Biochem. Cell. Arch. Vol. 22, No. 1, pp. 2333-2344, 2022	www.connectjournals.com/bca	*ISSN 0972-5075*
DocID: https://connectjournals.com/03896.2022.22.2333		*eISSN 0976-1772*

COMPARATIVE STUDIES OF PHYTOCHEMICAL SCREENING, ANTIOXIDANT, ANTI-INFLAMMATORY ASSAYS AND LC-MS/MS ANALYSIS OF *EUPHORBIA HIRTA* L. COLLECTED FROM MALAYSIA AND IRAQ

Dania F Alsaffar[1], Sura F Alsaffar[2], Ahmed Yaseen[1], Shaida Fariza Sulaiman[1], Roziahanim Mahmud[1] and Nur Hidayah Kaz Abdul Aziz[1]*

[1]School of Pharmaceutical Sciences, Universiti Sains Malaysia, 11800 Penang, Malaysia.
[2]Department of Biology, College of Science, University of Baghdad, Baghdad, Iraq.
*e-mail : hidayahkaz@usm.my

(Received 12 March 2021, Revised 3 May 2021, Accepted 12 May 2021)

ABSTRACT : *Euphorbia hirta* L, belonging to the Euphorbiaceae family is widely used in traditional remedies and have different pharmacological applications. Samples of the plant were collected in Malaysia and Iraq. The objectives of this study are to understand how variations in climate and geography might affect the plant's nutrient, secondary metabolite and antioxidant levels. The aerial part of *Euphorbia hirta* plant was simultaneously collected in Malaysia and Iraq. Hot and cold extraction methods involving the use of hexane, chloroform, methanol and water solvents were then undertaken. All extracts were subsequently screened for the presence of phytochemicals and total phenolic and flavonoid contents. In addition, in vitro antioxidant and anti-inflammatory activities were conducted. Finally, the optimum extracts (metabolic extracts) of the two species were subjected to LC-MS/MS analysis. The results showed that the highest phenolic and flavonoid content, antioxidant properties and anti-inflammatory activities were found in the methanolic extract of the Malaysian species obtained using hot methods (336.65±9.46 mg Gallic acid equivalent of dry plant material weight, 63.94±2.81mg (QE)/g dry wt. 80.80±0.53% and 88.81%, respectively). An LC-MS/MS analysis of the two species revealed the presence of various phenolic, flavonoids and steroid compounds. I can be conclude that differences in agro-climatic conditions, extraction methods and types of extraction solvent used have clear effects on the total phenolic and flavonoid content and the antioxidant and anti-inflammatory potential of the *E. hirta* plant.

Key words : *Euphorbia hirta*, antioxidant, anti-inflammatory effect, LC-MS/MS.

How to cite : Dania F Alsaffar, Sura F Alsaffar, Ahmed Yaseen, Shaida Fariza Sulaiman, Roziahanim Mahmud and Nur Hidayah Kaz Abdul Aziz (2022) Comparative studies of phytochemical screening, antioxidant, anti-inflammatory assays and LC-MS/MS analysis of *Euphorbia hirta* L. collected from Malaysia and Iraq. *Biochem. Cell. Arch.* **22**, 2333-2344. DocID: https://connectjournals.com/03896.2022.22.2333

INTRODUCTION

According to the World Health Organization (WHO), a huge number of people currently use medicinal plants to promote improvements in healthcare (Ekor, 2014 and Schwartz *et al*, 2016). Globally, four out of five people depend on plants used in primary health care such as *Panax ginseng, Ginkgo biloba* and *Chamiomilla recutita* (Ghosh *et al*, 2019; Alsaffar and Al-Kaissy, 2009). Furthermore, one in four of the remedial drugs are extracted directly from or constitute derivatives of plants (Brower, 2008). Therefore, medicinal plants should be studied appropriately to promote a more comprehensive understanding of their botanical attributes in addition to establishing their chemical properties, safety, and efficacy (Pranabesh Ghosh, 2019). *Euphorbia hirta* L., a member of the Euphorbiaceae family, has spread extensively throughout the temperate or tropical regions of India, Asia, Australia and Africa. It is also native to Central America, where it is commonly known as asthma weed and milk weed, often being found in rubbish dumps and at the roadside (Sinhababu and Banerjee, 2016; Asha, 2014; Linfang *et al*, 2012). In Malaysia, its commonly used Malay names include ara tanah, gelang and keremak susu; while in Iraq its Arabic common name comprises labeinah, em elhaleeb and euphorbia (Rajeh *et al*, 2010 and Asha 2014).

Pharmacological studies of *E. hirta* have revealed its antioxidant, antimicrobial, antiepileptic, sedative anxiolytic, anti-inflammatory, analgesic, antipyretic, anti-asthmatic, anti-histaminic, anti-diabetic, anti-cancer,

gastrointestinal, wound healing, diuretic, antiparasitic, immunological, hepatoprotective, galactogenic, angiotensin converting enzyme inhibiting and anti-dipsogenic properties (Al-Snafi, 2017). As is well known, the screening of plant chemicals prior to the conducting of isolation studies is essential in the well production of biologically active compounds. Screening and identification studies by LC-MS/MS spectrometry are insufficient and proper identification through NMR studies is required. Nevertheless, LC-MS/MS remains one of the important analytical techniques used in the separation, identification, and quantification of compounds. It combines the physical separation of the capabilities of liquid chromatography (LC) from the mass analysis capabilities of MS (Pang *et al*, 2016).

Climate change is causing observable effects on the distribution and phytochemical composition of plants, including aromatic and medicinal varieties, across the world (Das *et al*, 2016). Consequently, in this study, *E. hirta* plant samples collected in Malaysia and Iraq were screened phytochemically in order to determine both their total phenolic and flavonoid content, antioxidant and anti-inflammatory potency. The highest quality extracts were subsequently subjected to LC-MS/MS analysis. Malaysia's climate is characterized as equatorial; hot and humid throughout the year with an average maximum temperature of 32°C and an average minimum one of 24°C (Saw and Institute of Southeast Asian, 2015). In contrast, that of Iraq is of a predominantly hot desert variety. Average maximum temperatures at low elevations exceed 40°C during the summer months (June, July and August), whereas the average minimum temperature can fall below 0°C during the coldest winter month. Therefore, it is necessary to understand how variations in both climate and geography might affect the plant's nutrient intake, secondary metabolite levels and antioxidants. (Table 1) presents climatic and geographical information relating to the sampling area.

MATERIALS AND METHODS

Plant collection

The aerial parts of *E. hirta* L. (Iraqi species) were collected from the Al-Jadriya area at the University of Baghdad and subsequently authenticated by the Herbarium Center, Department of Biology, College of Sciences, University of Baghdad. A voucher specimen (No: 50351) of the plant has been preserved by the Herbarium Center for future reference. Samples of the Malaysian species were collected from various locations within the states of Penang and Kedah and subsequently authenticated at the Herbarium, School of Biological Sciences, Universiti Sains Malaysia. The voucher specimen (No: 11858) of the plant has been preserved in the Herbarium for future reference. The Iraqi and Malaysian plants were collected around the mid-point of 2019. The two plants were washed with tap water to remove any debris before being dried in the shade for three weeks at room temperature. The dried, powdered plant was packed in tightly closed containers and stored for further analysis.

Extraction of plant

Two extraction methods, hot and cold were used in this study for each plant species.

Cold extraction

A serial cold extraction method as described by Roopali M Sankeshwari AVA (Roopali M Sankeshwari, 2018) was carried out with slight modifications. 100 gm of dried plant powder was weighed and soaked with 500ml of hexane in a container and allowed to stand, although with frequent agitation, at room temperature for three days. After 72 hours, the mixture was filtered through a clean muslin cloth and then subjected to double filtration using Whatman No.1 filter paper. A rotary evaporator (Buchi, Switzerland) concentrated the filtrate under vacuum conditions. The marc-powdered plant was dried using the same procedure being repeated sequentially with chloroform, methanol and water. The aqueous extract was then dehydrated using a freeze dryer. By these methods, four concentrated cold extracts; hexane, chloroform, methanol and aqueous extracts, were obtained for each plant species.

