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PHYTOCHEMICAL ANALYSIS AND *IN VITRO* ENZYME INHIBITORY ACTIVITY ESSAY OF *EUPHORBIA HIRTA* LINN. EXTRACTS

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ABSTRACT

In this study, *in vitro* analysis of on *α-amylase* and *α-glucosidase* inhibitory activity, total phenolic content (TPC), total flavonoid content (TFC) and phytochemical analysis of three different extracts (petroleum ether extract, chloroform extract and ethyl acetate extract) from crude extract of *Euphorbia hirta* Linn. (*E.hirta*) were determined using spectrophotometer and Fourier-transform infrared spectroscopy (FTIR) methods. *α-amylase* and *α-glucosidase* inhibitory activity of extracts were expressed as percentage of inhibition and IC_{50} values. Value in percentage of standard acarbose, crude, petroleum ether extract, chloroform extract and ethyl acetate extract at a concentration 10mg/ml were 94.69%, 55.06%, 16.55% and 40.97% inhibitory effects on *α-amylase*. Percentage inhibition of α-glucosidase at a concentration 10mg/ml were 54.91%, 76.67% and 98.39% presence for standard acarbose, crude extract and ethyl acetate extract in which petroleum ether extract and chloroform extract did not show inhibitory effects on α-glucosidase. Total phenolic content value of crude extract, petroleum ether extract, chloroform extract and ethyl acetate extract were 194.55 ± 0.82, 51.85 ± 3.12, 81.56 ± 1.72 and 214.21±2.53 mg of GAE/ g extract, expressed as gallic acid equivalents. Crude extract, petroleum ether extract, chloroform extract and ethyl acetate extracts showed total flavonoids 40.56 ± 7.27, 29.49± 1.66, 64.99 ± 2.60 and 91.69± 1.67 mg of RU/g extract, as rutin equivalents. FTIR spectra analysis of four extracts were comparable and showed the presence of alkaloids, saponins, polyphenols, terpenoids, steroids, flavonoids, quinones and terpenes. Two bioactive compounds that are 5-methoxybenzene-1,2,3-triol and 2-(2,4-dihydroxyphenyl)-3,6-dihydroxy-8-methoxy-4H-chromen-4-one were identified in ethyl

acetate extracts by using column chromatography method, thin layer chromatography (TLC) and NMR spectroscopy.

KEYWORDS: *Euphorbia hirta* Linn., total phenolic content (TPC), total flavonoid content (TFC), *α-amylase*, α-glucosidase.

I. INTRODUCTION

Diabetes mellitus is a global public health problem which is metabolic disorder characterized by hyperglycemia resulting from defects in insulin secretion. Type II diabetes is known as characterized by insulin resistance that peripheral cells do not respond to insulin or β-cell dysfunction (Heise et al., 2004) which takes about 90-95% of all diabetes patients (American Diabetes Association, 2008). Currently, there are around 422 million people have diabetes in the world and this number is significantly increasing, expected that in 2040 this number will be reached 642 million (International Diabetes Federation, 2015). Vietnam has more than 3.5 million cases of diabetes and 5.6 million of the prevalence of diabetes (International Diabetes Federation, 2015). Current treatment for Type 2 diabetes is not clear, available modern medicines such as Metformin, Sulfonylureas, Thiazolidinediones, SGLT2 inhibitors and DPP-4 inhibitors can treat diabetes, but it gave some undesired effects (Fowler et al., 2007). In recent years, using herbal medicines or natural materials were be encouraged because they are safer, cheaper and more efficient than synthetic drugs. The effect of herbal medicines on hypoglycemic is usually inhibited the absorption of glucose by inhibition of carbohydrate-hydrolyzing enzymes such as alpha-amylase and alpha-glucosidase (Patel et al., 2012). The inhibition of these enzyme delay carbohydrate digestion, resulting in the reduction in glucose absorption rate (Tamil et al., 2010).

Euphorbia hirta Linn. is widely used in traditional medicine for the treatment of several ailments such as respiratory diseases, gastrointestinal disorders, skin diseases, diabetes and kidney stones (Kumar et al., 2010). Furthermore, *E. hirta* contain a large of phytochemicals including flavonoids, triterpenoids, alkaloids, and saponins with have antidiabetic potential (Sunil Kumar et al., 2010; Subramanian et al., 2011). In a recent study, an in *silico* research was found 8 of 27 bioactive compounds in *E. hirta* related to strong binding on four diabetes proteins (Quy & Le, 2014). Therefore, this study was carried out to evaluate the phytochemicals, total phenolic and flavonoid contents, *in vitro* inhibitory effect of various extracts (crude extract, petroleum ether extract, chloroform extracts and ethyl acetate extract) and to identify the bioactive compounds in ethyl acetate extract.

