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Aerial Parts of *Euphorbia hirta* L. in Polar and Non-Polar Solvents: Phytochemical, Antioxidant and Glucose Uptake Studies for Potential Source of Adjunct Drug for Diabetes

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ABSTRACT

Euphorbia hirta L. (Euphorbiaceae) is an herbal plant found in countries with tropical and subtropical climates. It contains biologically active components that have pharmacological properties related to progression of oxidative stress and hyperglycemia. In this study, the dried aerial parts of the plant were refluxed using different polar and non-polar solvents such as water, methanol, ethanol, acetone, ethyl acetate, dichloromethane, and hexane. The effect of solvents used during extraction on the number of phytochemicals, antioxidant properties using DPPH and FRAP assays as well as on the glucose uptake were investigated. Results showed that all extracts using various solvents have varied phytochemical contents while polar solvents exhibited higher antioxidant properties both in DPPH and FRAP assays. Likewise, glucose uptake results showed that extracts from water have higher glucose uptake compared to the commercial drug metformin. Thus, components from the water extract of *E. hirta L.* could be a potential adjunct drug for diabetes.

Keywords: Antidiabetic, Antioxidant, *Euphorbia hirta*, Flavonoid, Polyphenol.

Introduction

The study on natural products heavily relies on the existence of plant materials which contain secondary metabolites that exhibit valuable pharmacological applications.[1] There is a wide variety of plants in the plant kingdom and the genus *Euphorbia,* from the spurge family (Euphorbiaceae), is known to be the most varied. *Euphorbia* contains 2000 species and is widely spread out through the tropical and subtropical climate zones such as Philippines and Taiwan.[2] One of the species that belongs to this genus is the *Euphorbia hirta* L. (*E. hirta*), also known as Tawa-Tawa in the Philippines.[3] *E. hirta* can grow up to 80 cm tall, which is commonly erected, and is slender stemmed.[4] Preliminary phytochemical screening of the ethanol and methanol crude extracts showed that Tawa-Tawa contains notable components, such as alkaloids, flavonoids, terpenoids, tannins, phenols, and saponins, which have been proven to be biologically active in different studies, while the aqueous extract also indicated the presence of carbohydrates.[5] The constituents of a plant material play a major role in the pharmacological properties that it exhibits. For instance, polyphenols are known to contribute to anti-oxidation activity.[6] Folklore applications of Tawa-Tawa include treatment of gastro- intestinal diseases and disorder, asthma, and skin problems.[7] Previous studies involving *E. hirta* consider different biological properties such as anti-inflammatory, antioxidant, antidiabetic, and anticancer activities.[8] A current study revealed that the bioactive compounds isolated from *E. hirta* are flavonoids (quercetrin and myricitrin) and terpenoids (taraxerone and lupeol).[9] In addition, just recently, chemical constituents namely taraxerol, campesterol, astragalin, hymenoxin, luteolin-7-O-β-D-glucopyranoside and quercetin 3-O-α-L arabinofuranoside were isolated from the hexane and ethyl acetate extracts.[10] Although, phytochemical investigations and studies on pharmacological applications have been done, most of these studies used only one solvent and to the best of our knowledge no one has yet explored several types of solvents for extraction of *E. hirta* and tested it for phytochemical, antioxidant and glucose uptake by yeast as anti-diabetes assay.

Diabetes mellitus is a metabolic disease that is characterized by elevated level of sugar in the bloodstream. The disease is multifactorial; thus, no single drug can be used for medical treatment. One of the effective ways to reduce the elevated blood sugar to normal level is through enhancing the glucose uptake of cells. Metformin is a widely used drug and is considered as the first line of treatment for diabetic patients. The mechanism by which the drug acts physiologically is still not fully understood; thus far, it has been shown to reduce hepatic glucose production as a primary mode of drug action.[11] The drug also enhances the glucose uptake into cells.[12] Moreover, it has been reported that metformin increases the glucose uptake in peripheral tissues by regulating the Glucose Transporter (GLUT) trafficking.[13] Recently, it has been shown that metformin enhances the glucose uptake into cells by inhibiting the lipid