Hot extraction

A hot extraction method similar to that described by Nafiu MO (Nafiu *et al*, 2017), although with slight modifications, was carried out. The air-dried powder of the plant (100 gm) was extracted with hexane (1000 ml) using Soxhlet apparatus until exhausted. The marc was first air-dried, before being serially extracted using Soxhlet with chloroform (1000ml) and methanol (1000 ml) to the point of exhaustion. The extracts were then filtered to remove any plant ashes present. At that point, a rotary evaporator (Buchi, Switzerland) under vacuum conditions was employed to concentrate the filtrate. Ultimately, three concentrated hot extracts of hexane, chloroform and methanol were obtained for each plant species.

Phytochemical Screening

Phytochemical analysis of the extract was performed for each plant species in order to identify the various phytoconstituents such as steroids, alkaloids, saponins, sugar, flavonoids, tannins and carbohydrate; while

Table 1 : Climatic and geographical information of *E. hirta* L. sampling area.

Location	Lowest temperature (°C)	Highest temperature (°C)	Daylight hour (hr)	Above sea level (m)	Humidity (%)	Average rainfall (mm)
Penang, Malaysia	23	32	12 all the year	3	76	2670
Aljaderiah, Iraq	24	48	11 in December, 14 in June	34	0	0

Table 2 : Phytochemical test with observation.

No	Name of compounds	Test	Observation
1	Alkaloids	Dragendorff's Test	Orange color ppt
2	Flavonoids	Alkaline reagent test	Yellow colour
3	Tannins	Ferric Chloride Test	Deep blue or green ppt
4	Saponins	Froth Test	Foam formed
5	Terpenoid	Salkowski Test	Redish-brown intermediate layer
6	Steroids	Liebermann-Burchard Test	Blue- green colour
7	Carbohydrates	Molish's Test	Formation of red brown ring
8	Reducinging sugar	Benedict's test	Formation of red or green ppt
9	Anthraquinones glycosides	Borntrager's test	Rose-pink colour
10	Cardiac glycosides	Keller-Killiani's Test	Greenish colour
11	Proteins	Millons test	White ppt that changes to brick red on boiling

reducing sugar, anthraquinones glycoside, cardiac glycosides and protein. These qualitative phytochemical analyses were performed according to standard procedures (Vaghasiya *et al*, 2011; Evans *et al*, 2009) as shown in Table 2.

Total Phenolic Content

The total phenolic content of the plant extracts was carried out using Folin and Ciocalteu reagent as described by Singleton *et al* (1998) with slight modifications. The 200µl test sample was mixed with 500µl of distilled water (DW) and 500µl of 10% Folin-Ciocalteu's phenol reagent. After six minutes, 8µl of saturated sodium carbonate solution (7.5% w/v in water) was added to the mixture, with the volume being completed up to 3ml with distilled water. The mixture was allowed to stand for 30 minutes in a dark environment, with the absorbance of the sample and standard subsequently being read by spectrophotometer (Epoch, Bio Tek Microplate Spectrophotometer) at 765 nm in contrast to the reagent blank. The total phenolic content of the samples was calculated as the gallic acid equivalent GAE/g of dry plant material from the standard curve of gallic acid (10–400mg/L, $Y = 0.0021x+0.1157$, $R^2 = 0.9919$). Data was recorded as mean ± SD for three replications.

Total Flavonoids content

The total flavonoid content of the sample extracts was determined by application of the aluminum chloride reagent method as described by Alhawarri MB (Alhawarri *et al*, 2021). The quercetin standard was used to produce a standard curve. Stock quercetin solution was prepared by dissolving 1.0 mg of quercetin in 1.0mL of methanol. Standard solutions of quercetin were subsequently prepared in serial dilutions (10–100 µg/ml) using methanol as the solvent. 250 µl of crude extract (1 mg/ml methanol) was placed in a test tube prior to the addition of 1ml of distilled water and 75µl of 5% $NaNO_2$ solution. After a further six minutes of incubation 75µl of 10% $AlCl_3$ solution was added. The mixture was allowed to stand for another six minutes after incubation before 1ml of 4%NaOH solution was added. The final volume of the mixture was completed to 2.5ml with the addition of double-distilled water and allowed to stand for 15 minutes. Absorbance was measured at 510 nm using a spectrophotometer (Epoch, Bio Tek Microplate Spectrophotometer). The total flavonoid content was calculated based on the calibration curve ($Y = 0.00385x + 0.0257$, $R2 = 0.985$) and expressed as mg quercetin equivalent (QE)/g of the dried plant material. All samples were produced in triplicate (Pande *et al*, 2017 and Larit *et al*, 2019).

Antioxidant assay

In vitro antioxidant study by DPPH (2, 20-diphenyl-1- picrylhydrazyl), radical scavenging activity (Uppu *et al*, 2010). From each hexane, chloroform, methanol and aqueous extracts of each species plant, 200, 100, 50, 25, and 10µg/ml were placed in separate test tubes, before being mixed with 2.5 ml of methanol and 5 ml (100 µM) of DPPH-methanol solution. The reaction mixture was kept aside under dark conditions for 30 minutes at room

temperature, after which the absorbance was recorded at 517 nm using a spectrophotometer (Epoch, Bio Tek Microplate Spectrophotometer). The percentage inhibition of DPPH radical was calculated by comparing the test sample results with those of the control (untreated with the extract) using the following equation (Apak *et al*, 2018).

Percentage inhibition = (1 – absorbance of test/ absorbance of control) × 100

The results were compared to a standard solution of ascorbic acid at the same concentrations. The anti-radical activity was expressed in terms of IC50 (the amount of antioxidant necessary to reduce the initial DPPH absorbance by 50%). Five varying concentrations (10, 25, 50, 100, 200 µg/ml) of different solvent extracts derived from each species of *E. hirta* indicated contrasting percentages of inhibition. The IC50 value for each extract and ascorbic acid was graphically determined by plotting the percentage of DPPH scavenging activity of the extract against its corresponding concentration.

In vitro anti-inflammatory assay

The *in vitro* anti-inflammatory activity of plant extracts was analyzed by applying the denaturation of protein method as described by Mizushima (1968). 5ml of the reaction mixture consisting of 0.2ml of eggs albumin with 2.8ml of phosphate-buffered saline (PBS, pH 6.4) and 2ml of 200µg/ml concentration of each extract was prepared. The control was of a similar volume to that of the double-distilled water. At that point, the mixture was placed in an incubator for approximately 15 minutes at 37°C and then heated for five minutes to 70°C. After cooling, the absorbance of the mixtures was measured at 660 nm using a spectrophotometer (Epoch, Bio Tek Microplate Spectrophotometer) (Dharmadeva *et al*, 2018). Indomethacin was used as a reference drug. The percentage of inhibition of the protein denaturation of samples was calculated using the following formula:

% Anti-denaturation activity = [(Absorbance of control – Absorbance of sample)/Absorbance of control)] × 100

% Anti-Denaturation activity equal to the % inhibition of Protein Denaturation, which equals the % Anti-inflammatory activity.

Liquid Chromatography-Mass Spectrometry (LC-MS/MS) analysis

Chromatography was performed according to Yener *et al* (2019). The LC-MS/MS analyses of the plant compounds were carried out using a Nexera model Shimadzu UHPLC coupled with a tandem MS instrument. The injection volume was 10µL and separation achieved by an Agilent reverse-phase column (Zorbax Eclipse Plus C18 21.0 × 50 mm, 1.8 µm). The temperature of the column was maintained at 50°C with a constant flow rate of 0.4 mL/min and total LC run time of 40 minutes. The mobile phase comprised water containing 0.1% acetic acid (solvent A) and HPLC grade acetonitrile containing 0.1% acetic acid (solvent B). The mobile phase increased from 5.95 to 15.85 at 5-minute intervals, returning to the initial condition (95%) for five minutes for solvent A and maintaining that level for another five minutes. During the mass analysis, operation in negative ionization mode was set with the following parameters: a drying gas temperature of 350°C and nebulizer pressure of 40 psig. The drying gas flow was set at 12 L/min and MS/MS mass range at 150-1500 m/z. Data acquisition was performed using an Agilent MassHunter workstation with Acquisition MassHunter Qualitative Analysis software. MS/MS experiments were carried out in automatic multiple reaction monitoring (MRM) mode within which automatic MS/MS low energy collision dissociation (CID) was performed at 5 to 8 eV collision energy. Peak identification was conducted based on comparison with online databases and literature values.