II. MATERIALS AND METHODS

2.1 *Chemicals and reagents*

The chemicals used include absolute ethanol, petroleum ether, chloroform, ethyl acetate, methanol, purchased from Chemsol Company, Viet Nam. Aluminum chloride ($AlCl_3$), and sodium carbonate (Na_2CO_3), Folin-Ciocalteu reagent, dimethyl sulfoxide, starch, 5-dinitrosalicilic acid (DNS), thin layer chromatography (TLC) were purchased from Merck, Germany. Gallic acid, streptozotocin (STZ), nicotinamide (NAD), enzyme α-amylase and enzyme α-glucosidase, acarbose, *p*-nitrophenyl glucopyranoside (pNPG) were products of Sigma-Aldrich (St. Louis, MO, USA).

2.2 Plant material

The fresh *E.hirta* plant was collected in Ho Chi Minh City, Vietnam in October 2015. The whole plant without root was washed with tap water to remove all contamination and soaked in ethanol 70^0 to avoid microorganism. Then, samples were dried in oven at 60 ^{0}C for 8 hours to remove water content. After that, the sample was ground into powder and stored in a plastic bag for further uses.

2.3 Preparation of E.hirta extracts

The plant powder was macerated with methanol by the ratio 1:2 in 2 weeks. Then, the aqueous phase was filtered through Whatman No.1 filter paper, and the residue was added by fresh methanol. This process was repeated seven times until a clear colorless solution was obtained. Then, the extract solution was concentrated by a rotary evaporator at 60^0C, the concentrated extract called crude extract. Next, the crude extract was mixed with distilled water by ratio 1:1 and soaked with petroleum ether, chloroform, ethyl acetate respectively to produce the fractional extract. The rotary evaporator was used to evaporate all solvents to take the concentrated extracts.

The percentage of yield of extract was calculated as:

$$\%\,Yield\ (\%Y) = \frac{weight\ of\ dried\ extract\ (g)}{weight\ of\ sample\ (g)} x\ 100 \qquad (1)$$

2.4 Phytochemical analysis

2.4.1 Fourier Transform Infrared Spectrophotometer (FTIR)

Fourier Transform Infrared Spectrophotometer (FTIR) is a tool to identify chemical bonds within chemical functional groups. The characteristic of chemical bonds was determined by

the infrared absorption spectrum, and the individual compound was absorbed characteristic infrared radiation (Ashokkumar et al., 2014). The FTIR spectra were recorded in FTIR instrument by using Cary 600 Series FTIR spectrometer (Product No. K8000AA, Serial No. AU12460019). All extract samples were dissolved in dimethyl sulfoxide at 0.1mg/ml. Then the spectral data were compared with references to identifying the functional groups existing in the sample and the presence of phytochemicals in the sample.

2.4.2 Determination of total phenolic content (TPC)

Determination of total phenolic content was based on Folin-Ciocalteu assay method with some modification (Tambe & Bhambar, 2014; Quettier-Deleu et al., 2000). The reaction mixture consists of 0.2 ml of extract samples dissolving in methanol at 1 mg/ml. One milliliter of 10% Folin-Ciocalteu reagent was treated into the mixture and shaken well. After 5 minutes, adding 1.5 ml of 5% Na_2CO_3 solution into the mixture. Gallic acid was used as standard solutions (20, 40, 40, 60, 80 and 100 µg/ml). The absorbance was measured at 750 nm by spectrophotometer. Total phenol content was expressed as mg of GAE/gm of extract (Stankovi´c, 2011).

2.4.3 Dertermination of total flavonoid content (TFC)

Total flavonoid content was determined by spectrophotometric method (Tambe & Bhambar, 2014; Quettier-Deleu et al., 2000). The reaction mixture consists of 5 ml of extract samples dissolving in methanol at 0.4 mg/ml. Then, 5 ml of 2% AlCl3 solution was treated into the mixture. Rutin was used as a standard solution at different concentration (20, 40, 40, 60, 80 and 100 µg/ml). The absorbance was measured at 415 nm by spectrophotometer. The content of flavonoids in extracts was expressed in terms of rutin equivalent (mg of RU/g of extract) (Stankovi´c, 2011).

2.5 *In vitro* enzyme inhibitory activity essay

2.5.1 In vitro α -amylase inhibition

The α-amylase inhibitory activity was measured by the dinitrosalicylic acid method (M. A. Bhutkar, 2012). The 0.5 ml of extract samples were pre-incubated with α-amylase 2U/mL for 15 min. Then the mixture was added 0.5 mL of 1% w/v starch solution. The mixture was further incubated at 37°C for 10 min. Then the reaction was stopped by adding 1 mL DNS reagent and heated in a boiling water bath for 5 min. The control and blank was prepared

without plant extracts and the α-amylase enzyme. Acarbose was used as positive control. The absorbance was measured at 540 nm. Percentage inhibition is calculated as:

$$\%Inhibition = \frac{(Abs_{control} - Abs_{extract})}{Abs_{control}} \times 100 \qquad (2)$$

Concentrations of extracts resulting in 50% inhibition of enzyme activity (IC_{50}) were determined graphically (Kazeem el al., 2013).

