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PHYTOCHEMICAL PROPERTIES AND EFFECTS OF CRUDE EXTRACTS OF SARABAT FIDDLEHEAD FERN SHOOTS IN HYPERGLYCEMIA INDUCED NORMAL MICE (*Mus musculus*)

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Abstract: This study provides scientific-based evidence on the potential pharmacological and nutritional value of the Fiddle head fern or Sarabat consumed as vegetable in southern part of Region 02. The study determined the general phytochemical constituents of two species of Sarabat (*Diplazium polypodioides* Blume and *Diplazium sp.*) fiddlehead fern vegetables. One of the Sarabat (*D. polypodiodes* Blume) was evaluated for its postprandial glucose lowering effect in ICR mice. Phytochemical screening was undertaken using standard qualitative methods. Thirty (30) ICR albino mice were grouped into five treatments of six (6) mice each and 24 subjected for oral glucose tolerance test (OGTT) to evaluate the glucose lowering effect of Sarabat. The plant extracts and commercial anti-glycemic drug were administered in vivo and observation periods was accomplished at 0, 30, 60, 90 and 120 minute post-treatments. The study utilized the following treatments: T_1-Negative control (plain pellets and water; T_2- two (2) grams glucose/kg body weight (bwt); T_3-two (2) grams glucose/kg bwt plus 60mg Glicazide/kg bwt; T_4-two (2) grams glucose/kg bwt plus 80mg/kg bwt Sarabat methanolic extract and; T_5-two (2) grams glucose/kg bwt plus 40mg/kg bwt Sarabat methanolic extract. Qualitative phytochemical screening of both Sarabat species revealed the presence of sterols, flavonoids alkaloids mainly in methanolic extracts and tannins as traces. There is remarkable predominance of saponins in all the solvents utilized and a remarkable abundance of glycosides and triterpenes in the aqueous extract of Sarabat (*D. polypodiodes* Blume). Oral glucose tolerance test reveals that at 60 minutes of post treatment the Glicazide took effect immediately by significantly lowering the glucose level when compared to Treatment 4 but its effect is found to be statistically comparable to Treatment 5 at 90 to 120min postprandial glucose administration indicating the glucose stabilization effect of Sarabat.

Key Words: phytochemical; fiddlehead fern; posprandial glucose, antihyperglycemic

1. INTRODUCTION

Diabetes mellitus is a syndrome of impaired carbohydrate, fat, and protein metabolism caused by either lack of insulin secretion or decreased sensitivity of the tissues to insulin (Hall, 2011). Four well-established groups of oral antidiabetic drugs are used most commonly to treat type 2 diabetes. These include insulin secretagogues, the biguanide metformin, thiazolidinediones, and alpha-glucosidase inhibitors (Trevor, 2013). Ethnobotanical information indicates that more than 800 plants are used as traditional remedies for the treatment of diabetes (Alarcon-Aguilar *et al.*, 1998). The hypoglycemic activity of a large number of these plants has been evaluated and confirmed in different animal models. Among the five ferns studied by Chai et al (2015a), they highly recommend *Diplazium esculentum* as a potential source of an antidiabetic agent and is recommended for further investigation. The current pharmacologic regimen for management of diabetes also includes supplements of plant origin due to the chronic and complex nature of the disease and with

the attendant serious side effects of the four well established drug groups patients and physicians turn to herbal drugs of known efficacy. Based on the WHO recommendations hypoglycemic agents of plant origin used in traditional medicine are important (WHO, 1980).

The attributed anti hyperglycemic effects of these plants is due to their ability to restore the function of pancreatic tissues by causing an increase in insulin output or inhibit the intestinal absorption of glucose or to the facilitation of metabolites in insulin dependent processes. Hence treatment with herbal drugs has an effect on protecting beta-cells and smoothing out fluctuation in glucose levels. Most of these plants have been found to contain substances like glycosides, alkaloids, terpenoids, flavonoids etc. that are frequently implicated as having antidiabetic effects (Loew and Kaszkin, 2002). Flavonoids, sterols, triterpenoids, alkaloids and phenolic are known to be bioactive antidiabetic principles (Kameswara *et a.,l* 1999). It is in this context also that this study will also seek to undertake a phytochemical qualitative assay on the presence of the aforementioned compounds.