Statistical analysis

All experiments were carried out in triplicate with values being expressed as the mean ± standard deviation (SD) of the three separate experiments using an Excel and Graph pad prism 8 computer program.

RESULTS

Phytochemical screening

The phytochemical screening results of the n- hexane, chloroform, methanol and aqueous extracts of the aerial parts of each Iraqi and Malaysian species are listed in Table 3.

Determination of Total phenolic content

The total phenolic content of plant extracts obtained through the use of Folin-Ciocalteu reagent was expressed in terms of Gallic acid equivalents GAE/g of dry plant material, GAE. (Table 4) showed the total phenolic content for varying polarity extracts of *E. hirta* obtained by cold and hot methods. Fig. 1 indicated that the total phenolic content was higher when using hot extraction methods compared to cold ones for all extracts of the two species. For the Malaysian species, the concentrations of total phenols obtained ranged from 15.75±7.36 to 336.65±9.46 mg GA/g. The highest total phenol content was found in the methanolic extract, followed by chloroform and hexane extract, with aqueous

Table 3 : The phytochemical screening of *E. hirta* plant extracts of both Iraqi and Malaysian species.

No	Test	I HEX	I CL	I MeOH	I AQ	M HEX	M CL	M MeOH	M AQ
1	Alkaloids	+	+	+	+	+	+	+	+
2	Flavonoids	-	+	+	-	-	+	+	+
3	Tannins	-	-	+	+	-	-	+	+
4	Saponins	-	-	+	+	-	-	+	+
5	Terpenoid	+	+	+	-	+	+	+	-
6	Steroids	+	+	+	-	+	+	+	-
7	Carbohydrates	-	-	-	+	-	-	-	+
8	Reducinging sugar	-	-	+	+	-	-	+	+
9	Anthraquinones glycosides	-	-	-	-	-	-	-	-
10	Cardiac glycosides	-	-	+	+	-	-	+	+
11	Proteins	-	-	+	+	-	-	+	+

I HEX: Iraqi species hexane extract,I CL: Iraqi species chloroform extract, IMeOH: Iraqi species methanolic extract, I AQ: Iraqi species aqueous extract, M HEX: Malaysian species hexane extract, M CL: Malaysian species chloroform extract, M MeOH: Malaysian species methanolic extract M AQ: Malaysian species aqueous extract.

Table 4 : Total phenolic contain, total flavonoid contain, Dpph IC50, antioxidant and anti-inflammatory of both Malaysian and Iraqi species extracts (n = 3).

Sample	Total phenolic	Total flavonoid	DPPH	IC 50	Anti-inflammatory
IC HEX	15.22±3.17	24.64±2.24	48.85±0.3	113.78±6.84	76.80±2.24
IC Cl	62.84±3.17	13.24±7.30	45.25±1.31	164.76±5.76	67.11±3.22
IC MeOH	296.17±2.79	37.67±1.84	80.35±3.09	24.75±2.43	79.93±.0.78
IH HEX	8.08±9.46	35.30±3.42	38.63±2.05	164.98±11.93	75.17±5.22
IH CH	81.89±7.13	28.85±1.75	30.76±3.29	190.33±5.34	56.53±6.79
IH MeOH	317.48±9.92	57.27±1.45	80.8±1.81	16.75±5.63	84.83±3.10
I AQ	58.08±7.13	18.72±0.53	46.75±1.34	96.61±1.11	72.01±2.02
MC HEX	31.89±4.86	47.01±1.71	39.69±1.42	184.94±12.96	57.21±1.74
MC CL	86.65±7.13	29.03±5.53	39.62±1.31	186.81±5.71	23.47±6.89
MC MeOH	262.84±7.13	59.77±2.90	81.99±1.28	36.94±3.59	74.15±5.31
MH HEX	79.51±9.46	60.82±1.58	43.76±0.71	137.77±16.77	50.41±4.51
MH CL	105.70±3.17	32.27±2.91	38.55±2.19	195.04±17.33	22.11±5.81
MH MeOH	336.65±9.46	63.94±2.81	84.92±3.49	12.93±3.64	88.81±6.49
M AQ	19.75±7.36	15.63±0.22	65.42±11.91	46.86±15.09	64.29±5.31
A A	---	---	85.06±3.15	10.47±2.86	--
Ind	---	---			91.77 ±2.85

IC HEX: Iraqi species cold hexane extract, IC CL Iraqi species cold chloroform extract IC MeOH, Iraqi species cold methanolic extract, IH HEX: Iraqi species hot hexane extract, IH CL Iraqi species hot chloroform extract, IH MeOH: Iraqi hot methanolic extract, I AQ: Iraqi species aqueous extract, MC HEX: Malaysian species cold hexane extract, MC CL: Malaysian species cold chloroform extract, MC MeOH: Malaysian species cold methanolic extract, MH HEX: Malaysian species hot hexane extract, MH CL: Malaysian species hot chloroform extract, MH MeOH: Malaysian species hot methanolic extract, M AQ: Malaysian species aqueous extract, A A: ascorbic acid, Ind: indomethacin. Each value in this table was obtained by calculating the average of three analyses (n=3), ± mean standard deviation.

extracts as the lowest. In the Iraqi species, the total phenols ranged from 8.08±9.46 to 314.84±9.92 mg GA/g, with the highest levels occurring in the methanolic extract followed by chloroform, aqueous, and hexane extract as the lowest. The highest phenolic content of all the extracts was found in the Malaysian methanolic extract produced by hot method (336.65±9.46mgGAE/g dry wt.).

Determination of flavonoids content

The total flavonoid content of varying polarity extracts of *E. hirta* obtained by cold and hot methods is expressed in terms of mg quercetin equivalent per g dry plant weight (QE)/g dry wt. The results shown in (Table 4) indicate clearly that, of all the extracts, the highest level of flavonoid content was found by means of hot methanolic extract (63.94±2.81mg (QE)/g dry wt) in the Malaysian species. The total flavonoid content in the same species ranged from 15.63±0.22 to 63.94±2.81 (QE)/g dry wt, while in its Iraqi counterpart the total flavonoid content ranged from 13.24±7.30 to 57.27±1.45 (QE)/g dry wt, as shown in Fig. 1.

Antioxidant activity

The results of DDPH (2,2-diphenyl-1-picrylhydrazyl) assay confirmed significant differences in the antioxidant activity as shown in Table 4. For Malaysian plant species, radical scavenging activity ranged from 38.63±2.05 to 84.92±3.49% in the following descending order; methanolic > aqueous > hexane extract > chloroform ($p < 0.05$). In contrast, in the Iraqi species, it spanned 30.76±3.29 to 80.8±1.81% in the descending order of