2.5.2 In vitro α -glucosidase inhibition

The effect of the plant extracts on α-glucosidase activity was determined according to the method described by Li et al. (Li et al., 2005) with some modification. The reaction mixture consists of 112 µL potassium phosphate buffer (PPS, pH 6.8), 20 µL enzyme solution (1U/mL), and 8 µL of the sample solution, the mixture then incubated at 37 °C for 15 min. After that, 20 µL of pNPG (2.5 mmol/L) was treated and pre-incubated at 37 °C for 15 min. The reaction was terminated by adding 80 µL of Na2CO3 solution (0.2 mol/L). The control and blank were prepared without plant extracts and the amylase enzyme. Acarbose was used as positive control. Absorbance was measured at 405 nm. Percentage inhibition is calculated as:

$$\%Inhibition = \frac{(Abs_{control} - Abs_{extract})}{Abs_{control}} \times 100 \qquad (3)$$

The α-glucosidase inhibitory activity was expressed as the IC_{50} according to the percentage inhibition (Liu et al., 2016).

2.6 Isolation of bioactive compounds from ethyl acetate extract

Bioactive compounds were separated by column chromatography from ethyl acetate extract fraction by using several solvent mixtures. Thin layer chromatography (TLC) was used to analyze the fractions of compounds by column chromatography. Silica gel in column chromatography and thin-layer chromatography (TLC) have been used to separation of bioactive molecules (Altemimi et al., 2017). UV-visible, 25% sulfuric acid, vanillin solution are used for qualitative analysis and identification of classes of compounds in both pure and mixtures. Pure bioactive compounds are shown one dot in TLC when checked with some indicators such as UV-visible, 25% sulfuric acid, vanillin solution.

Nuclear Magnetic Resonance (NMR) spectroscopy is to determine the structure molecules by using the data from a wide range of spectroscopic technique. The basic principle of NMR is based on the spins of atomic nuclei. The spectroscopy means passing electromagnetic

radiation through an organic molecule that absorbs some of the radiation to produce a spectrum (Rosen & Tj., 1983; Altemini et al., 2017). NMR spectrum includes ^{1}H-NMR, ^{13}C-NMR, DEPT90, DEPT135, H-H COSY, HSQC and HMBC was measured by NMR Bruker Avance II equipment at Central Laboratory for Analysis of Ho Chi Minh City University of Science. The spectrum is specific to certain bonds in a molecule, and the structure of the organic molecule was identified depending on the spectrum (Altemimi et al., 2017).

2.7 Statistical analysis

Statistical analysis was performed using SPSS 22.0 software and obtained mean±standard deviation at $p < 0.05$. Values are presented as means ± standard deviation (n=3).

III. RESULTS AND DISCUSSION

3.1 Preparation of *E.hirta* extracts

9.5 kg of fresh *E. hirta* produced around 1.5 kg of dried powder, 184.56 g of crude extract, 61.25g of petroleum ether extract, 2.1 g of chloroform extract and 32.95 g of ethyl acetate extract. The results are shown in figure 1.

