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Bioassay-Guided Fractionation Of Antioxidants From Crataegus -Monogyna (Common Hawthorn) And Its Role In Treating Cardiovascular Disease Using H9c2 Rat Myoblast Cardiac Cell Line

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Abstract

Crataegus monogyna plants are well known for their medicinal property. Extraction and identification of compounds rich in antioxidant present in C. monogyna is the main focus of this research paper. Firstly, solid-liquid extraction of different plant parts (leaves, flowers and fruits) of C. monogyna using different solvents (aqueous, ethanol and methanol) was conducted to identify the suitable plant part and solvent type that contributed to the maximum yield of total phenolic content (TPC) and antioxidant activity (2,2-diphenyl-1-picrylhydrazyl (DPPH) and 2,2'-azino-bis (3-ethylbenzothiazoline-6-sulfonic acid) (ABTS)). Upon extraction, liquid-liquid partition using solvent with different polarities (water, n-hexane and chloroform) was conducted prior to fractionation of antioxidant compounds. Bioassay-guided fractionation of antioxidant compounds from the most antioxidant-rich plant part of C. monogyna was conducted using chromatographic methods and the antioxidant compounds were identified via Fourier transform infrared spectroscopy (FTIR) and gas chromatography-tandem mass spectrometry (GC-MS/MS) analysis. Lastly, the cardiac activity of the most antioxidant-rich fraction was investigated using the H9c2 rat myoblast cardiac cell line. As results, solid-liquid extraction of different plant parts of C. monogyna using different solvents revealed that the fruit of C. monogyna extracted with ethanol had the highest TPC (0.070 mg GAE/g), antioxidant activity (DPPH 90.35% and ABTS 71.37%) and crude extract yield (0.49±0.05g). Bioassay-guided fractionation of this extract afforded twelve fractions (F1 to F12) and F9 presented the highest antioxidant values (DPPH 46.14% and ABTS 45.06%) and TPC (28±0.002 mg GAE/g). GC-MS/MS profiling of F9 revealed eleven antioxidant compounds and five major compounds as 2-(3,4 dihydroxy phenyl)-3,5,7-trihydroxychromen-4-one (19.46%), 1H-1,2,3,4-Tetrazol-5-amine,1-ethyl-N-[(1-methyl-1H-pyrrol-2-yl)methyl]-) (11.24%), benzenesulfonamide, N-[[5-(aminomethyl)-2-furanyl]methyl]- (8.21%), 4-Nonylphenol (8.01%) and 1,3,2-Dioxathiolane-4-methanol, 2-oxide (7.49%). The findings of current research conclude that the antioxidant compounds present in the most antioxidant-rich plant part of C. monogyna (fruit) are polar in nature and are majority phenols. Fraction F9 shows an effective cell viability percentage (99.5%) against H9c2 cells on a dose-dependent manner, suggesting that C. monogyna ethanol fruit extract possesses therapeutic potential against rat myoblast cells. In conclusion, C. monogyna fruit is an excellent alternative of natural antioxidant.

Keywords— C. monogyna; antioxidant activity; profiling; separation; identification; H9c2 cell line; cardiovascular activity.

INTRODUCTION

In the past two decades, there is a drastic boom in research and usage of natural products such as medicinal plants and herbs for human ailments (Nabavi *et al.*, 2012, 2013; S. F. Nabavi, Habtemariam, *et al.*, 2015; S. F. Nabavi, Russo, *et al.*, 2015; S. M. Nabavi *et al.*, 2015). One such herb with high nutraceutical potential is *Crataegus monogyna*, belonging to the family *R*osaceae, an endemic herb grown in Europe, Asia and Africa (Chang *et al.*, 2002). *C. monogyna* is also known as common hawthorn, an edible plant which possesses various biological and medicinal properties (Prinz *et al.*, 2007). In *C. monogyna*, leaves, flowers and fruits are habitually utilised for treating heart alignments (Konyalioglu, Cebe and Aktar, 2017). *C. monogyna* is also used to treat digestive ailments, kidney stones and dyspnea. In the late 1800s, Europe first recorded hawthorn for the treatment of various cardiac disorders (Zhang *et al.*, 2001; Rigelsky and Sweet, 2002). Currently, country such as Malaysia uses hawthorn berry incorporated drink for cardiac health.