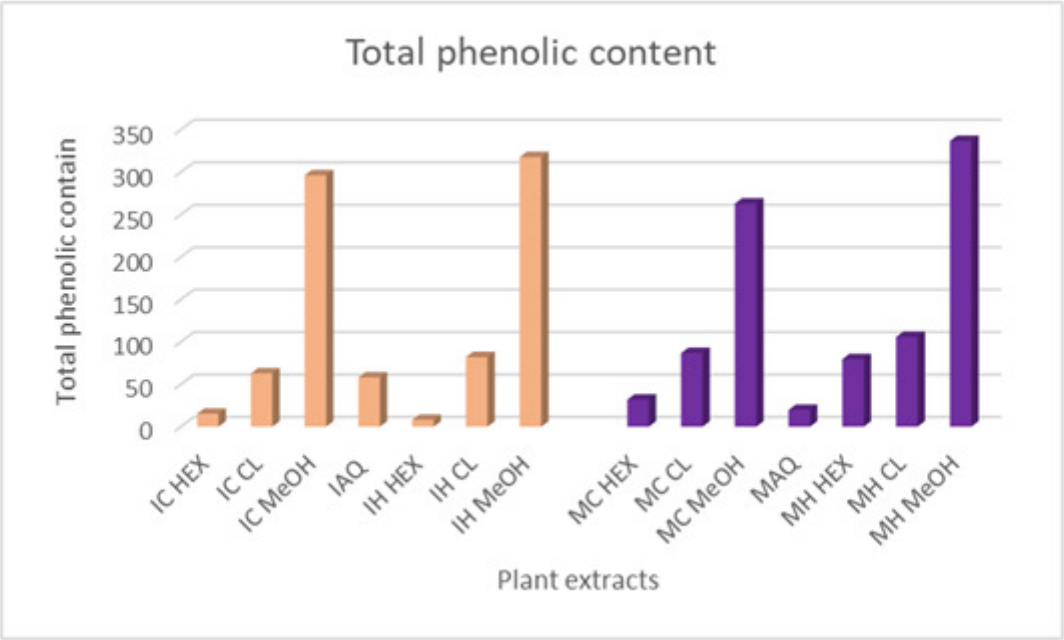

Fig. 1 : Total phenolic content (Abs.765nm), IC HEX: Iraqi species cold hexane extract, IC CL Iraqi species cold chloroform extract IC MeOH, Iraqi species cold methanolic extract, IH HEX: Iraqi species hot hexane extract, IH CL Iraqi species hot chloroform extract, IH MeOH: Iraqi hot methanolic extract, I AQ: Iraqi species aqueous extract, MC HEX: Malaysian species cold hexane extract, MC CL: Malaysian species cold chloroform extract, MC MeOH: Malaysian species cold methanolic extract, MH HEX: Malaysian species hot hexane extract, MH CL: Malaysian species hot chloroform extract, MH MeOH: Malaysian species hot methanolic extract, M AQ: Malaysian species aqueous extract.

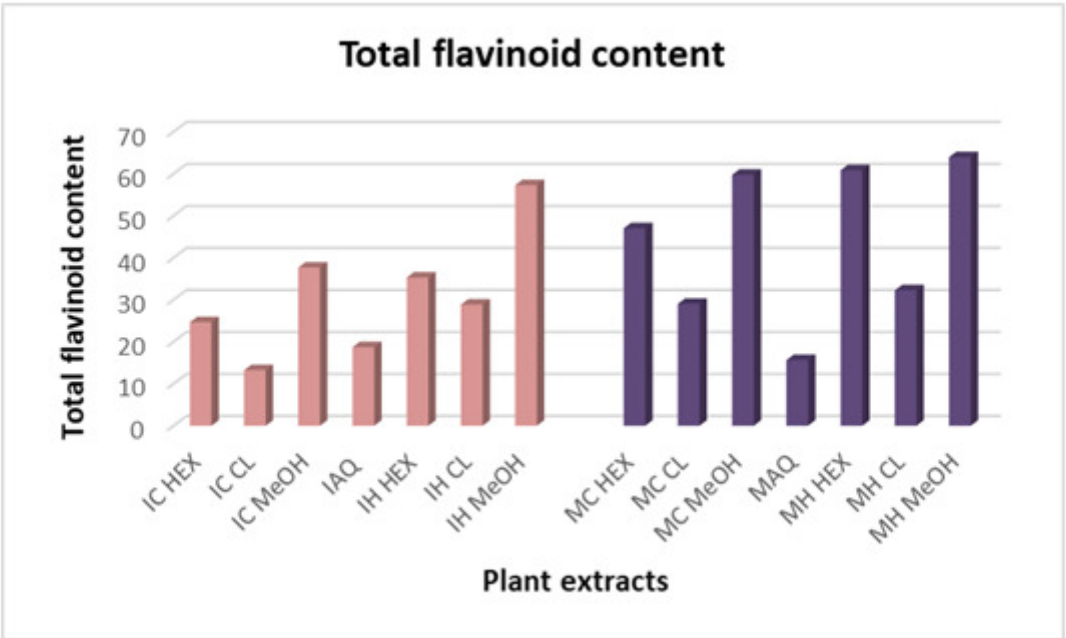

Fig. 2 : Total Flavonoid content (Abs.510 nm), IC HEX: Iraqi species cold hexane extract, IC CL Iraqi species cold chloroform extract IC MeOH, Iraqi species cold methanolic extract, IH HEX: Iraqi species hot hexane extract, IH CL Iraqi species hot chloroform extract, IH MeOH: Iraqi hot methanolic extract, I AQ: Iraqi species aqueous extract, MC HEX: Malaysian species cold hexane extract, MC CL: Malaysian species cold chloroform extract, MC MeOH: Malaysian species cold methanolic extract, MH HEX: Malaysian species hot hexane extract, MH CL: Malaysian species hot chloroform extract, MH MeOH: Malaysian species hot methanolic extract, M AQ: Malaysian species aqueous extract.

methanolic > aqueous > hexane > chloroform extracts ($p < 0.05$). As shown in Fig. 3, the highest levels of antioxidant activity were found in the hot Malaysian methanolic extract at 84.92±3.49% when compared to the ascorbic acid standard of 85.06±3.15% at the same concentration.

The IC50 value was designed to determine the concentration of the sample required to inhibit 50% of

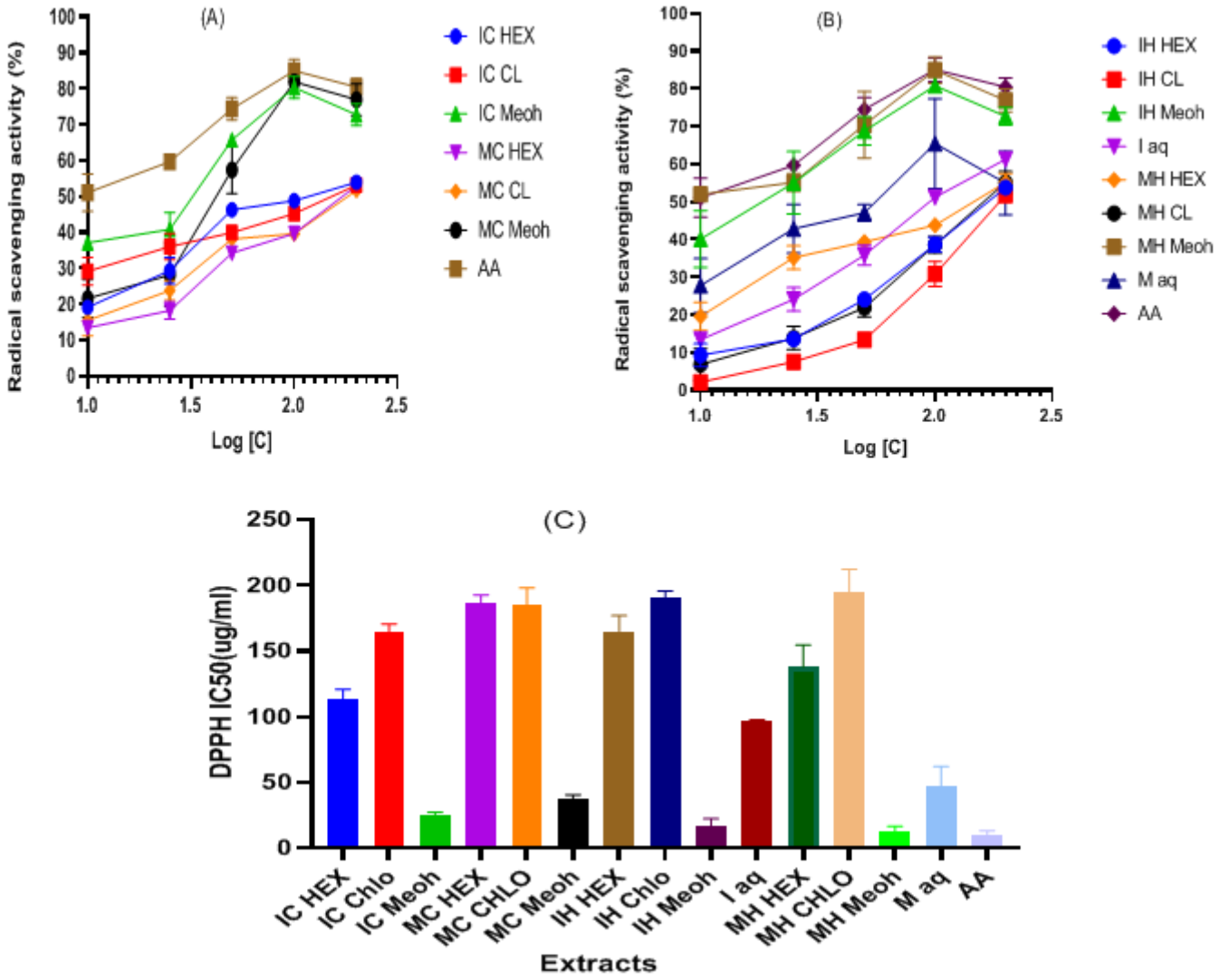

Fig. 3 : The radical scavenging activity (percentage of inhibition) of the two species solvent extracts compared to ascorbic acid. The percentage of inhibitions of extracts by cold extraction method; B, percentage of inhibition of extracts by hot extraction method. C; DPPH IC50 of extracts in ug/ml. IC HEX: Iraqi species cold hexane extract, IC CL Iraqi species cold chloroform extract IC MeOH, Iraqi species cold methanolic extract, IH HEX: Iraqi species hot hexane extract, IH CL Iraqi species hot chloroform extract, IH MeOH: Iraqi hot methanolic extract, I AQ: Iraqi species aqueous extract, MC HEX: Malaysian species cold hexane extract, MC CL: Malaysian species cold chloroform extract, MC MeOH: Malaysian species cold methanolic extract, MH HEX: Malaysian species hot hexane extract, MH CL: Malaysian species hot chloroform extract, MH MeOH: Malaysian species hot methanolic extract, M AQ: Malaysian species aqueous extract.