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phosphatase Src homology 2 domain-containing inositol-5-phosphatase 2 (SHIP2) activity.[14]

In screening natural products as potential candidate for cure in diabetes, experiment involving glucose uptake in yeast cell can provide insights into their potential efficacy in vascular organisms. In addition, yeast has been used as a model to understand the glucose transport across cell membrane.[15] However, the exact mechanism of action of metformin in the uptake of glucose in yeast cell has not been reported. The use of metformin as a reference drug in screening natural products for increase glucose uptake is justified because it has been shown to act in such a manner where Glucose Transporter plays a role and is nearly resembles that of membrane transporters present in yeast cells (i.e. Hexose Transporter, Hxt). Moreover, several studies have used metformin as a reference drug that involves glucose uptake experiment.[16]

Thus, the present study aims to assess the chemical constituents present in the extracts of *E. hirta* from non-polar solvents (hexane, dichloromethane, ethyl acetate, and acetone) to polar solvents (ethanol, methanol, and double-distilled water) and to evaluate the pharmacological profiles of the seven extracts by using the *in vitro* antioxidant and anti-diabetes assays using glucose uptake.

Materials and Methods

Materials and equipment

The aerial part of *E. hirta* was collected from Changhua County, Taiwan, in September 2019, and was authenticated by Dr. Chia-Jung Lee, Ph.D. Program in Clinical Drug Development of Herbal Medicine, College of Pharmacy, Taipei Medical University, and comparison with images from online photos (West African plants: a photo guide; Senckenberg). A voucher specimen # CJCU-EH-001 of *Euphorbia hirta* was deposited at Department of Medical Sciences Industry, Chang Jung Christian University, Taiwan. All chemicals and reagents together with the yeast cells were bought from Sigma-Aldrich. The double-distilling water equipment was obtained from Millipore. All analytical measurements were performed using ELISA reader (BioTek Synergy HT).

Sample preparation and extraction

The aerial part of *E. hirta* was oven dried at 40°C for three days and was pulverized using a blender. Approximately 50 g of the powdered leaves and stems was weighed and extracted through a 2-h reflux at 65°C in a solid-solvent ratio of 1:20. The following non-sequential extraction solvents used were double-distilled water (DD H_2O), methanol (MeOH), ethanol (95% EtOH), acetone, ethyl acetate (EtOAc), dichloromethane (DCM), and hexane The crude extract was filtered out of the mixture and the solvent was evaporated under vacuum conditions for freeze-drying. Except for DD H_2O extract, which was dissolved with DD H_2O, all dried extracts were easily dissolved with 95% EtOH to make a concentration of 10 mg/mL. The sample solutions were then stored at -20°C.

Total polyphenol content analysis

The polyphenol content of the plant material was assessed using a slightly modified procedure of Folin Ciocalteu method reported by Slinkard and Singleton (1965).[17] Gallic acid was used as the standard and was subjected to 2-fold serial dilution to obtain five concentrations (0.03125 – 1 mg/mL in 95% EtOH) while the samples were dissolved in 1 mL 95% EtOH to obtain a concentration of 1 mg/mL. The reaction mixtures were prepared by mixing 100 µL of each of the extract and the standard solutions with 500 µL of $FeCl_3$ and 400 µL 7.5% Na_2CO_3 and were allowed to react at room temperature for 30 min. In a 96-well plate, 200 µL of the solution was transferred in three replicates and the absorbance was measured at 600 nm.

Total flavonoid content analysis

The flavonoid content of *E. hirta* was determined using a modified aluminum trichloride method described in Rebaya (2014).[18] Samples were prepared by dissolving 1 mg of extract in 1 mL of 95% EtOH while rutin, the standard compound, was subjected to 2-fold serial dilution to obtain five concentrations (0.025 - 0.400 mg/mL in 95% EtOH). A volume of 500 µL of each of the extract and standard solutions were mixed with 500 µL of 2% $AlCl_3$ in MeOH and were allowed to react at room temperature for 60 minutes. In a 96-well plate, 200 µL of the solution was transferred in three replicates and the absorbance was measured at 430 nm.