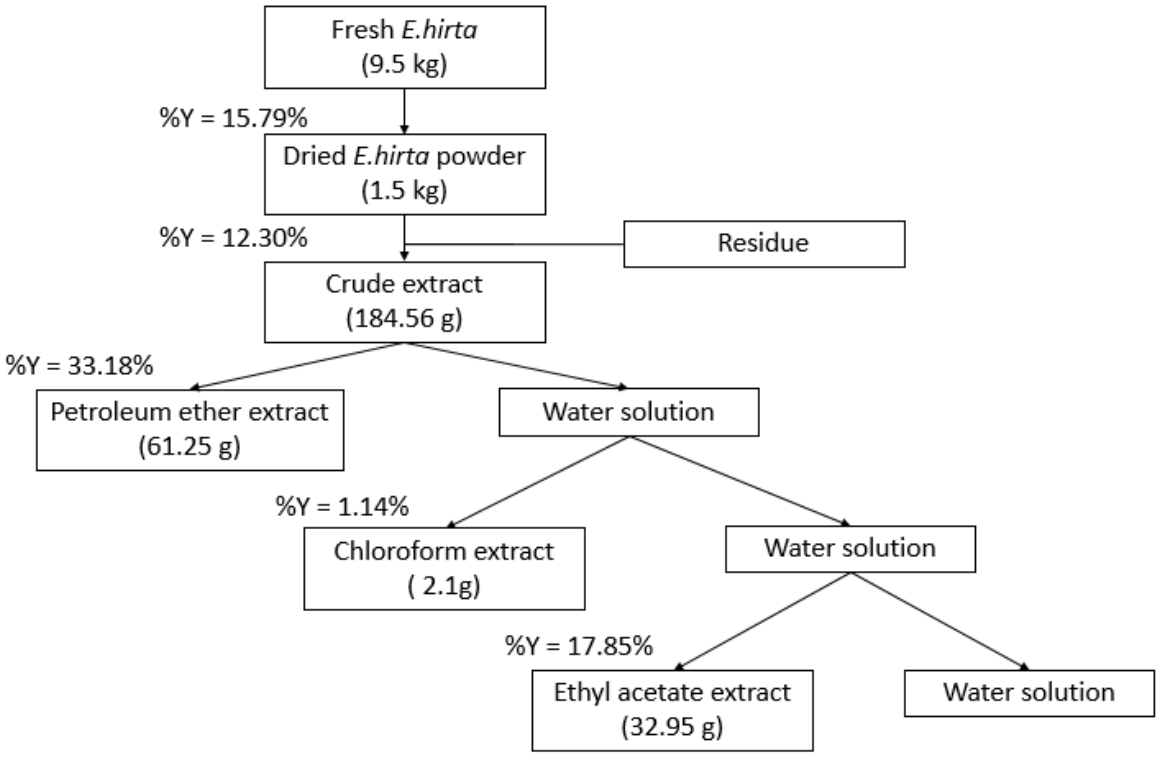


Figure 1: Percentage of extracts from *E.hirta*.

3.2 Phytochemical analysis

3.2.1 Fourier Transform Infrared Spectrophotometer (FTIR) analysis

The FTIR spectroscopic analysis showed the presence of phytoconstituents (Moses et al., 2013) [Figure 2, Table 1].

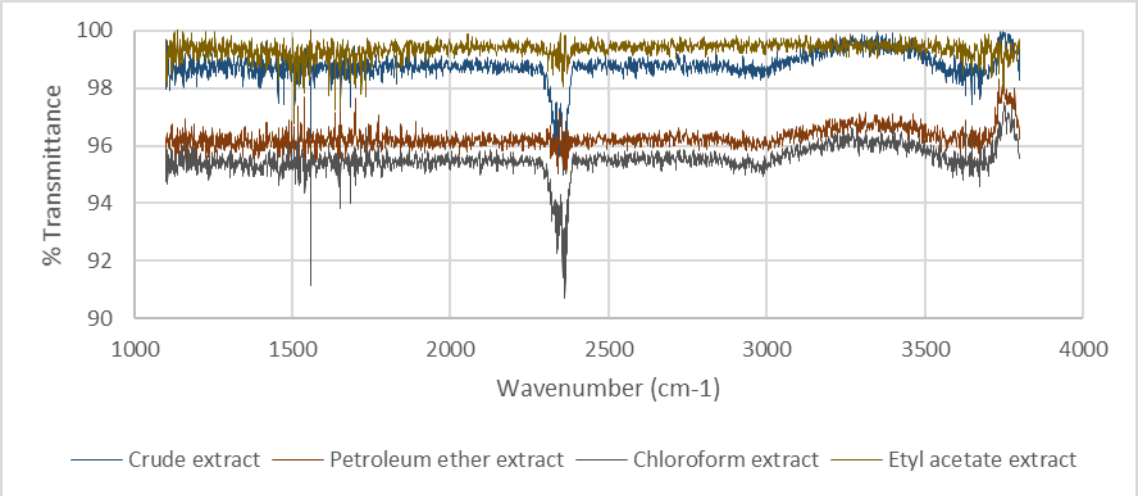


Figure 2: FTIR spectra of *Euphorbia hirta* Linn. Extracts.

The presence of alkaloids due to N-H stretch at the 3279.33, 3285.60, 3291.39, 3259.08, 3350.68 cm-1, C-N stretch of aromatic amine at the 1344.08, 1319.06, 1314.72, 1374.03 cm-1and N-H bend at 1683.17, 1665.53, 1684.55 ,1683.34 cm^{-1} in the primary, secondary and tertiary amines in all extracts. The saponins were obtained in the present due to C=O stretch at the 1750.03 cm^{-1} and 1235.66 cm^{-1} C-O stretch in the ethyl acetate extract. The polyphenols were found to be present with O-H stretch at the 3486.64, 3441.28, cm^{-1} and C=O stretch at the 1772.25, 1780.92, cm^{-1} for crude extract, and ethyl acetate extract. The esters peak for C=O stretch at the 1780.64, 1781.95, 1780.92, 1750.03 cm^{-1} and C-O stretch at the 1236.14, 1227.94, 1235.66, 1235.66 cm^{-1} were due to the presence of terpenoids and steroids for all extract. The presence of flavonoids were present with O-H stretch at the 3441.28, 3486.64 cm^{-1} and C=O stretch at the 1683.55, 1683.34 $cm^{-1}$for crude extract, chloroform extract and ethyl acetate extract. The terpenes were present with C-H stretch at the 2980.87, 2967.89, 2932.21, 2996.91 cm^{-1}, C=C stretch at the 1653.17, 1645.53, 1652.68, 1652.68 $cm^{-1}$for all extracts. The Cyanogenic glycosides were present due to the presence of C=N (stretch) at the 2358.07, 2348.25, 2361.42, 2355.60 cm-1 for all extrats while N=C=S (stretch) 2028.25, 2094.30 cm-1 were for Isothiocyanate glycosides for petroleum ether extract and chloroform extract.