radicals. The higher the IC50 value, the lower the antioxidant activity of samples (Jadid *et al*, 2017). The observed IC50 value indicated that methanolic extracts in both plants exhibited the highest antioxidant activity followed by the aqueous, hexane and chloroform extracts (Table 2).

According to Ibrahim and Asri (2020), extracts that have IC50 values ranging from 10-50 µg/mL are considered to demonstrate strong antioxidant activity. Meanwhile, extracts with an IC50 range from 50 to 100 µg/ mL are regarded as exhibiting intermediate antioxidant activity. The extracts with an IC50 value exceeding 100 µg/mL are considered to show weak antioxidant activity. Thus, the methanolic extracts of both plants obtained by hot and cold extraction methods exhibited strong antioxidant activity. Meanwhile, the chloroform and hexane extracts of both plants obtained by hot and cold extraction methods demonstrated weak antioxidant activity, and the aqueous extracts exhibited such activity at an intermediate level.

Anti-inflammatory activity

The *in vitro* anti-inflammatory activity of the plant extracts was studied to establish their ability to inhibit protein denaturation. As shown in Table 4, of all the extracts, the maximum inhibition of 88.81±6.49% was observed in the Malaysian methanolic hot variety.

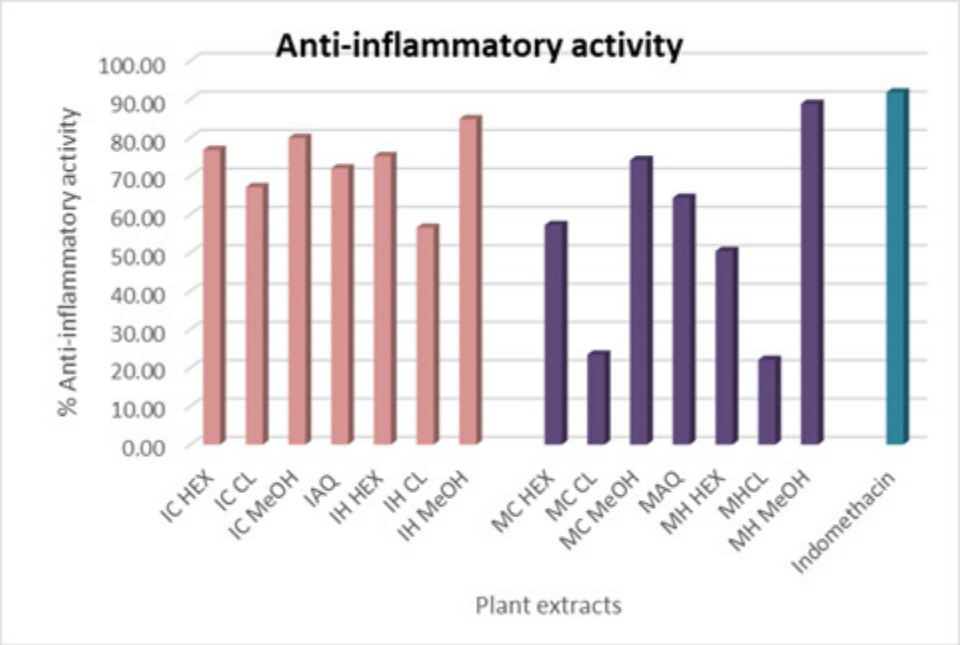

Fig. 4 : Anti-inflammatory Activity (Abs.517 nm), IC HEX: Iraqi species cold hexane extract, IC CL Iraqi species cold chloroform extract IC MeOH, Iraqi species cold methanolic extract, IH HEX: Iraqi species hot hexane extract, IH CL Iraqi species hot chloroform extract, IH MeOH: Iraqi hot methanolic extract, I AQ: Iraqi species aqueous extract, MC HEX: Malaysian species cold hexane extract, MC CL: Malaysian species cold chloroform extract, MC MeOH: Malaysian species cold methanolic extract, MH HEX: Malaysian species hot hexane extract, MH CL: Malaysian species hot chloroform extract, MH MeOH: Malaysian species hot methanolic extract, M AQ: Malaysian species aqueous extract.

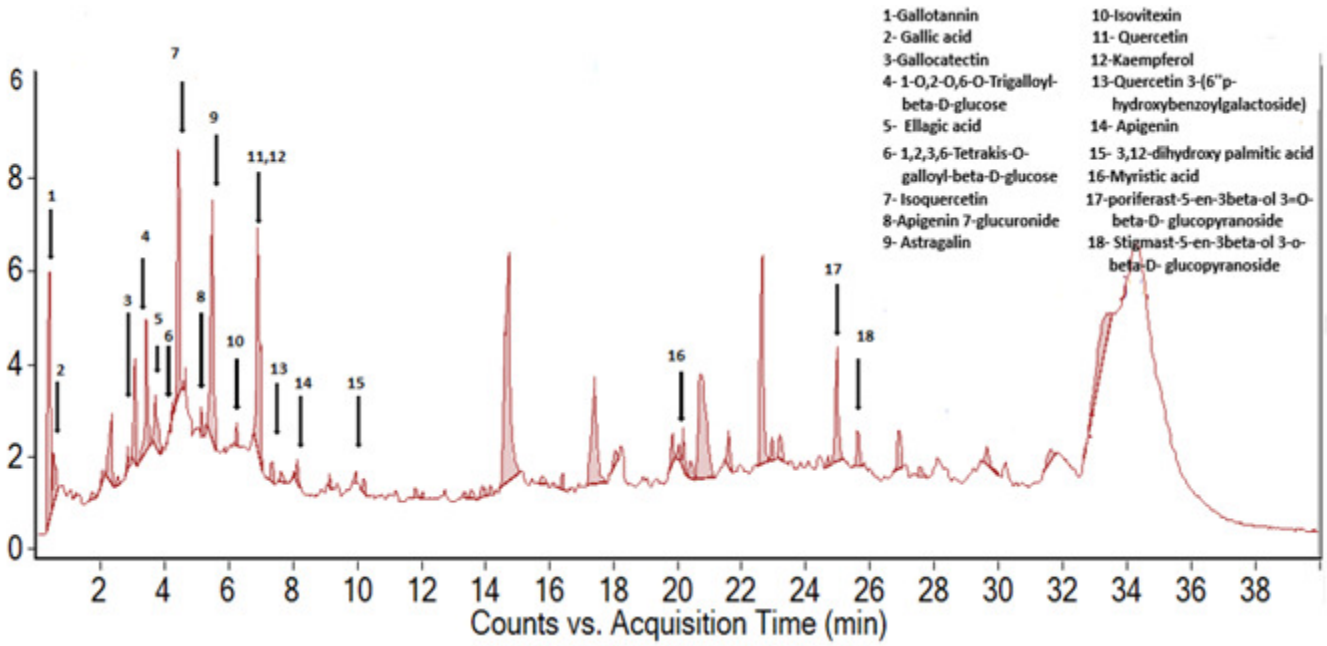

Fig. 5 : LC-MS/MS chromatogram of *E. hirta* Malaysian species, where peak labelling characterizes the compounds identified and listed in (Table 5).