Total condensed tannin content analysis

Condensed tannin of *E. hirta* extracts were determined by preparing 1 mg/mL of each of the extract in 95% EtOH. The standard catechin was subjected to a 2-fold serial dilution to obtain five concentrations (0.01 – 0.16 mg/mL in DD H_2O). For the reaction mixture, 300 µL of each of the extract and standard solutions were mixed with 600 µL of 0.01 g/mL vanillin powder in 80% H_2SO_4 and were allowed to react at room temperature for 15 min. Three replicates of 200 µL of the solutions were transferred in a 96-well plate and the absorbance was measured at 530 nm.

Total polysaccharide content analysis

Polysaccharides in the sample were extracted by setting aside 1.25 mg/mL of each of the extract in 95% EtOH for 24 h. The solutions were centrifuged at 1300 rpm at 10°C for 10 mins. The supernatant was discarded, and the polysaccharide extract was washed with 80% EtOH for 30 mins. The washing was decanted after centrifugation and 1 mL of DD H_2O, 0.5 mL of 5% phenol solution, and 5 mL of concentrated H_2SO_4 were added and the reaction mixtures were allowed to react in a 95°C water bath for 15 min. After cooling down, 200 µL of the solution was transferred to a 96-well plate with three replicates and the absorbance was measured at 485 nm. The same chemicals were added to each of the standard solutions of glucose (50, 40, 30, 20, 10, 5 µL) obtained from the glucose stock solution (0.01 mg/mL in DD H_2O) and the same steps were performed starting from dilution with 1 mL of DD H_2O.

DPPH free radical scavenging assay

The free radical scavenging activityof the extracts were evaluated using the same procedure we used before.[19] A 200 µM of 1,1-diphenyl-*β*-picryl hydrazine (DPPH) was prepared in 95% EtOH under dark condition. A volume of 150 µL of 200 µM DPPH solution was added to 50 µL serial diluted extracts (six concentrations: 0.1563 – 10 mg/mL) on 96-well plates and incubated under dark condition for 30 min. Absorbance was measured at 517 nm in three replicates and the concentration at 50% inhibition was computed using the equation below:

$$IC_{50} = \frac{(Abs_{control} - Abs_{sample})}{Abs_{control}} \times 100\%$$

Ascorbic acid in 95% EtOH was used as a positive control (six concentrations: 0.00781 - 5 mg/mL).

Ferric reducing ability power (FRAP) assay

The procedure of Lin (2013)[20] was utilized for the evaluation of FRAP activity of the extracts with slight modification. 300 mM of sodium acetate buffer, 5 mM of 2,4,6-tris(2-pyridyl)-s-triazine (TPTZ) in MeOH, and 20 mM of $FeCl_3 \cdot 6H_2O$ in DD H_2O solutions were prepared. The reaction reagents were then mixed in proportions of 10:1:1, respectively. 1450 µL of the mixed reaction reagent was added to 50 µL of 10 mg/mL of each of the extract. 200 µL of the solution was placed on 96-well plates in three replicates and the absorbance was measured at 593 nm. A standard solution was prepared by mixing 10 mg of Trolox, 2 mL of 95% EtOH and 3 mL of DD H_2O. The 1 mM Trolox solution was subjected to 2-fold serial dilution to obtain five concentrations (0.078 – 1 mg/mL) and the same steps were conducted. The FRAP value (%) was calculated using the equation:

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$$\text{FRAP value (\%)} = \frac{(\text{Abs}_{\text{sample}} - \text{Abs}_{\text{blank}})}{(\text{Abs}_{\text{control}} - \text{Abs}_{\text{blank}})} \times 2$$

Determination of Glucose Uptake by Yeast Cell Assay

The assay, which was focused on the determination of the increase in glucose uptake by yeast cells, was conducted using the well-established method of Cirillo (1962)[21] with some modifications. Yeast cells were dissolved in DD H_2O (10% w/v) and suspension was kept overnight inside the refrigerator. Suspended yeast cells were repeatedly centrifuged (1000 rpm) and washed until a clear supernatant was obtained. 500 µL of washed yeast suspension (10% v/v) was poured into 1 mL Eppendorf tubes and was supplemented with 100 µL of extract with varying concentrations (1, 2 and 5 mg/mL in 95% EtOH). The mixture was then incubated for 10 min at 37°C. 250 µL of glucose with different weights (0.5, 1.0 and 2.5 mg in DD H_2O) was added and another incubation was done for 60 minutes at 37°C. The final mixture was centrifuged for 10 min (10000 rpm, 10°C) and was set aside until it reached room temperature. The glucose uptake was determined by analyzing glucose through addition of dinitrosalicylic acid (DNS) reagent.

