contention that traditional medicinal plants remain a valuable source in the potential discovery of natural products in pharmaceuticals. Moreover, the four

ecotypes showed significant differences in its phytochemical properties. Further, the ecotypes of 3 leaflets showed a significant difference compared to the other three ecotypes. Two ecotypes of 7 and 9 leaflets were not significantly different when compared to antioxidants and total polyphenol compounds. However, in antifungal activity, the 7 leaflets behaved differently than that of 9 leaflets. Ecotype with 5 leaflets showed the weakest activity among four ecotypes of *Munronia pinnata* for antioxidant and total polyphenolic activity. As all ecotypes were collected from the same agro-ecological region, the influence of the climatic factors would be at a minimum level. Further, the results of this study suggest that ecotypes have different phytochemical constituents in its composition due to the changes in its genetic material as visible in its morphological differences.

Diabetes mellitus is a clinical ailment characterized by hyperglycemia when the elevated amount of glucose circulates in the blood plasma, and that affects the metabolism of carbo-hydrates, proteins, fat, electrolytes and water simultaneously. Sequentially, the pancreas does not produce enough insulin or cells do not respond to the produced insulin, and that instantaneously affects diabetes.

Alpha-amylase and alpha-glucosidase inhibitors are used to achieve greater control over hyperglycemia in type II diabetes mellitus. Alpha-amylase is involved in the breakdown of long-chain carbohydrates, and alpha-glucosidase breaks down starch and disaccharides to glucose and serves as an important digestive enzyme of carbohydrates. Targeting on inhibition of these two enzymes is the key point in treating diabetes.

However, natural alpha-amylase and glucosidase inhibitors from the dietary plants are preferable as an effective therapy for treating postprandial hyperglycemia with minimal side effects [38]. The inhibition of pancreatic alpha-amylase is one of the therapeutic targets of reducing the absorbable monosaccharides in the intestinal brush border, result in reduced postprandial hyperglycemia [39]. The results of the present study is indicated that *Munronia pinnata* could not be used in the control of type II diabetes mellitus as it showed no significant inhibition of alpha-amylase and alpha-glucosidase enzymes.

CONCLUSION: The results of the present study indicate that four ecotypes of *Munronia pinnata* consist of different phytochemical activities. This species is a good source of antioxidants and poly-phenols. However, out of the four ecotypes, ecotype with 3 leaflets showed the highest antioxidant activity and highest polyphenol content while having slight antifungal activity. However, there is no any significant inhibitory activity of alpha-amylase or alpha-glucosidase in all four ecotypes of Munronia. Furthermore, it can be concluded that different ecotype of *Munronia pinnata* has different phytochemical activities and ecotype with 5 leaflets is greatly different from that of the other three ecotypes.

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