Indomethacin, a standard anti-inflammatory drug, demonstrated the maximum inhibition, 91.77±2.85%, at the same concentration. As shown in Fig. 3, for the Malaysian species of the plant its anti-inflammatory activity, in terms of inhibition of protein denaturation, is presented in the following descending order of extracts ($p < 0.05$); methanolic > aqueous > hexane > chloroform. In the case of the Iraqi species, the anti-inflammatory activity is shown in the following descending order of extracts ($p < 0.05$); methanolic > hexane > aqueous > chloroform.

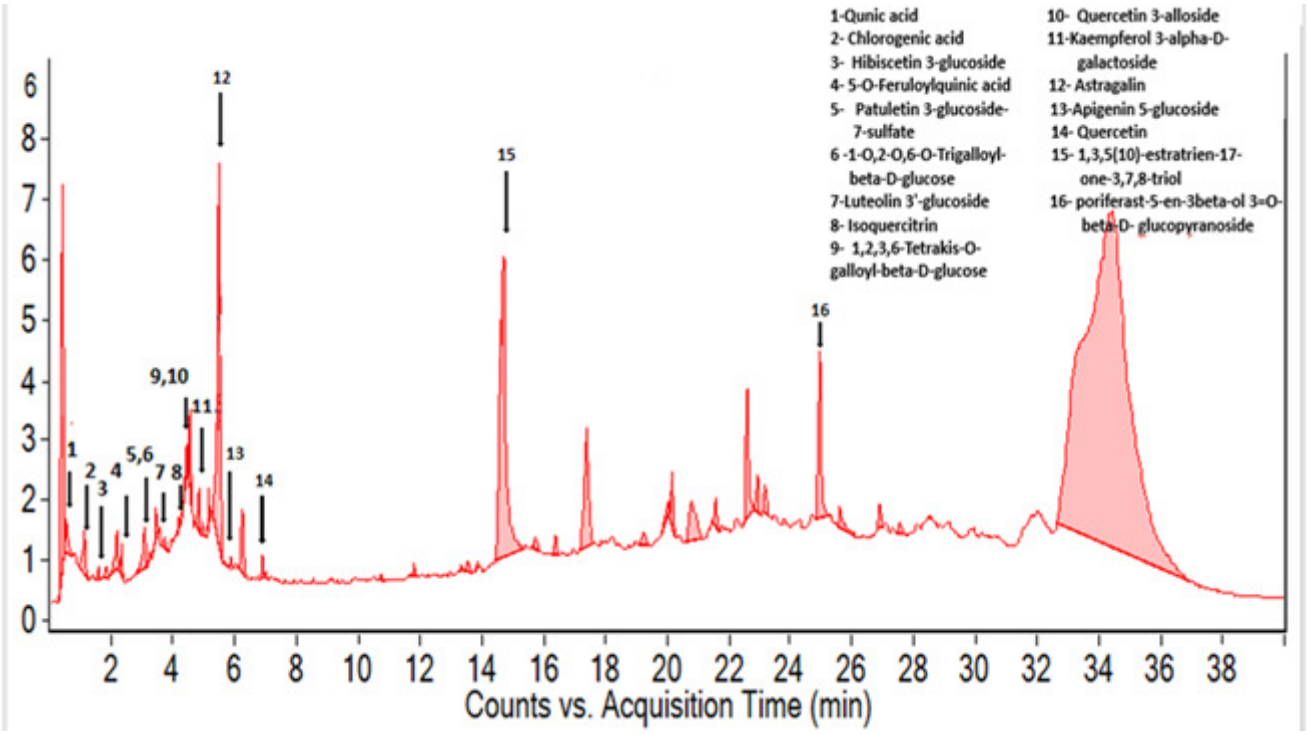

Fig. 6 : LC-MS/MS chromatogram of *E. hirta* Iraqi species, where peak labelling characterizes the compounds identified and listed in (Table 6).

Table 5 : Tentative identification of chemical constituents in the Malaysian species *E. hirta* methanolic extract of by (LC-MS/MS) analysis.

No	Compound	RT (min)	Mass	Molecular formula	Precursor type	Precursor m/z
1	Gallotannin	0.3587	636.1004	C27 H24 O18	[M – H]–	635.0931
2	Gallic acid	0.5836	170.0236	C7 H6 O5	[M – H]–	169.0164
3	(+)-Gallocatechin	3.0265	306.0782	C15 H14 O7	[M – H]–	305.0712
4	1-O,2-O,6-O-Trigalloyl-beta-D-glucose	3.7817	636.0974	C27 H24 O18	[M – H]–	635.091
5	Ellagic acid	3.9079	302.0071	C14 H6 O8	[M – H]–	301.0004
6	1,2,3,6-Tetrakis-O-galloyl-beta-D-glucose	4.4105	788.1082	C34 H28 O22	[M – H]–	787.1018
7	Quercetin3'-glucoside (isoquercetin)	4.6241	464.0957	C21 H20 O12	[M – H]–	463.0883
8	Apigenin 7-glucuronide	5.0445	446.087	C21 H18 O11	(M+HCOO)-	491.0854
9	Astragalin	5.4669	448.1008	C21 H20 O11	[M – H]–	447.0934
10	Isovitexin	6.235	432.1066	C21 H20 O10	[M – H]–	431.0994
11	Quercetin	6.872	302.0431	C15 H10 O7	[M – H]–	301.0357
12	Kaempferol	6.9649	286.0486	C15 H10 O6	[M – H]–	285.0413
13	Quercetin 3-(6''-p-hydroxybenzoylgalactoside)	7.632	584.118	C28 H24 O14	[M – H]–	583.1108
14	Apigenin	7.9956	270.0546	C15 H10 O5	[M – H]–	269.0473
15	3,12-dihydroxy palmitic acid	10.1871	288.231	C16 H32 O4	[M – H]–	287.2238
16	Myristic acid	20.0245	228.2102	C14 H28 O2	[M – H]–	227.2029
17	poriferast-5-en-3beta-ol 3=O-beta-D-glucopyranoside	24.6671	576.4401	C35 H60 O6	(M+HCOO)-	621.4383
18	stigmast-5-en-3beta-ol 3-0-beta-D-glucopyranoside	25.6099	576.4415	C35 H60 O6	(M+CH_3COO)-	635.4555

Liquid Chromatography Mass Spectrometry (LC-MS/MS) analysis

The *E. hirta* optimized methanolic extracts of both species were subjected to LC-MS/MS analysis in order to achieve a qualitative description of their chemical constituents. Although, the Malaysian species of *E. hirta*, LC-MS/MS, had been detected previously, to the best of the authors' knowledge, this constitutes the first occasion on which active compounds in the *E. hirta* methanolic extract of Iraqi species were detected by means of LC-MS/MS analysis. Moreover, this represents the first comparison between the two species. Compounds were characterized by their retention times, forms of detected

Table 6 : Tentative identification of chemical constituents in the Iraqi species *E. hirta* methanolic extract of by (LC-MS/MS) analysis.

No	Compound	RT (min)	Mass	Molecular formula	Precursor type	Precursor m/z
1	Quinic acid	0.6923	192.0641	C7 H12 O6	[M – H]–	635.0931
2	Chlorogenic Acid	0.9616	354.0967	C16 H18 O9	[M – H]–	169.0164
3	Hibiscetin 3-glucoside	1.5811	496.0863	C21 H20 O14	[M – H]–	305.0712
4	5-O-Feruloylquinic acid	2.3372	368.1119	C17 H20 O9	[M – H]–	367.1047
5	Patuletin 3-glucoside-7-sulfate	3.0358	574.0622	C22 H22 O16 S	(M+CH3COO)-	633.075
6	1-O,2-O,6-O-Trigalloyl-beta-D-glucose	3.3413	636.097	C27 H24 O18	[M – H]–	635.0898
7	Luteolin 3'-glucoside	3.9716	448.1026	C21 H20 O11	[M – H]–	301.0004
8	Isoquercitrin	4.2812	464.0965	C21 H20 O12	[M – H]–	463.0883
9	1,2,3,6-Tetrakis-O-galloyl-beta-D-glucose	4.4458	788.1084	C34 H28 O22	[M – H]–	787.1013
10	Quercetin 3-alloside	4.6167	464.0965	C21 H20 O12	[M – H]–	463.0891
11	Kaempferol 3-alpha-D-galactoside	5.2512	448.1017	C21 H20 O11	[M – H]–	447.0944
12	Astragalin	5.4861	448.1017	C21 H20 O11	[M – H]–	431.0994
13	Apigenin 5-glucoside	5.7275	432.1075	C21 H20 O10	[M – H]–	431.1002
14	Quercetin	6.8962	302.0439	C15 H10 O7	[M – H]–	301.0357
15	1,3,5(10)-estratrien-17-one 3,7,8-triol	14.6633	300.1725	C19 H24 O3	[M – H]–	299.1652
16	poriferast-5-en-3beta-ol 3=O-beta-D-glucopyranoside	24.6824	576.4402	C35 H60 O6	(M+HCOO)-	621.4384