DNS was prepared from the previously reported procedure made by Highley (1997).[22] 60% of analyte from the treated samples was placed in 2 mL Eppendorf tubes (600, 300 and 120 µL from 0.5, 1.0 and 2.5 mg glucose weight, respectively; balanced with DD H_2O) and 40% of DNS was added resulting to a total mixture of 1 mL. The tubes were then heated at 80°C for 15 min and cooled down to room temperature. 250 µL of the cooled solutions were placed on 96-well plates in three replicates. Absorbance was measured at 575 nm and the percent increase in glucose uptake of yeast cells was calculated using the formula below:

$$\%\ \text{increase in glucose uptake} = \frac{(\text{Abs}_{\text{control}} - \text{Abs}_{\text{sample}})}{\text{Abs}_{\text{control}}} \times 100\%$$

where control is the solution of yeast cells and glucose only. Reagent blank (40% v/v DNS in DD H_2O) and method blank (extracts only) were deducted from the absorbance of control and sample, respectively. Metformin was used as the reference drug with concentrations 1, 2 and 5 mg/mL in 95% EtOH.

Statistical analysis

Results were presented as average ± standard deviation of the three replicates. The readings were acquired with the application of Analysis of Variance (ANOVA) at significant level of $p < 0.05$ using Microsoft Excel © software.

Results and Discussion

Chemical constituents of E. hirta

The results of phytochemical investigation of the aerial parts of *E. hirta* extracts using various polar and nonpolar solvents are shown in Figure 1. The chemical constituent analysis performed in the study includes total polyphenol, total flavonoid, total polysaccharide, and total condensed tannin content. However, the total polysaccharide content was not included in the graph because it was present in the water extract only. The data were calculated using the calibration curve method and was reported as milligram component per gram extract. Acetone extract possessed the highest polyphenol content with 266.6 mg/g extract followed by EtOH with 203.8 mg/g extract. Notably, non-polar extracts (DCM and hexane) have more polyphenols as compared to polar extracts (H_2O and MeOH) by roughly 2 folds. On the other hand, ethyl acetate extract contains the highest flavonoids with 274.0 mg/g extract followed by acetone with 239.9 mg/g extract. Comparatively, non-polar extracts (DCM and hexane) contain more flavonoids than polar extracts (EtOH, MeOH and H_2O) with H_2O extract being the lowest. Thus, these results showed that the total polyphenols and total flavonoids are relatively higher in slightly polar solvents like acetone, ethyl acetate and ethanol compared to polar solvents (water and methanol) and nonpolar solvents (DCM and hexane). Although the extraction method used in this study was refluxing with various solvents, the results was in accordance with the previous reports that species under the genus *Euphorbia* contains polyphenols.[23-25] On the other hand, the total condensed tannins content of *E. hirta* sample extracts were relatively the same in all solvents with water extract being the lowest.

Antioxidant activity of E. hirta L.

The antioxidant potential of a substance maybe determined using two approaches. One is via direct assessment of the sample's individual constituent that is known to contribute to the antioxidant activity, and the other one is by analysis of its integral content, which covers a wider range of compounds and also takes into account the possibility of synergistic effects caused by the active constituents. The antioxidant property of the aerial parts of *E. hirta* using different polar and nonpolar solvents were assessed using the radical scavenging assay (DPPH) and ferric reducing antioxidant power (FRAP) assay. These methods were among the most popular methods for examining the antioxidant capacity.[26] The results in terms of %DPPH and %FRAP value are shown in Figure 2. It can be observed that in both assays the antioxidant property increases with increasing concentration of extracts. Likewise, extracts from polar and slightly polar solvents showed higher activities than extracts from nonpolar solvents in FRAP assay while in DPPH assay extracts from nonpolar solvents showed no activity.