Table 1: FTIR spectrum analysis of E.hirta extracts.

***Euphorbia hirta* Linn.**	**Absorption spectrum, Frequency (cm^{-1})**				
Functional groups	**Component (Peaks)**	**Crude extract**	**Petroleum ether extract**	**Chloroform extract**	**Ethyl acetate extract**
Alkyl aryl ether	C-O (stretching)	1236.14	1227.94	1235.66	1235.66
Aromatic amine	C-N (stretching)	1344.08	1319.06	1314.72	1374.03
Sulfone	S=O (stretching)	1344.08	1343.17	1314.72	-
Phenol	O-H (bending)	1384.74	1377.40	-	1374.03
Sulfate	S=O (stretching)	-	1394.27	-	-
Fluoro compound	C-F (stretching)	1401.98		1459.95	-
Alkane (methyl group)	C-H (bending)	1455.04	1462.25	1458.87	1457.91
Nitro compound	N-O (stretching)	1507.54	1522.51	1540.35	1506.63
Alkene (vinylidene)	C=C (stretching)	1653.17	1645.53	1652.68	1652.68
Amine (Primary, secondary, tertiary)	N-H (bending)	1683.17	1665.53	1684.55	1683.34
Conjugated aldehyde	C=O (stretching)	1683.17	-	1684.55	1683.34
Esters (6-membered lactone)	C=O (stretching)				1750.03
Vinyl / phenyl ester	C=O (stretching)	1780.64	-	1780.92	1772.25
Isothiocyanate	N=C=S (stretching)	-	2028.25	-	-
Cyanate	C=N (stretching)	2358.07	2348.25	2361.42	2355.60
Alkane	C-H (stretching)	2980.87	2967.89	2932.21	2996.91
Secondary amine	N-H (stretching)	3279.33	3285.60	3291.39	3259.08
Alcohol (intramolecular bonded)	O-H (stretching)	3441.28	3379.13	-	-
Alcohol (free)	O-H (stretching)	3682.47	3674.47	3674.67	3668.11

3.2.2 Total phenolic content (TPC) and total flavonoid content (TFC)

According to previous studies, *E. hirta* contain high amount of phenolic and flavonoid components (Asha et al., 2016). In the present study, total phenolic and flavonoid contents were shown in table 2. The total phenolic content in the examined extracts was expressed in terms of gallic acid equivalent (mg of GAE/g extract) by the standard curve equation: $y = 8.659x – 0.0153$, $R^2 = 0.998$. The value of the phenolic content were 194.55 ± 0.82, 51.85 ± 3.12, 81.56 ± 1.72 and 214.21±2.53 mg of GAE/ g extract for crude extract, petroleum ether extract, chloroform extract and ethyl acetate extract respectively. The concentration of flavonoids was expressed as rutin (mg of RU/g extract) by standard curve equation: $y = 12.905x – 0.0515$, $R2 = 0.9888$. The concentration of flavonoids in various plant extracts were 40.56 ± 7.27, 29.49± 1.66, 64.99 ± 2.60 and 91.69± 1.67 mg of RU/g extract. The highest concentration of phenolic and flavonoid were measured in ethyl acetate extract, and the lowest concentration of phenolic and flavonoid were measured in petroleum ether extract.

The concentration of phenolic and flavonoid in the plant extracts depend on the solvent polarity which used in the extract preparation (Stankovi´ c, 2011).

Table 2: Total phenolic and flavonoid contents in *E.hirta* extracts.

Extracts	Total phenolic content (mg of GAE/g extract)	Total flavonoid content (mg of RU/g extract)
Crude extract	194.55 ± 0.82 [1]	40.56 ± 7.27
Petroleum ether extract	51.85 ± 3.12	29.49± 1.66
Chloroform extract	81.56 ± 1.72	64.99 ± 2.60
Ethyl acetate extract	214.21±2.53	91.69± 1.67

[1]Each value is the average of three analyses ± standard deviation

3.3 In vitro α-amylase and α-glucosidase inhibitory activity essay

The inhibition of carbohydrate-hydrolyzing enzymes such as α-amylase and α-glucosidase activity is known as delay the digestion and absorption of carbohydrates from the small intestinal tract. By inhibition of these enzyme are minimized the absorption of glucose into blood (Tamil et al., 2010 and Chipiti et al., 2015).