ions, mass spectrums and fragmentation profiles. The results derived from the LC-MS/MS analysis of the Malaysian species confirm the presence of 18 compounds of steroids, phenolics, flavonoids and their glucosides compounds (Gallotannin, Gallic acid, (+)-Gallocatechin, 1-O,2-O,6-O-Trigalloyl-beta-D-glucose, Ellagic acid, 1,2,3,6-Tetrakis-O-galloyl-beta-D-glucose, Quercetin3'-glucoside (isoquercetin), Apigenin 7-glucuronide, Astragalin, Isovitexin, Quercetin, Kaempferol, Quercetin 3-(6''-p-hydroxybenzoylgalactoside), Apigenin, 3,12-dihydroxy palmitic acid, Myristic acid, poriferast-5-en-3beta-ol 3=O-beta-D-glucopyranoside and stigmast-5-en-3beta-ol 3-0-beta-D-glucopyranoside) as shown in Table 5 and Fig. 5. The Iraqi species indicated the presence of 16 important steroids, phenolics, flavonoids and their glucosides compounds (Quinic acid, Chlorogenic Acid, Hibiscetin 3-glucoside, 5-O-Feruloylquinic acid, Patuletin 3-glucoside-7-sulfate, 1-O,2-O,6-O-Trigalloyl-beta-D-glucose, Luteolin 3'-glucoside, Isoquercitrin, 1,2,3,6-Tetrakis-O-galloyl-beta-D-glucose, Quercetin 3-alloside, Kaempferol 3-alpha-D-galactoside, Astragalin, Apigenin 5-glucoside, Quercetin, 1,3,5(10)-estratrien-17-one 3,7,8-triol and poriferast-5-en-3beta-ol 3=O-beta-D-glucopyranoside) as displayed in Table 6 and Fig. 6.

DISCUSSION

The phytochemical constituents of plants have significant potential application in the domain of human health care. Each secondary metabolite present in *E. hirta* areal part extract justifies the traditional uses of the plant in treating various health complications. It is of considerable importance to know that the production and distribution of bioactive constituents in the plant may vary according to seasonal changes, environmental factors, time of harvest and stage of plant development. Other factors that affect the capture of active phytochemicals include; extraction methods, the types of solvents used, extraction conditions, and length of extraction period.

By comparing the two different sampling locations, the concentration of phenol and flavonoid content decreased in the following order: Malaysian species > Iraqi species. This variation in the quantity of phenolic compounds and flavonoids in E. hirta could be related to the differences in soil nutrition, climatic conditions, type, and elevation above sea level, as shown in Table 1. Numerous previous studies have shown that phytochemical composition, the total phenolic and flavonoid content of plants, is significantly influenced by a variety of environmental factors including; the climate, geography, seasonal changes, soil type, and exposure to sunlight, among others (Ganskopp and Bohnert, 2003; Khan *et al*, 2006).

The contrasting total phenolic and flavonoid content, which is higher in hot extraction methods than cold ones and for all the extracts of the two species, could be attributable to the fact that some phenolic elements, such as tannins, are naturally more soluble in hot conditions than cold ones (Laurent, 1975 and Hmidani *et al*, 2019). According to this finding, hot extraction by means of Soxhlet apparatus appears to constitute the most effective extraction method for obtaining the highest quality phenolic and flavonoids content from this plant.

Antioxidants, through their free radical scavenging

qualities, have the potential to prevent and manage various human diseases such as cancer, diabetes, cataracts, neurodegenerative disorders, cardiovascular conditions and liver disease (Alsaffar *et al*, 2017). In this study, the results of DDPH assay showed minor contrasts in the antioxidant activity between the two species and significant differences between various types of solvent extracts. Several previous studies have indicated that phenol, which is the main antioxidant component, and the total contents were directly proportional to their antioxidant activity. Another sizeable body of research has established that the quality and quantity of the phenolic and flavonoid compounds are strongly correlated with the antioxidant activities of plants (Jing *et al*, 2015).

Several researchers have stated that *E. hirta* extract produces various pharmacological effects one of them being that of an anti-inflammatory agent. The *in vitro* egg albumin method provides an inexpensive alternative method of assaying the anti-inflammatory activity of herbal medicine using a denaturation technique. Inflammatory activity can be impeded through inhibition of protein denaturation, a method which could be confirmed by further studies (Sarveswaran *et al*, 2017). Numerous recent studies have shown that the anti-inflammatory activity of many plants contributes significantly to the production of polyphenols and flavonoids (Okoli and Akah, 2004). Hence, in this study, the presence of phenol and flavonoids in the methanol extract of *E. hirta* in the two species may have contributed to its anti-inflammatory activity. In the LC-MS/MS chromatogram of the Malaysian E. hirta species the major two compounds were kaempferol-3-O-glucoside (astragalin) and Quercetin3'-glucoside (isoquercetin) with respective retention times of 5.4669 minute and 3.9079 minutes. This was also reported in a previous study of E. hirta methanolis extract of the Malaysian species (Abu Bakar *et al*, 2020). In the case of Iraqi E. hirta methanolic extract, the kaempferol-3-O-glucoside (astragalin) with (RT = 5.4861 minutes) and 1,3,5(10)-estratrien-17-one 3,7,8-triol (RT=14.6633 minutes) were the major compounds obtained This result suggests that the antioxidant and anti-inflammatory potency of *E. hirta* methanolic extracts in the two species results mainly from the contribution of phenolic compounds, tannins and flavonoids (Abu Bakar *et al*, 2020).

CONCLUSION

This study demonstrated that the levels of secondary metabolites in *E. hirta* plants differ in the Malaysian and Iraqi species. The former contain higher levels of phenolic and flavonoid compounds than the latter. It can, therefore, be concluded that agro-climatic locations, in combination with geography have significant effects on the *E. hirta* plant phytoconstituents and their antioxidant potential. In addition, the present study clearly demonstrated that the solvent type and extraction methods have a fundamental effect on the level of antioxidant compounds in the *E. hirta* areal parts. Finally, it can be concluded that *E. hirta* has demonstrated the existence of strong correlations between antioxidant and anti-inflammatory activities and their phenolic and flavonoid contents. Further investigation of the secondary metabolites by LC-MSMS analysis of *E. hirta* methanolic extracts revealed the presence of phenolic, flavonoid and steroid compounds. Hence, *E. hirta* could represent a natural source of polyphenol compounds, which can be used in pharmaceuticals for the treatment of a range of diseases. This research has raised numerous questions which require further investigation.

ACKNOWLEDGEMENT

We would like to thank Universiti Sains Malaysia for funding this project through the Incentive Bridging Grant (304.PFARMASI.6316549).

REFERENCES

Abu Bakar F I, Abu Bakar M F, Abdullah N, Endrini S, Fatmawati S J E-B C and Medicine A (2020) Optimization of extraction conditions of Phytochemical compounds and Anti-Gout activity of *Euphorbia hirta* L.(Ara Tanah) using Response surface Methodology and Liquid Chromatography-Mass Spectrometry (LC-MS) Analysis.

Al-Snafi P D A E (2017) Pharmacology and therapeutic potential of *Euphorbia hirta* (Syn: Euphorbia pilulifera)- A review. *IOSRPHR IOSR J. Pharmacy (IOSRPHR)* **07**, 07-20.

Alhawarri M B, Dianita R, Razak K N A, Mohamad S, Nogawa T and Wahab H A (2021) Antioxidant, anti-Inflammatory, and inhibition of Acetylcholinesterase Potentials of *Cassia timoriensis* DC. *Flowers* **26**, 2594.