Sarabat is the shoot and stalk of two species of fiddlehead vegetable ferns occasionally sold in the market places of Nueva Vizcaya in Cagayan Valley and neighboring provinces. *Diplazium polypodioides* Blume has larger succulent and dark green hairy fiddlehead and edible stalk and another *Diplazium* sp. which is smaller and slender also called by the same name Sarabat. It is also used as medicinal herb according to the local folks for ulcer, abdominal colic, cancer, diarhhea, and diabetes. *Diplazium esculentum and Stenochlaena palustris (*Chai *et al.*, 2015b) were demonstrated to have alpha glucosidase inhibition and antioxidant activity hence have antidiabetic properties as well. Yet while " pako" fern exhibits alpha glucosidase inhibition and possibly useful as antidiabetic It was also shown by another research to have immunosuppressant and hemolytic activity (Roy *et al.*, 2013). These controversial studies surrounding a common edible vegetable fern may as well extend to the other fiddlehead edible fern vegetables such as Sarabat and which warrants further investigations.

Currently, most researches on Pteridophytes as medicines are limited and mostly in relation to Chinese herbal Medicine (de Winter and Amoroso, 2003). The present study sought to clarify the use of Sarabat as both a functional food with possible nutritive and pharmacologic effects in health maintenance particularly as an adjunct to diabetes management particularly glucose modulation. Phytochemical assay was undertaken to document the phytochemical constituents likely implicated in its blood glucose lowering activity as well as establish the presence or absence of constituents that pose hazards to human health with overconsumption.

Objectives

The study investigated the phytochemical constituents and antihyperglycemic effects of extracts of the larger " Sarabat" fiddleheads in ICR mice (*Mus musculus*). Specifically,

1. the major phytochemical constituents of Sarabat fiddlehead fern were qualitatively identified using three solvents namely, water, ethanol and methanol.
2. the blood glucose lowering effect of fiddlehead fern locally known as Sarabat sold in marketplaces in Nueva Vizcaya was evaluated on hyperglycemia induced mice by way of oral glucose tolerance test and;
3. the initial dosage of the Sarabat noted to effect blood glucose lowering and stabilization in the experimental mice was tested.

2. METHODOLOGY

2.1 Procurement of Plant samples

"Sarabat" Fiddlehead Fern Shoots and stalks were purchased in Bayombong and Solano markets in Nueva Vizcaya and brought to Biology Department DLSU for identification by Dr. Esperanza Maribel Agoo. Two types of sarabat fiddlehead ferns were used in this study for purposes of phytochemical tests. Sarabat 1 (*D. polypodiodes* Blume) stouter with big fiddleheads and Sarabat2 (*Diplazium* sp.)of smaller stalks and fiddleheads.

2.2. Preparation of plant treatments

aAir-dried sarabat fern shoots was pulverized and was extracted with the Soxhlet technique using three solvents. The " Sarabat" fiddlehead fern shoots were extracted by aqueous, ethanolic and methanolic extraction process at the Cagayan State University Natural Science lab. About 200 ml each of the methanolic, ethanolic and water extracts of the fiddlehead ferns were brought to the STD DOST Bicutan for phytochemical assays using qualitative chemical assays and the majority of the

extracts was brought to DLSU and lyophilized at the Department of Chemistry and stored until further use.

2.3. Phytochemical Screenings

The procedures for phytochemical screening was based on Trease and Evans, (2002). To test the presence of Saponins, Froth test was employed wherein alcoholic extract was dissolved in hot water then filtered. The aqueous filtrate extract was vigorously shaken became frothy; and persisted for at least 30 minutes. For tannin, Ferric Chloride Test was used. The dried extract was dissolved in hot water and filtered. About 1-2 drops of Ferric Chloride was added. The production of dark coloration that may either be black, dark blue, blue-black, indicate the presence of tannins. The test for glycosides was done using Fehling's Test. The alcoholic extract was dissolved in hot water, and then filtered. The filtrate was used for the test. 2 test tubes were obtained. 2 mL of sample was placed in each test tube. To the tube 1, 1.0 ml dilute HCl was added; to the tube 2, nothing was added (control tube). The two test tubes were placed in a boiling waterbath for 5 minutes, then allowed to cool. The samples were neutralized with anhydrous sodium carbonate until no effervescence was produced. Then 1.0 mL Fehling's solution was added and the formation of brick red precipitate indicate the presence of glycosides. For flavonoid, the Mg^{+} turning test was used to screen for the presence of flavonoids, 1 mL dried alcoholic extract is treated with 1.0 mL 10% HCl and magnesium turnings. Finally, a red coloration was observed.