The result of the present study showed that crude extract is a better α-glucosidase inhibitor than the standard acarbose as evidently shown by the lower IC_{50} values [Figure 4, Table 3]. However, the crude extract is significantly lower α-amylase inhibitor than the standard acarbose [Figure 3, Table 3]. Acarbose (at a concentration 10mg/ml) showed 94.69% and 54.91% inhibitory effects on α-amylase and α-glucosidase activity with IC_{50} value 3.998 and 8.342, respectively. The crude extract (at a concentration 10mg/ml) exhibited 55.06 and 76.67% inhibitory effects on α-amylase and α-glucosidase activity with IC_{50} value 8.192 and 5.791, respectively. The petroleum ether and chloroform extract (at a concentration 10mg/ml) showed 16.55% and 40.97% inhibitory effects on α-amylase, however, these extract did not show α-glucosidase inhibitory activity. The ethyl acetate extract (at a concentration 10mg/ml) showed 85.41% and 98.39% inhibitory effects on α-amylase and α-glucosidase with IC50 value 4.892 and 3.542, respectively. The results also indicated the ethyl acetate extract has highest α-amylase and α-glucosidase inhibitor with IC_{50} lowest comparing to the other extracts and appreciable α-amylase and α-glucosidase inhibitory effect when compared with acarbose.

Previously, the antidiabetic activity of *E. hirta* has been reported in the literature. The methanolic extract of *E. hirta* whole plant shown strong-α-glucosidase inhibitory activity and mid- α-amylase inhibitory activity (Sheliya et al., 2016). The result of the study showed that

crude extract and ethyl acetate extract moderately inhibited α-amylase and strongly inhibited α-glucosidase activity which is the same as antidiabetic drug model (Chipiti et al., 2015).

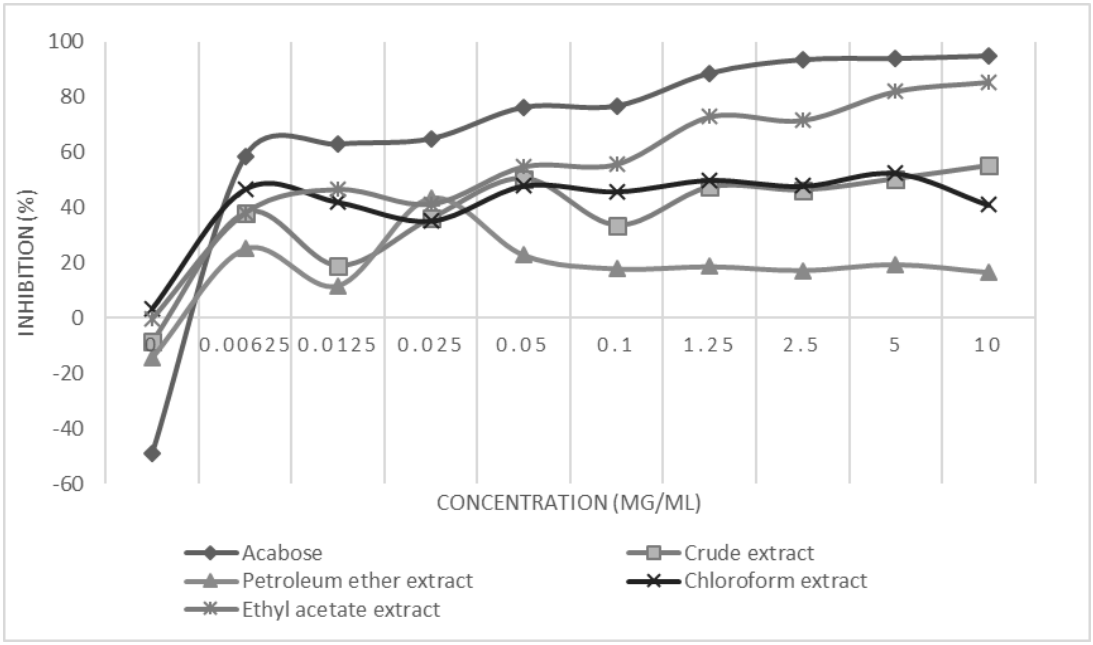


Figure 3: The inhibitory potency of *E. hirta* extracts against α-amylase activity.