Alsaffar D F, Ali K H, Alsaffar S F and Dawood A H (2017) Hepatoprotective effects of London Rocket (*Sisymbrium irio* L) Extract against CCL4 induced Hepatotoxicity in Albino rats.

Alsaffar S and Al-Kaissy K (2009) *Histological study of aqueous extract of chamomile effect On respiratory system in male albino mice ATHESIS A Partial Fulfillment of The Requirements For the Degree of Master in Biology / Histology.*

Apak R A, C'Apanoglu E and Shahidi F (2018) Measurement of antioxidant activity and capacity : recent trends and applications.

Asha S (2014) *Euphorbia hirta* Linn - A review on traditional uses, phytochemistry and pharmacology. *World J. Pharmaceutical Res.*

Brower V (2008) Back to Nature: Extinction of Medicinal Plants Threatens drug Discovery. *JNCI J. National Cancer Institute JNCI Journal of the National Cancer Institute* **100**, 838-839.

Das M, Jain V and Malhotra S J (2016) Impact of climate change on medicinal and aromatic plants. *I J A S* **86**, 1375-1382.

Dharmadeva S, Galgamuwa L, Prasadinie C and Kumarasinghe N (2018) *In vitro* anti-inflammatory activity of *Ficus racemosa* L.

bark using albumin denaturation method. *Ayu* **39**, 239-242.

Ekor M (2014) The growing use of herbal medicines: issues relating to adverse reactions and challenges in monitoring safety. *Frontiers in pharmacology* **4**.

Evans W C, Evans D and Trease G E (2009) *Trease and Evans pharmacognosy*. Edinburgh; New York, Saunders/Elsevier.

Ganskopp D and Bohnert D (2003) Mineral Concentration Dynamics among 7 Northern Great Basin Grasses. *J. Range Manage.* **56**, 174-184.

Ghosh P, Chatterjee S, Das P, Banerjee A, Karmakar S and Mahapatra S (2019) Natural habitat, phytochemistry and pharmacological properties of a medicinal weed- *Cleome rutidosperma* DC. (Cleomaceae): A comprehensive review. *Int. J. Pharm. Sci. Res.* **10**, 1605-1612.

Hmidani A, Bouhlali E D T, Khouya T, Ramchoun M, Filali-Zegzouti Y, Benlyas M and Alem C (2019) Effect of extraction methods on antioxidant and anticoagulant activities of *Thymus atlanticus* aerial part. *Scientific African* **5**, e00143.

Ibrahim M A and Asri N A A (2020) The Study of Antioxidant activities of *Piper sarmentosum* and *Piper nigrum*. *M J T A* **1**, 1-3.

Jadid N, Hidayati D, Hartanti S R, Arraniry B A, Rachman R Y and Wikanta W (2017) Antioxidant activities of different solvent extracts of *Piper retrofractum* Vahl. using DPPH assay. *AIP Conference Proceedings*, 2017. AIP Publishing LLC, 020019.

Jing L, Ma H, Fan P, Gao R and Jia Z (2015) Antioxidant potential, total phenolic and total flavonoid contents of Rhododendron anthopogonoides and its protective effect on hypoxia-induced injury in PC12 cells. *BMC Complementary and Alternative Medicine* **15**, 287.

Khan Z, Ashra M, Ehsan A and Valeem E E (2006) Forage mineral status evaluation: The influence of pastures. *Pak. J. Bot.* **38**, 1043-1054.

Larit F, Leon F, Benyahia S and Cutler S J (2019) Total phenolic and Flavonoid content and Biological activities of extracts and isolated compounds of *Cytisus villosus* Pourr. *Biomolecules* **9**, 732.

Laurent S (1975) Eìtude comparative de diffeìrentes meìthodes d'extraction et de dosage des tannins chez quelques pteìridophytes. *Archives Internationales de Physiologie et de Biochimie* **83**, 735-752.

Linfang H, Shilin C and Meihua Y (2012) *Euphorbia hirta* (Feiyangcao): A review on its ethnopharmacology, phytochemistry and pharmacology. *J. Medicinal Plants Res*.

Mizushima Y K M (1968) Interaction of anti-inflammatory drugs with serum proteins, especially with some biologically active proteins. *The J. Pharmacy and pharmacology* **20**, 169-73.

Nafiu M O, Hamid A A, Muritala H F and Adeyemi S B (2017) Chapter 7 - Preparation, Standardization and Quality Control of Medicinal plants in Africa. In: KUETE V (ed.). *Medicinal Spices and Vegetables from Africa.* Academic Press.

Okoli C and Akah P (2004) Mechanisms of the anti-inflammatory activity of the leaf extracts of *Culcasia scandens* P. Beauv (Araceae). *Pharmacology, biochemistry, and behavior* **79**, 473-81.

Pande H, Kumar B, Varshney V J W and Valorization B (2017) Phenolic composition and antioxidant capacity of biomass residue (leaves) generated from *Bambusa tulda* plantations **8**, 2349-2362.

Pang B, Zhu Y, Lu L, Gu F, Chen H J E-B C and Medicine A (2016) The applications and features of liquid chromatography-mass spectrometry in the analysis of traditional chinese medicine. 2016.

Pranabesh Ghosh C G, Shaktijit Das, Chandrima Das, Suprodip Mandal, Sirshendu Chatterjee (2019) Botanical description, Phytochemical Constituents and Pharmacological properties of *Euphorbia hirta* Linn: A review. *Int. J. Health Sci. Res.* **9**, 273. ISSN: 2249-9571.

Rajeh M A B, Zuraini Z, Sasidharan S, Latha L Y and Amutha S J M (2010) Assessment of *Euphorbia hirta* L. leaf, flower, stem and root extracts for their antibacterial and antifungal activity and brine shrimp lethality **15**, 6008-6018.

Roopali M, Sankeshwari A V A, Kishore Bhat and Kirankumar Hullatti (2018) Soxhlet versus cold maceration: Which method gives better antimicrobial activity to licorice extract against *Streptococcus mutans*? *J. Scientific Society* **45**, 67-71.

Sarveshwaran R, Jayasuriya W J A B and TS S (2017) *In vitro* assays to investigate the anti-inflammatory activity of herbal extracts: A review. *World J. Pharmaceutical Res.* **6**, 131-141.

Saw S-H and Institute of Souteast Asian S (2015) *The population of Malaysia*.

Schwartz J, Padmanabhan A, Aqui N, Balogun R A, Connelly-Smith L, Delaney M, Dunbar N M, Witt V, Wu Y and Shaz B H (2016) Guidelines on the use of Therapeutic Apheresis in clinical Practice-Evidence-based Approach from the Writing Committee of the American Society for Apheresis: The Seventh Special Issue. *JCA J. Clin. Apheresis* **31**, 149-338.

Singleton V L, Orthofer R and Lamuela-Raventos R M (1998) Section III. Polyphenols and Flavonoids - 14 - Analysis of Total Phenols and Other Oxidation Substrates and Antioxidants by Means of Folin-Ciocalteu Reagent. *Methods in Enzymology* **152**.

Sinhababu A and Banerjee A (2016) Ethno-medicinal Plants of the Family Euphorbiaceae in Southern Bankura of West Bengal. *Research & Review: Journal of Botany* **5**, 36-39.

Uppu R M, Murthy S N, Pryor W A And Parinandi N L (2010) *Free radicals and antioxidant protocols*, Springer.

Vaghasiya Y, Dave R and Chanda S (2011) Phytochemical analysis of some medicinal plants from western region of India. *Res. J. Med. Plant* **5**, 567-576.

Yener Ý, Ertaþ A, Yilmaz M A, Tokul Ölmez Ö, Köseoðlu Yýlmaz P, Yeþil Y, Kaplaner E, Öztürk M, Temel H, Kolak U and Topçu G (2019) Characterization of the Chemical Profile of *Euphorbia* species from Turkey by Gas Chromatography–Mass Spectrometry (GC-MS), Liquid Chromatography–Tandem Mass Spectrometry (LC-MS/MS) and Liquid Chromatography–Ion Trap–Time-of-Flight–Mass Spectrometry (LC-IT-TOF-MS) and Chemometric Analysis. *Analytical Letters* **52**, 1031-1049.