C. monogyna plant parts are loaded with antioxidant compounds. Phenols, flavonoids and phenolic acids had been identified and isolated from different parts of *C. monogyna* typically using polar solvents (Miliauskas *et al.*, 2005; Cai *et al.*, 2006; Bernatoniene *et al.*, 2008; Martino *et al.*, 2008; Shortle *et al.*, 2013; Simirgiotis, 2013). Recent research in *C. monogyna* leaf extract also assessed the chemical composition, antioxidant, and antibacterial activity (Belabdelli *et al.*, 2021). However, there is no study to date which focuses on extracting the suitable plant part of *C. monogyna* that contributes to the highest antioxidant activity and subsequently identifying the dominant antioxidant compounds that are responsible for this highest antioxidant activity via bioassay-guided fractionation of *C. monogyna*.

Recently, ultrasound-assisted extraction (UAE) is recognised as a "Green Extraction Technique" (Ibáñez and Cifuentes, 2020). The advantage of using UAE is to enhance the yield of bioactive compounds such as anthocyanins, polyphenolics, polysaccharides aromatic compounds and volatile compounds from plant and animal materials (Martínez-Patiño *et al.*, 2019; Santos *et al.*, 2019). The higher yield obtained is mainly due to the effect of cavitation. The cavitation phenomena enhance the extraction yield by an intense explosion of bubbles by increasing the flow of solvent between the plant material (Vilkhu *et al.*, 2008). The main advantage of cavitation-based extraction is to reduce the extraction time, to reduce the degradation of bioactive compounds and to achieve a good extraction yield with high purity (Panda and Manickam, 2019). Thus, UAE is used for the extraction of *C. monogyna* due to its efficacy in the extraction of bioactive compounds (Chemat *et al.*, 2017).

After the extraction process, isolation is carried out from the obtained crude extract using chromatographic techniques (Pradeep Kumar and Sachin, 2013). The selection of an appropriate isolation technique is based on the property and volatility of the compounds to be isolated (Devika, 2017). Column chromatography techniques are widely used for the isolation of organic as well as macromolecules. Among which, silica gel chromatographic column is commonly used due to its high adsorption ability. However, to avoid co-elution of two or more chemical compounds, a wise selection of chromatographic absorbent column based on the solvent system which is to be identified is important (Zhao *et al.*, 2018).

Therefore, this study aimed to extract and identify the compounds present in the most antioxidant-rich plant part of *C. monogyna* using UAE extraction followed by bioassay-guided fractionation. The plant parts (flowers, fruits and leaves) of the *C. monogyna* plant were examined to identify the most antioxidant-rich plant part through TPC and antioxidant activity (DPPH and ABTS). Next, the bioassay-guided fractionation was conducted on the selected plant part with a combination of chromatographic techniques to

determine the fraction with the highest antioxidant activity. The profiling of the compounds present in the most antioxidant-rich fraction was conducted via FTIR and GC-MS/MS analysis.

Different plant parts of *C. monogyna* were reported to possess therapeutic effects in treating various illnesses especially cardiovascular diseases (CVD) (Chang *et al.*, 2002; Rigelsky and Sweet, 2002), bioactive compounds like chlorogenic acid, rutin, catechin, epicatechin, quercetin, hyperoside, vitexin, oleanolic acid and oligomeric procyanidins were reported to have cardiac action (Bahorun *et al.*, 2003; Urbonavičiute *et al.*, 2006; Salehi *et al.*, 2009; Attard and Attard, 2019). Therefore, the last phase of the study was to investigate the cardiovascular activity of *C. monogyna* extract. The antioxidant-rich fraction obtained through the bioassay-guided fractionation of the identified plant part of *C. monogyna* was tested for its role in treating CVD using the H9c2 rat myoblast cardiac cell line via 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) assay. This is the first study to extract and identify the compounds present in the most antioxidant-rich *C. monogyna* plant fraction through bioassay-guided fractionation and subsequently to enhance the cardiovascular activity of *C. monogyna* to ascertain its role in treating cardiovascular diseases.