The test for alkaloids was done using the Mayer's Test. In this test, the dried alcoholic extract is extracted with 1% HCl then filtered. To the filtrate 2 drops of the Mayer's reagent was added. A cream colored precipitate was observed. Wagner's Test A small amount of dried extract was dissolved in 1.0 mL of dilute acetic acid. A white of cream colored precipitate is formed. Lastly, sterols and triterpenes were detected using Liebermann-Burchard Test in which a small amount of dried extract was dissolved in acetic anhydride. The soluble portion was decanted and added 1-2 drops of concentrated sulfuric acid. A green color, either immediately or slowly going to red or blue tones, was formed. A pink to red color is indicative of triterpenoids, while a blue color indicates the presence of steroids. For triterpenoids and steroids, Salkowski's Test was used. Concentrated sulfuric acid was added to several mg of the substance, and 2 drops of acetic anhydride to its solution in chloroform. Production of red color indicates presence of triterpenoids, blue for steroids.

2.4 Test animals and set-up

The experimental set-up followed the protocol as adopted by Sasikala and Sudhakar (Sasikala et al 2006) in antidiabetic and Hypolipidemic effects of methanolic extract of *Filicium decipiens* in streptozotocin induced diabetic rats and in antidiabetic effect of methanolic extract of *Decalepsis hamiltonii* root in normal and Alloxan induced diabetic rats. A total of 30 12-week old (approximately 25-35 g) ICR mice *(Mus musculus*) of either sex was obtained from institutionally bred and raised albino mice at the Philippine Institute for Traditional and Alternative Health Care Region 2, Philippines. These were kept in separate, standard-sized polypropylene cages. All cages were sanitized and bedded with autoclaved paddy husk. The plates and bottles were thoroughly washed, sanitized and dried twice a week. Prior to experimentation, mice were acclimatized for one week to adjust to a 12 h light, 12 h dark cycle at 28-30 °C. They were fed with standard pellet diet and water ad libitum. Proper handling and maintenance of the mice was observed all throughout the experiment. The experiment was approved by the Institutional Animal Care and Use Committee of PITAHC in Tuguegarao city.

2.5. Oral Glucose Tolerance Test (OGTT)

The oral glucose tolerance test was performed in overnight fasted (18 h) normal mice. The mice were divided into five treatment groups of six mice each. Treatment 1 will serve as normal control receive regular feed and water only. Treatment 2; Normal mice fasted. Glucose given 2gm/kg. Treatment 3; Normal mice fasted given glucose 2gm/kg then Glicazide 60mg/kg and Treatments 4 and 5 received orally by gavage feeding 80 mg and 40 mg/ kg of methanolic extract of Sarabat respectively. After 30 min, treatment groups were orally loaded with 2 g/kg of glucose. Blood samples were collected just prior to glucose administration and at 30, 60, 90 and 120 min after glucose loading. Blood glucose levels was measured using commercial kit (Acuchek).

2.6. Experimental procedures/ Hypoglycemic Activity in Normal Mice

Healthy ICR White mice weighing 25-30 g were fasted overnight and were divided into five groups of six mice each.

T_1 -Negative control (plain pellets and water

T_2 - two (2) grams glucose/kg body weight (bwt)

T_3 -two (2) grams glucose/kg bwt plus 60mg Glicazide/kg bwt

T_4- two (2) grams glucose/kg bwt plus 80mg/kg bwt Sarabat methanolic extract

T_5- two (2) grams glucose/kg bwt plus 40mg/kg bwt Sarabat methanolic extract. 3.5

2.7 Statistical analysis

The results were expressed as mean ± sd. Statistical comparisons were done with the use of univariate and multivariate analysis of variance (ANOVA) and the results are considered statistically significant when $p < 0.05$.

3. RESULTS AND DISCUSSION

a. Phytochemical Constituents of Sarabat

In the present study, the phytochemical constituents of Sarabat were determined. Results of the phytochemical characters of all the samples are summarized in Table 1. Presence of sterols, triterpenes, flavonoids, alkaloids, saponins, glycosides, tannins were recorded and noted in the samples.