In addition, the antioxidant properties were also reported in terms of the inhibition of free radicals by a concentration of extract at 50% (IC_{50}) as well as milligrams trolox equivalent per milligram of extract. These results are tabulated in Table 1. These results indeed showed that extracts from polar solvents have higher antioxidant property compared to extracts from nonpolar solvents. This can be correlated with the phytochemical contents of the extracts. Using both the DPPH and FRAP assay, the extracts from polar and slightly polar solvents have higher antioxidant property since they also have high phytochemical contents. However, extracts from nonpolar solvent have relatively high phytochemical contents but the antioxidant property was not detected in DPPH assay while minimal in FRAP assay. This may be attributed to the nature of phytochemicals present in the extracts. It may be possible that the antioxidant components in those extracts have slow or inert reactivity to those assays.[27] It is worth noting that DPPH assay is based on the hydrogen atom donating ability of the antioxidants towards the nitrogen radical of DPPH and thus, there is a possibility of the reactions between them to be slow or inert leading to a low or even no detectable activity. This may be true also with FRAP assay which is based on electron transfer reaction that measures the reducing ability of the antioxidants against oxidative stress of the reactive oxygen species. Thus, the high FRAP value of extracts obtained from polar and slightly polar is due to the higher phenolics and flavonoids content. The significant FRAP values obtained for extracts from nonpolar solvents DCM and hexane could be ascribed to its significant phenolics and flavonoids contents as well. Comparing both results, it was evident that extracts from slightly polar solvents have the highest antioxidant properties followed by the nonpolar and lastly with polar solvents; water and methanol. These findings may be due to the nature of the antioxidants present in the extracts.

Antidiabetic activity of E. hirta via glucose uptake

The antidiabetic activity of *E. hirta* extracts were assessed by determining the glucose uptake by yeast cells in the presence of the extracts. The process by which the transportation of glucose across the yeast cell membrane take place has been given attention as an *in vivo* screening method for hypoglycemic effect.[28] Facilitated diffusion is considered as the mechanism of transportation and different factors affect this such as succeeding glucose metabolism or glucose concentration within the cells. High glucose uptake is favoured when the condition of the cell is low in internal glucose concentration because of metabolism of sugars into other metabolites.[29] Hence, stereospecific membrane carriers facilitate effective transport of non-metabolized sugars when the concentration gradient of glucose between cell membranes are high. The transport of glucose across the cell membrane of yeast cells is assisted by the extracts of *E. hirta*.

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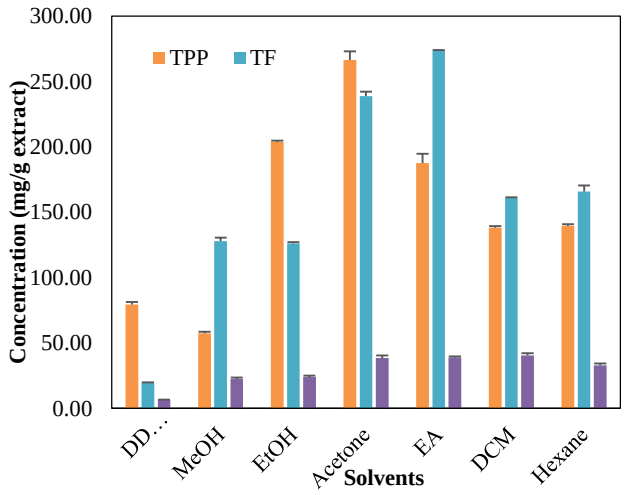

Figure 1: Chemical constituents of *E. hirta* extracts using various solvents.