Materials and Methods

Chemicals

Methanol, ethanol, n-hexane, chloroform, formic acid, gallic acid, acetic acid and ethyl acetate of HPLC grade, DPPH and ABTS of analytical grade and other chemicals, including sodium hydroxide solution, Folin-Ciocalteu (FC) reagent, potassium persulphate and potassium bromide were acquired from Sigma-Aldrich (Sigma, St. Louis, MO, USA and Sigma Aldrich Chemicals Pvt. Ltd - Research and Biotech Production, Merck India). For cell line, H9c2 - Rat heart myoblast cell line (NCCS, Pune), cell culture medium: DMEM- High Glucose - (#AL111, Himedia), fetal bovine serum (#RM10432, Himedia), MTT Reagent (5 mg/ml) (# 4060 Himedia), DMSO (#PHR1309, Sigma), Doxorubicin (#D1515, Sigma) and D-PBS (#TL1006, Himedia).

Plant materials

Plants of *C. monogyna* (common hawthorn) were procured from Berries and Exotic Farm Enterprise, Perak, Malaysia. The leaves, flowers and fruits were removed separately from the plants, rinsed and cut into small pieces. These plant parts were pre-treated (chilled at 4°C for 24 h and pre-froze at -20°C for 24 h) and freeze-dried at -100°C for 48 h. After drying, the samples were crushed into powder, sieved with 800 µm sieve mesh, and stored in airtight containers.

Ultrasound-assisted extraction for identification of C. monogyna plant part

Powdered leaf, flower and fruit samples of 1 g each were added to 30 mL of 90% ethanol in aqueous, 90% methanol in aqueous and 30 mL of distilled water as extraction solvents in 50 mL glass bottles separately. These mixtures were kept in an ultrasound water bath (P120H, Elmasonic, Germany) and heated to 50°C (Shortle *et al.*, 2014). All the extraction was performed at a frequency of 37 kHz for 30 min at a constant power of 400 W. The extracts were filtered using Whatman No. 1 filter paper and subjected to vacuum rotavapor with required temperature, (78.38°C – ethanol extract, 64.8°C - methanol extract and freeze-dried for the extracts from distilled water)) separately to obtain the crude extracts. All the extractions performed were repeated thrice and the crude extracts were labelled and stored at 4°C.

Total phenolic content (TPC)

About 1 mL of Folin's phenol reagent was mixed with 1 mL of 20% sodium carbonate solution and 1 mL of the extract sample solution was added. This mixture was set aside for 45 min at 45°C (Choo *et al.*, 2020). The absorbance was measured at 765 nm with a spectrophotometer (Labtronics LT 291, USA Model), gallic acid

was used as standard and all the TPC calculations were carried out with gallic acid calibration curve and equation $y= 0.0045x + 0.0309$ and R^2 value = 0.9921. All the tests were performed in triplicates and signified as mg GAE/g ± standard deviation.

*A*ntioxidant activity

DPPH radical scavenging activity

Approximately 0.5 mL of the extract sample solution was dissolved in 1 mL of 0.1 mM DPPH solution and 0.4 mL of 50 mM Tris-HCl solution. This mixture was stored at room temperature for 30 min. The absorbance of the mixture was measured at 517 nm using a spectrophotometer (LT 291, Labtronic, USA) (Ravikumar *et al.*, 2021). All the tests for antioxidant activity were performed in triplicates and the DPPH % inhibition was calculated from Equation 1.

$$\text{DPPH \% inhibition} = \frac{Abs_{control} - Abs_{sample}}{Abs_{control}} \times 100\% \quad \textit{(Eq. 1)}$$

Where, $Abs_{control}$ is the absorbance of blank solution and Abs_{sample} is the absorbance of the extract sample solution with DPPH.