Table 1 also shows that the fiddlehead extracts of Sarabat is consistently rich in Saponins and sterols hence can be valuable agents for lowering hypercholesterolemia. Glycosides are likewise notably present in the aqueous extract fraction of Sarabat. Triterpenes are likewise visibly seen in Sarabat 1 aqueous extract as well as an abundance of glycosides. The ethanolic and methanolic extracts of Sarabat 1 shows moderate amounts of alkaloids as shown by turbidity while flavonoids are moderately present as shown by formation of red precipitates in the ethanolic and methanolic extracts. Sterols are moderately seen in the ethanolic and methanolic extracts of sarabat 2 as shown by bluish color change. Tannins are found in all as traces signified by black color changes. Overall it can be shown that methanolic extraction positively yielded more of the phytochemical constituents.

The aforementioned chemical constituents of Sarabat parallels some of the constituents of *Diplazium esculentum* as determined by Tongco *et al.*, (2014) although a wider spectrum of phytochemical constituents was assessed in *D. esculentum*. This is consistent with the presence of phytochemical constituents such as alkaloids, flavonoids especially in the methanolic extracts, triterpenes in the aqueous extract and phytosterols as shown in Table 1 These phytochemical constituents of the fern plant may act synergistically to produce the desired effect of blood glucose lowering or enhance, influence and modulate the various key physiologic processes such as enzyme activity or transport of metabolites across membrane channels or absorption in the gastrointestinal tract particularly in regard to glucose metabolism .

Table 1. Qualitative phytochemical screening of ethanolic, methanolic and aqueous extracts of Sarabat fiddlehead ferns

Phytochemical Components	Aqueous Extract		Ethanolic Extract		Methanolic Extract	
	Sample 1	Sample 2	Sample 3	Sample 4	Sample 5	Sample 6
Sterols	(-)	(+)	(+)	(++)	(+)	(++)
Triterpenes	(++)	(+)	(+)	(-)	(+)	(-)
Flavonoids	(+)	(+)	(+)	(++)	(++)	(++)
Alkaloids	(+)	(+)	(++)	(+)	(++)	(++)
Saponins	(+++)	(+++)	(+++)	(+++)	(+++)	(++)
Glycosides	(+++)	(+)	(+)	(+)	(++	(+)
Tannins	(+)	(+)	(+)	(+)	(+)	(+)

Table 2. Analysis of mice glucose levels as affected by treatment extracts at varying observation periods.

Treatment	Period of observation	Type I Sum of Squares	df	Mean Square	F	Sig.*
Treatment	0min	1010.021	4	252.505	1.218	.333
	30min	13939.813	4	3484.953	6.140	.002
	60min	6609.754	4	1652.438	3.711	.020
	90min	1527.405	4	381.851	.612	.659
	120min	2360.513	4	590.128	1.486	.242

*Level of significance is at 5%.

Table 2a. Posthoc analysis showing treatment means having significant differences (p-value: 0.05) using Bonferroni test.

Period after post treatment	(I) Treatment	(J) Treatment	Mean Difference (I-J)	Std. Error	Sig.*
30min	Treatment 1	**Treatment 2**	**-62.13***	**14.426**	**0.003**
		Treatment 3	**-56.73***	**14.426**	**0.008**
		Treatment 4	**-49.33***	**14.426**	**0.026**
		Treatment 5	**-43.73**	**14.426**	**0.063**
60min	Treatment 3	**Treatment 4**	**-42.20***	**13.346**	**0.047**

*Level of significance is at 5%.

3.2 Effect of Sarabat on Blood Glucose

The effects of plant extracts on mice blood glucose level at varying period of observations were determined. The noted presence of phytochemical constituents with antidiabetic effects together high Saponins and presence of sterols of the experimental

test extracts triggered the researchers to further study the plant extracts on its potential effects to lower postprandial blood glucose level. This was accomplished by artificially increasing the glucose level of mice blood with oral glucose tolerance test via oral gavage of 2gm/kg glucose in overnight fasted mice. After which, an in vivo assay of the plant extracts and the commercial anti-glycemic drug were administered and observation periods were accomplished at 0, 30, 60, 90 and 120 minute post-treatments. Results reflected in Table 2 shows that at 30 minutes and 60 minutes, the treatments manifested effects on mice glucose level. In particular, at 30 minutes and 60 minutes post treatment, the treatments used manifested statistical difference at 5% and 1% level of significance with p-values of 0.002 and 0.02, respectively. Further analysis employing Bonferroni test revealed that the non-induced blood glucose and Treatment1 had significant difference on the induced treated and non-treated mice in terms of the significantly low blood glucose levels. Also reflected in Table 2 is the observation that at 60 minutes of post treatment the Glicazide took effect immediately by significantly lowering the glucose level especially when compared to Treatment 4 but its effect is found to be statistically comparable to Treatment 5. The effect of commercial drug manifested its effect on lowering blood glucose at 60-minute post-treatment yet, its significant effect against Treatment 4 was not sustained at 90- and 120-minute post-treatment as noted by the non-statistical differences in mean glucose levels reflected in Treatments 4 and 5 (Table 2a). Interestingly, the high dose plant extract (Treatment 5) showed decreasing pattern of mean glucose level in all observation periods which is consistently noted to be comparable to the effect of the commercial drug used as positive control.