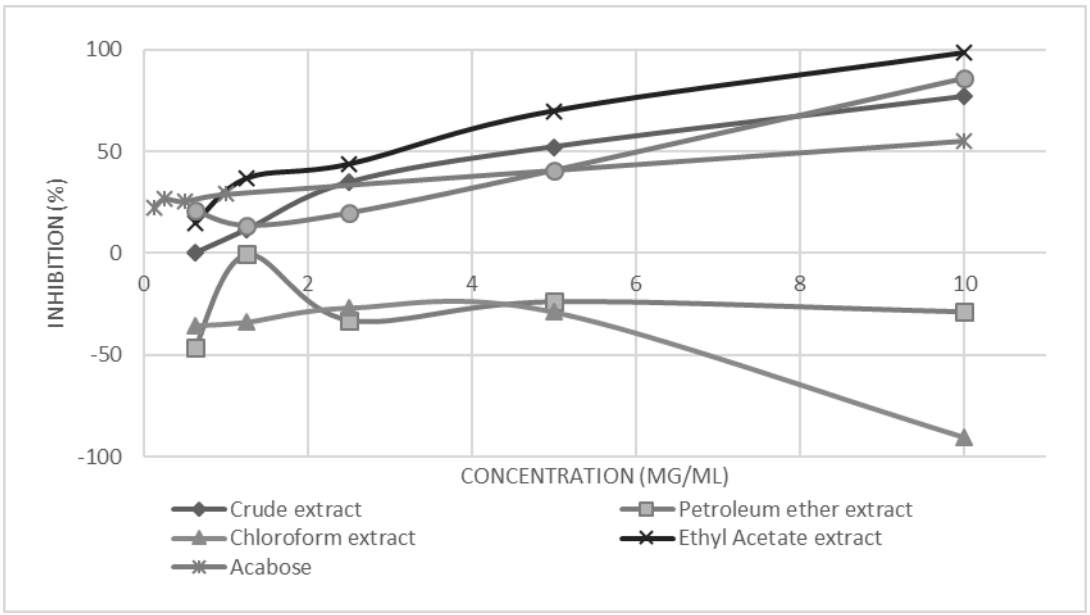


Figure 4: The inhibitory potency of *E. hirta* extracts against α-glucosidase activity

Table 3: IC50 of *E. hirta* extracts of α-amylase and α-glucosidase.

Sample	α-amylase equation (y = ax + b)	α-amylase IC50 (mg/ml)	α- glucosidase equation (y = ax + b)	α-glucosidase IC50 (mg/ml)
Acabose	y = 10.687x + 7.2743	3.998	y = 3.0437x + 24.608	8.342
Crude extract	y = 4.921x + 9.6882	8.192	y = 7.7811x + 4.9399	5.791
Petroleum ether extract	y = 1.1331x + 11.599	33.980	-	-
Chloroform extract	y = 2.7157x + 26.332	8.715	-	-
Ethyl acetate extract	y = 7.8836x + 11.437	4.892	y = 8.1988x + 20.957	3.542

3.4 Isolation of compounds from ethyl acetate extract

Representative silica TLC separations of *E. hirta* ethyl acetate extract from column-chromatography are shown in Figure 5. Separation of extract in Chloroform:Acetone:Methanol (7:2:1, v/v/v) under 25% sulfuric acid indicator, over 5 cm, resulted in many bands at different Rf values. These bands showed green, yellow, gray and almost orange color which indicated that this extract contains many flavonoid compounds. Then, nine smaller fractions were separated independently based on the characteristic of the bands occurring in the TLC plate. In this particular case, column-chromatography and TLC were used again and again until only one compound appearing.