2,2'-azino-bis (3-ethylbenzothiazoline-6-sulfonic acid (ABTS) radical-scavenging activity

The radical cation ($ABTS^+$) was produced by the reaction of diluted ABTS solution (ABTS dissolved in water to 7 µM concentration) with 2.45 µM potassium persulphate at 20°C in dark (12–16 h). Then the absorbance value of the $ABTS^+$ solution was modified to 0.700 ± 0.02 at 734 nm by diluting with water, followed by the inclusion of 3.0 mL of diluted $ABTS^+$ solution to 100 µL of extract sample solution. The absorbance was recorded at 734 nm after 6 min (Dhanani *et al.*, 2017). The activity was given as percentage $ABTS^+$ scavenging that was the same as DPPH activity.

Bioassay-guided Fractionation of C. monogyna Extract

Thin layer chromatography (TLC) analysis

Prior to TLC analysis, separation was carried out. For separation, water, *n*-hexane and chloroform were used as separating solvents. The antioxidant rich plant part of *C. monogyna* was partitioned with water, *n*-hexane and finally chloroform (each 250 mL approximately). The ethanol fruit extract was mixed with water (250 mL) in a separating funnel to form an aqueous layer followed by the above-mentioned steps. This afforded three partition factions from the layers of aqueous, n-hexane and chloroform.

The fractionation procedure was monitored using thin-layer chromatography (TLC) analysis. The ethanol fruit extract sample solution of *C. monogyna* was loaded on silica-coated TLC silica gel of 60 F254 Merck sheet (Germany) and placed in a beaker with the predetermined solvent systems (water: formic acid: acetic acid: methanol (2:4:2:2), water: acetic acid: methanol (4:2:4) and water: ethyl acetate: formic acid: acetic acid (2:6:2:2) (Li, Jiang and Chen, 2004)). Next, the paper was removed, dried and incubated in iodine chamber for 5 to 10 min and the compounds were visualised under UV light.

Bioassay-guided fractionation of C. monogyna ethanol fruit extract via open column chromatography

A glass column was washed and cleaned with distilled water and followed by 70% ethanol to set a stain-free and pure column. About 2 to 3 cm of dust-free absorbent cotton was fixed in the bottom as a stationary phase and the prepared silica gel (100- 200 mesh) was added. The prepared column was left for 3-4 hr for the complete saturation and the removal of bubbles to make the bed static. The aqueous extract was chromatographed over gravity liquid chromatography with the selected solvent system (water; ethyl acetate; formic acid; acetic acid (2:6:2:2)) by isocratic separation. The eluants were collected separately and were subjected to TLC analysis. The analysed eluants with similar compositions were pooled together to get twelve fractions of *C. monogyna* extract. All the fractions were subjected to antioxidant activity analysis, FTIR and GC-MS/MS for the identification of antioxidant compounds (Br Hemavathy and Sundari, 2019).

Fourier Transform Infrared Spectroscopy (FTIR) Analysis

A small amount of *C. monogyna* ethanol fruit extract fraction was blended with potassium bromide (dry) and the mixture was mixed with a mortar and pressed at 6 bars within 2 min to create a fine disc of potassium bromide. In the sample cup of a diffuse reflectance accessory, the disc was placed. The IR spectrum was obtained using Bruker, Germany Vertex 70 infrared spectrometer. The sample was scanned from 4000 to 400 cm^{-1}. The FTIR peak values were recorded (Jain *et al.*, 2016).

Gas Chromatography-Tandem Mass Spectroscopy (GC-MS-MS) Analysis

The GC-MS-MS analysis was carried out with an Agilent CH-GCMS-MS-02 Innowax FSC column (60 m x 0.25 mm, 0.25 µm film thickness) with carrier gas helium at 0.8 mL/min. GC oven temperature was retained at 60°C for 10 min and assigned to 220°C at a rate of 4°C /min for 10 min and then assigned to 240°C at a rate of 1°C /min. The split ratio was adjusted at 40:1. Later, the injector temperature was set at 250°C. Mass spectra were recorded at 70 eV. Mass range was from 35 to 450 m/z. The sample was dissolved with 10% n-hexane and 1µL was injected. The mass spectra of unknown peaks with those stored in the NIST (National Institute of Standards and Technology) and Wiley mass spectral electronic libraries 2020 were compared for identification of the compounds (Bazgir *et al.*, 2020).