Different concentrations of extracts (1, 2 and 5 mg/mL) were applied with different glucose weights (0.5, 1.0 and 2.5 mg) as shown in Figure 3. At 0.5 mg of glucose, only acetone extract effectively transports glucose into yeast cells at all concentrations in increasing manner. DCM and hexane extracts show glucose uptake starting at 2 mg/mL concentration while EtOH, MeOH, and EtOAc extracts start glucose uptake at 5 mg/mL concentration. Hexane extract possesses the highest glucose uptake with 53.71 ± 1.19% followed by DD H_2O extract with 47.75 ± 1.38%. At 1.0 mg of glucose, 5 mg/mL DCM extract exhibited the highest glucose uptake with 36.07 ± 2.70% and the lowest was 5 mg/mL EtOH extract with 5.72 ± 0.28%; whereas, MeOH extract showed no transport of glucose into yeast cells. Notably, acetone and EtOAc extracts have relatively the same glucose uptake for both 0.5 mg and 1.0 mg glucose in a decreasing manner from 44 mg/mL to 27 mg/mL. Likewise, EtOH and hexane extracts decrease yeast glucose uptake with increasing glucose weights from 0.5 mg to 1.0 mg, whereas DCM extract increases yeast glucose uptake in the same range of the said glucose weights. Overall, only DD H_2O extract assisted yeast cells for glucose uptake at all concentrations and results are comparable to metformin's glucose uptake

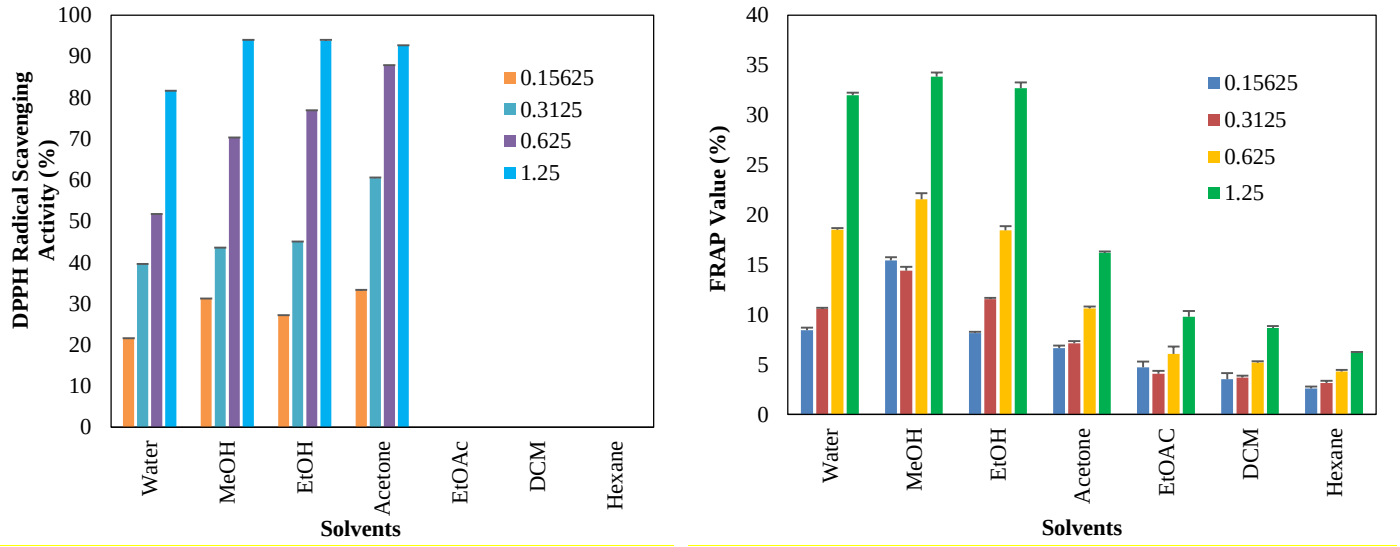

Figure 2: Antioxidant activities of *E. hirta* extracts with different solvents using DPPH and FRAP assays

Meanwhile, at 5 mg/mL extract concentration, DD H_2O, acetone, EtOAc, and hexane extracts showed higher glucose uptake than metformin in 0.5 mg glucose weight while acetone, EtOAc, and DCM at the same concentration showed higher glucose uptake than metformin in 1 mg glucose weight. Other extracts showed comparable glucose uptake with metformin at different glucose weights.