Discussion

The preliminary findings in this study are consistent with the findings of Menon *et al.* (2015) in their study on the glucose lowering effect of *Kalanchoe pinnata* leaves. Since the leaves of *K. pinnata* are known to contain flavonoids, polyphenols, triterpenoids and phytosterols, it is generally believed that these chemical constituents might be responsible for the observed hypoglycemic activity of the herb. Similarly, the findings of Manickam and Periyasamy (2012) on the antihyperglycemic activity of *Decalepsis hamiltonni* which was found to contain flavonoids, sterols, triterpenoids, alkaloids and phenolics exhibited blood glucose lowering activity both on oral glucose tolerance tests and alloxan induced diabetes in mice. In their study they proposed that the antidiabetic activity of the methanolic extract of *D. hamiltonni* leaves is that " it promotes insulin secretion by closure of K^+ ATP channels, membrane depolarization and stimulation of calcium influx which is a key step in insulin secretion (Manickam and Periyasamy., 2012)

Gliclazide is an oral hypoglycemic 2nd generation sulfonylurea. It reduces blood glucose levels by correcting both defective insulin secretion and peripheral insulin resistance (extra pancreatic effects). This occurs by closure of K+ channels in the B□5f cells of pancreas, subsequently calcium channels open leading to an increase in intracellular calcium and induction of insulin release. Gliclazide also increases sensitivity of B5f cells to glucose. It also restores peripheral insulin sensitivity such as decreasing hepatic glucose production and increasing glucose clearance. Although it is well tolerated with low incidence of side effects, it can cause hypoglycemia if meal times are irregular or if meals are skipped. (Getz Pharma, 2015)

The closest explanation on the observed hyperglycemia lowering activity of Sarabat is in relation to its potential activity as an alpha glucosidase inhibitor. Chai *et al.* (2015a) have successfully demonstrated the alpha glucosidase inhibition activity of two other edible ferns namely *S. palustris* and *D. esculentum* (Chai *et al.*, 2015b) they have correlated these observed alpha glucosidase inhibitions with the presence of phenolic compounds, particularly hydroxycinnamic acids, which may have contributed to the antiglucosidase and antioxidant activities detected in *S. palustris*.. Alpha-Glucosidase inhibitors delay or inhibit gastrointestinal digestion of oligosaccharides and the resulting release of glucose. As a result, glucose absorption into the bloodstream is reduced or delayed. Despite their effectiveness in dampening after-meal hyperglycemia, oral antiglucosidase drugs have undesirable side effects, such as flatulence and diarrhea.

4. CONCLUSIONS

General phytochemical tests conducted have shown the presence of sterols, triterpenes, flavonoids, alkaloids, saponins, glycosides, tannins in the crude

extracts of the edible vegetable fern Sarabat(*Diplazium polypodiodes* Blume and *Diplazium sp*) with a preponderance of saponins. The aqueous extract likewise indicates the strong presence of glycosides and triterpenes. Alkaloids and flavonoids are also noted to be present in all solvents utilized but more prominently noted in the methanolic solvent. Oral glucose tolerance test shows that at 30 minutes and 60 minutes, the treatments manifested effects on mice with statistical difference at 5% and 1% level of significance with p-values of 0.002 and 0.02, respectively. Further analysis employing Bonferroni test revealed that the non-induced blood glucose and no-treatment (Treatment 1) was significantly different from the induced treated and non-treated mice in terms of the significantly low blood glucose levels. It was also observed that at 60 minutes of post treatment, the glycalizide took effect immediately by significantly lowering the glucose level especially when compared to Treatment 4 (low dose sarabat extract) but its effect is found to be statistically comparable to Treatment 5 (high dose sarabat extract). The effect of commercial drug manifested its effect on lowering blood glucose at 60-minute post-treatment yet, its significant effect compared to Treatment 4 was not sustained at 90- and 120-minute post-treatment as noted by the non-statistical differences in mean glucose levels reflected in Treatments 4 and 5 of the experimental set-up.

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