H9c2 rat myoblast cardiac cell line assay

Preparation of control assay

(i) Medium control (medium without cells)

(ii) Negative control (medium with cells but without the experimental fraction)

(iii) Positive control (medium with cells and 10µg/ml of Doxorubicin)

The control assay was prepared by seeding 200 µl cell suspension in a 96-well plate at required cell density (20,000 cells per well), without the test agent. The cells were allowed to grow for about 24 hours. The appropriate concentrations of the test fraction (0, 12.5, 25,50,100,200ug/ml) were prepared and incubated for 24 hrs at 37°C in a 5% CO_2 atmosphere. After the incubation period, the MTT reagent was added to give a final concentration of 0.5mg/ml. The plate was wrapped with aluminium foil to avoid exposure to light. The plates were then incubated for 3 hours. The MTT reagent was removed and 100 µl of solubilisation solution (DMSO) was added and stirred in a gyratory shaker to enhance dissolution. The absorbance at 570nm and 630nm was recorded using a spectrophotometer. The IC_{50} value was determined using a linear regression equation ($Y = Mx + C$). Here, Y = 50, M and C values were derived from the viability graph (Jain *et al.*, 2016; Br Hemavathy and Sundari, 2019). The percentage of cell viability was calculated from equation 2.

$$\text{Percentage of cell viability} = \left[\frac{\text{Abs of treated cells}}{\text{Abs of Untreated cells}}\right] \times 100\% \qquad \textit{(Eq. 2)}$$

Statistical analysis

All the experiments were performed in triplets and the results were reported as average ± standard deviation. All statistical calculations were performed using SPSS IMB 2019 software. The accuracy of the results was determined by the coefficient of determination (R^2).

Results and discussion

Identification of C. monogyna plant part and extraction solvent with high antioxidant activity

To determine the *C. monogyna* plant part which possessed the highest antioxidant activity, different extraction solvents (i.e., aqueous, ethanol and methanol) were applied to extract antioxidant compounds from each plant part (i.e., leaves, flower and fruit) of *C. monogyna* using the UAE method. Table 1 demonstrates the TPC and the antioxidant activity obtained from *C. monogyna* plant extracts

Table 1: TPC and antioxidant activity (DPPH and ABTS) of C. monogyna leaves, flowers and fruits

Extract	*C. monogyna* leaves	*C. monogyna* flower	*C. monogyna* fruit

	TPC (mg GAE/g)	DPPH (%)	ABTS (%)	TPC (mg GAE/g)	DPPH (%)	ABTS (%)	TPC (mg GAE/g)	DPPH (%)	ABTS (%)
Aqueous extract	0.064 ± 0.003	74.59 ± 0.003	30.11 ± 0.005	0.044 ± 0.002	80.9 ± 0.002	21.08 ± 0.003	0.059 ± 0.002	73.46 ± 0.003	65.19 ± 0.005
Ethanol extract	**0.066** ± 0.002	**87.77** ± 0.003	**56.25** ± 0.003	**0.053** ± 0.003	**84.8** ± 0.001	**33.35** ± 0.002	**0.070** ± 0.003	**90.35** ± 0.001	**71.37** ± 0.003
Methanol extract	0.059 ± 0.002	73.91 ± 0.001	17.89 ± 0.004	0.043 ± 0.004	78.4 ± 0.002	13.71 ± 0.002	0.065 ± 0.002	87.09 ± 0.002	38.41 ± 0.002

For the *C. monogyna* leaf extract, higher TPC, DPPH and ABTS percentage were attained in ethanol extract (0.066 mg GAE/g, 87.77% and 56.25%) compared to *C. monogyna* aqueous and methanol extract. Similarly, DPPH and ABTS activity was higher in ethanol extract (87.77% and 56.25%), whereas comparatively lower in aqueous (74.59% and 30.11%) and methanol extract (73.91% and 17.89%). For the *C. monogyna* flower extract, results in Table 1 shows that higher TPC value of 0.053 mg GAE/g was attained in ethanol extract and the lowest TPC value of 0.043 mg GAE/g was reported for methanol extract. The TPC of *C. monogyna* fruit extracts showed higher TPC in ethanol extract of 0.070 mg GAE/g and the lowest in aqueous extract with 0.059 mg GAE/g. Similarly, DPPH and ABTS of *C. monogyna* ethanol extract were higher (90.35% and 71.37%, respectively) when compared to *C. monogyna* methanol (87.09% and 38.41%, respectively) and aqueous extracts (73.46% and 38%, respectively). Higher values in TPC and antioxidant activity from ethanol extract might due to the presence of compounds which are polar in nature. Typically, ethanol as solvent is used for extraction of polar and some non-polar metabolites since it is less toxic, and degradation of metabolites is lower when compared to other solvents. However, further investigation on these ethanol extract can help better in identification on type of compounds.