In the present study, increase glucose concentration decreases the effectiveness of most extracts to transport glucose at all concentrations. This suggests that reaction may occur between glucose and extracts, or the synergistic effect of the organic compounds in the extracts may hinder the diffusivity of yeast cell membranes. With this intention, cytotoxicity of the extracts on yeast cells can provide a plausible insight for this scenario and can be raised as a point to look into. Also, the saturation of glucose experienced by the yeast cells during the exchange across its membrane may be too much for the yeast cells to accommodate. Conversely, DD H_2O extract manifest potency to transport glucose at a certain level in all concentrations. Further exploration of the *in vivo* activity of natural extracts would be interesting to study as it may aid to enhance glucose uptake by adipose tissues and muscle cells of the body through increase binding of glucose to the carriers.

Table 1. Antioxidant activities of *E. hirta* extracts in polar and nonpolar solvents

Extract	DPPH assay IC_{50} (mg/mL)	FRAP assay mg Trolox/mg extract
H_2O	0.6131 ± 0.0298	0.143 ± 0.000
MeOH	0.4149 ± 0.0417	0.142 ± 0.001
EtOH	0.4038 ± 0.0135	0.138 ± 0.002
Acetone	0.2143 ± 0.0142	0.059 ± 0.000
EtOAc	ND	0.034 ± 0.000
DCM	ND	0.029 ± 0.001
Hexane	ND	0.023 ± 0.001

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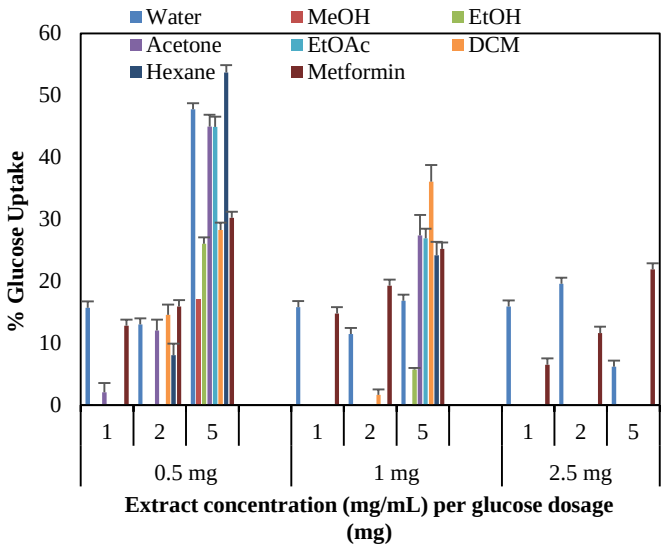

Figure 3: Glucose uptake (%) of *E. hirta* extracts in polar and nonpolar solvents at various concentration and glucose dosage

Conclusion

In this study several polar and nonpolar solvents were considered to extract components from *E. hirta*. All crude extracts from polar and nonpolar solvents showed significant amount of polyphenols, flavonoids and condensed tannins wherein extracts from slightly polar (ethyl acetate, acetone and ethanol) solvents had the highest amount followed by the extracts from nonpolar solvents (DCM and hexane) and the least from polar solvents (water and methanol). The antioxidant properties of the extracts using both DPPH and FRAP assays showed that extracts from polar solvents have higher activity compared to extracts from nonpolar solvents. It was presumed that even though the amounts of flavonoids and polyphenols in the extracts from nonpolar solvents were higher than the extracts from polar solvents, the nature of the antioxidant present dictates its activity. Lastly, the potential of these extracts to act as adjunct drug for diabetes was tested using glucose uptake by yeast cell assay. Results showed that among the extracts, those from the water has higher glucose uptake at 1 mg/mL and 2 mg/mL compared to metformin at 2.5 mg glucose dosage. Thus, this extract could be a potential source of adjunct drug for diabetes.

Conflict of interest

The authors declare no conflict of interest.

Authors' Declaration

The authors hereby declare that the work presented in this article is original and that any liability for claims relating to the content of this article will be borne by them.

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