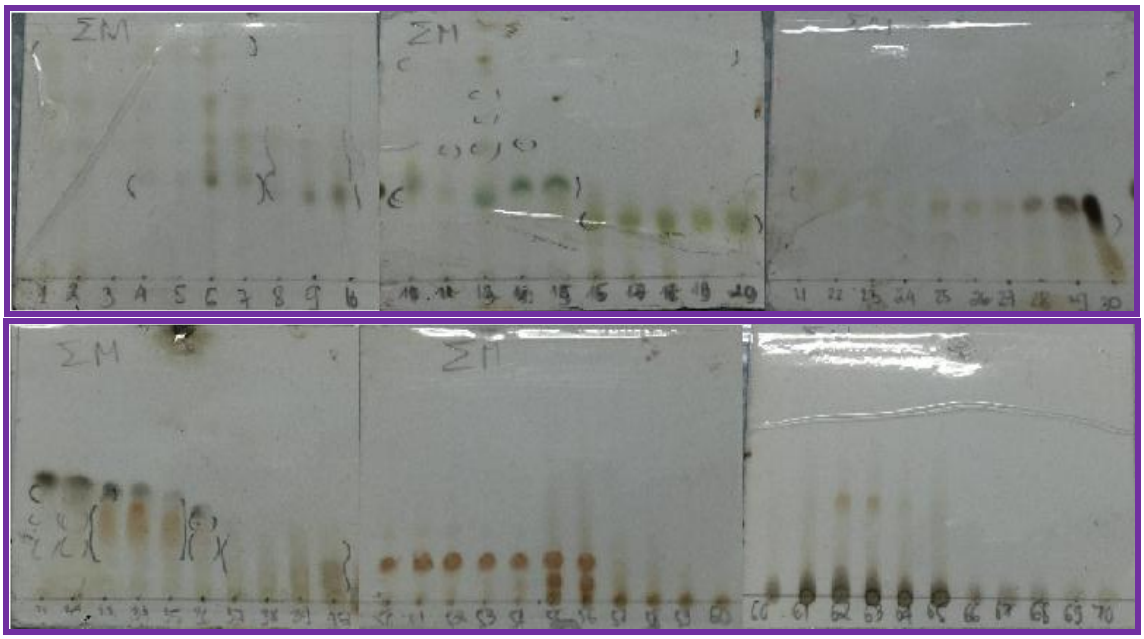


Figure 5: TLC result of ethyl acetate extract fractions with solvent mixture (Chloroform: Acetone: Methanol = 7:2:1) under 25% sulfuric acid indicator.

The TLC results of two pure compounds from ethyl acetate extract were shown in figure 6. A pure compound in Chloroform: Acetone: Methanol (7:2:1, v/v/v) under a UV-visible

indicator, over 5 cm, resulted in only one band. Figure 7 shows the appearance of two pure compounds after extract in open-column chromatography.

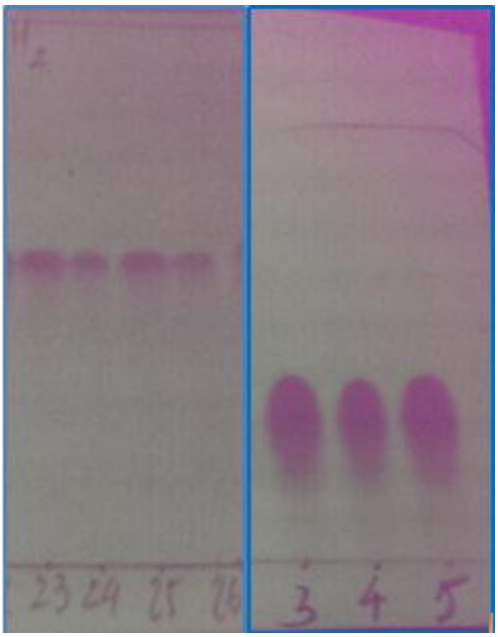


Figure 6: TLC result of Compound 1 and Compound 2 with solvent mixture (Chloroform:Acetone:Methanol=7:2:1, v/v/v) under UV-visible indicator (Compound 1-CSE1_right, Compound 2-CSE2_left).

Figure 7: Two pure compounds (Compound 1-CSE1_left, Compound 2-CSE2_right).

Nuclear Magnetic Resonance (NMR) spectroscopy is to determine the structure molecules by NMR spectrum include ^{1}H-NMR, ^{13}CNMR, DEPT90, DEPT135, H-H COSY, HSQC and HMBC. The structure of two pure compounds was identified and showed in Figure 8.

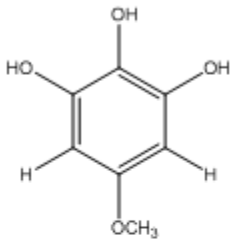
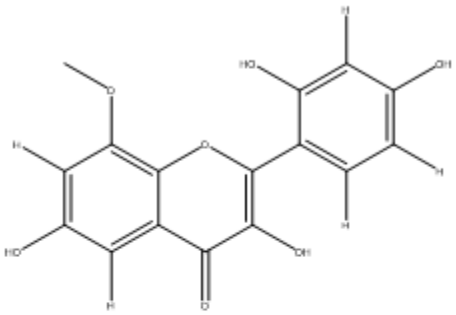
	CSE1	CSE2
Structure		
IUPAC name	5-methoxybenzene-1,2,3-triol	2-(2,4-dihydroxyphenyl)-3,6-dihydroxy-8-methoxy-4H-chromen-4-one
Chemical formula	$C_7H_8O_4$	$C_{16}H_{12}O_7$
Appearance	White crystalline solid	Yellow crystalline solid

Figure 8: Structure, IUPAC name and chemical formula of two compounds.

IV. CONCLUSION

The results of the study indicate that the potential antidiabetic activity for *Euphorbia hirta* Linn. extracts, particularly ethyl acetate extract contain the high amount of phenolic and flavonoid, and against α-amylase and α-glucosidase activity. Further large number of isolates of bioactive compounds are necessary to investigate the inhibitory of carbohydrate-hydrolyzing enzyme further. In addition, this study gives evidence that *E. hirta* could be useful in the management of diabetes.

V. ACKNOWLEDGEMENT

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