C. monogyna ethanol fruit extract exhibited a high TPC value of 0.070 mg GAE/g while the lowest TPC value of 0.043 mg GAE/g was found in *C. monogyna* methanol flower extract. *C. monogyna* ethanol fruit extract had higher DPPH scavenging activity of 90.35% and lowest DPPH scavenging activity of 73.91% in *C. monogyna* methanol leaf extract. ABTS radical scavenging activity percentage was also high in *C. monogyna* ethanol berry extract (71.37%) and lowest in *C. monogyna* methanol flower extract (13.71 %). Higher TPC content corresponds to higher values of antioxidant activity. Thus, the *C. monogyna* ethanol fruit extract exhibited an overall high value of DPPH and ABTS among the investigated plant parts, followed by leaf extract and flower extracts of *C. monogyna*.

Table 2: Crude extract yield of Crataegus monogyna plant parts

***Crataegus monogyna* plant part**	**Solvent Extract**	**Crude extract yield (g)**
Leaves	Aqueous	0.13 ± 0.05
	Ethanol	0.26 ± 0.05
	Methanol	0.19 ± 0.05
Flower	Aqueous	0.098 ± 0.05
	Ethanol	0.20 ± 0.05
	Methanol	0.16 ± 0.05
Fruit	Aqueous	0.10 ± 0.05
	Ethanol	**0.49 ± 0.05**
	Methanol	0.14 ± 0.05

Table 2 shows the crude extract yield of the *C. monogyna* plant part for all extracts. *C. monogyna* fruit ethanol extract had the highest crude extraction yield of 0.49 ± 0.05 g and the lowest was found in *C. monogyna* aqueous fruit extract (0.10 ± 0.05 g). When comparing overall results (from Tables 1 and 2), *C. monogyna* ethanol fruit extract demonstrated the highest TPC, antioxidant activity and crude extract yield. Therefore, chromatographic analysis was run to

extract and identify the antioxidant compounds that were present in *C. monogyna* ethanol fruit extract.

Bioassay-guided fractionation of C. monogyna ethanol fruit extract

Results of paper and thin layer chromatography (TLC) analysis

In most of the qualitative analysis techniques, paper chromatography is commonly used technique for the separation of coloured mixtures. In the current research, *C. monogyna* ethanol fruit extract was subjected to separating solvents (i.e., water, chloroform and n-hexane) using paper chromatography. Based on the results obtained, no spot was observed for all the three extracts in the first and second solvent systems (water: formic acid: acetic acid: methanol (2:4:2:2) and water: acetic acid: methanol (4:2:4)). This is due to lack of tendency of the compound existing in the extract to dissolve into the mobile phase. The third solvent system (water: ethyl acetate: formic acid: acetic acid (2:6:2:2)) showed better separation of compounds as varied spots for all three separated extracts were visualised. Therefore, the third solvent system was used for the subsequent TLC analysis for all three separation extracts (water, n-hexane and chloroform). Better separation was achieved in water, suggesting that majority of compounds that were present in *C. monogyna* ethanol fruit extract were polar in nature (Abarca-Vargas, Peña Malacara and Petricevich, 2016).

TPC and antioxidant activity of C. monogyna ethanol fruit extract from partitioned solvents

Antioxidant activity (DPPH and ABTS) and TPC were investigated for *C. monogyna* ethanol fruit extract partitioned with all three solvents (water, chloroform and n-hexane).

Table 3: TPC and antioxidant activities of C. monogyna fruit ethanol extract

Extracts	TPC (mg GAE/g)	DPPH %	ABTS %
Water	**86.00± 0.002**	**74.84 ± 0.002**	**47.86 ± 0.002**
Chloroform	43.00 ± 0.001	65.72 ± 0.002	31.42 ± 0.002
n- hexane	4.00 ± 0.003	37.62 ± 0.002	10.84 ± 0.003

From Table 3, the *C. monogyna* fruit ethanol extract separated from water had high value for TPC (86 mg GAE/g) and demonstrated the highest antioxidant activities (74.84% and 47.86% for DPPH and ABTS), thereby indicating (Altemimi *et al.*, 2017) that the dominant antioxidant compounds that were present in *C. monogyna* ethanol fruit extract were polar compounds (Altemimi *et al.*, 2017).

Bioassay-guided fractionation of C. monogyna ethanol fruit extract via open column chromatography

The aqueous layer of the *C. monogyna* fruit ethanol extract was then subjected to column chromatography using the third solvent system (water: ethyl acetate: formic acid: acetic acid at a ratio of 2:6:2:2, respectively) by isocratic separation to furnish twelve fractions. All twelve fractions (F1 to F12) were subjected to two antioxidant activity assays (DPPH and ABTS) and TPC to identify the fraction with the highest TPC and antioxidant activity.

Antioxidant activity and TPC analysis of C. monogyna ethanol fruit extract fractions

Table 4 shows the TPC and antioxidant activity of all twelve fractions of *C. monogyna* ethanol fruit extract.

Table 4: TPC and Antioxidant test for separated fractions

Fraction	TPC (mg GAE/g)	DPPH %	ABTS %
F1	2.01 ± 0.002	12.32 ± 0.002	7.01 ± 0.002
F2	5.21 ± 0.002	15.43 ± 0.002	9.89 ± 0.001
F3	9.21 ± 0.002	28.91 ± 0.002	10.11 ± 0.002
F4	11.74 ± 0.002	29.26 ± 0.002	24.95 ± 0.002

F5	18.56 ± 0.002	33.78 ± 0.002	27.31 ± 0.002
F6	21.02 ± 0.002	37.21 ± 0.002	30.02 ± 0.002
F7	21.91 ± 0.002	38.22 ± 0.002	30.78 ± 0.002
F8	24 ±.0 ± 0.002	42.29 ± 0.002	42.50 ± 0.002
F9	**28 ±.0 ± 0.002**	**46.14 ± 0.002**	**45.06 ± 0.001**
F10	8 .89 ± 0.003	26.87 ± 0.002	33.86 ± 0.002
F11	8.43 ± 0.002	23.58 ± 0.002	30.89 ± 0.002
F12	5.24 ± 0.002	15.58 ± 0.002	10.21 ± 0.002

Based on the antioxidant activity (Table 4), Fraction F9 possessed the highest DPPH (46.14%) and ABTS (45.06%) scavenging activities along with a high TPC value of 28 mg GAE/g. Therefore, Fraction F9 which had the highest antioxidant activity was proceeded with FTIR analysis (identification of functional groups) and GC-MS-MS to identify the antioxidant compounds that were present predominantly in F9.

FTIR Analysis

FTIR spectrum peak value was used to determine the functional groups of *C. monogyna* ethanol fruit extract F9 fraction (Taylor and Halmer, 2014). The FTIR spectrum of *C. monogyna* fruit ethanol for the Fraction F9 is shown in Figure 1. The peaks at 2970.38 and 2885.51 cm^{-1} were due to C-H stretching as medium, alkane or alkene compound, 1390 to 1310 cm^{-1} was due to medium O-H bending of phenol compounds, 1050 to 1040 cm^{-1} was due to strong broad CO-O-CO stretching anhydride (Kalaichelvi and Dhivya, 2017). FTIR study predicted the presence of O-H, C-H and CO-O-CO groups in Fraction F9 of *C. monogyna* ethanol fruit extract. The presence of characteristic functional groups of alkane, phenol and anhydride may contribute to the antioxidant property of *C. monogyna* ethanol fruit extract.

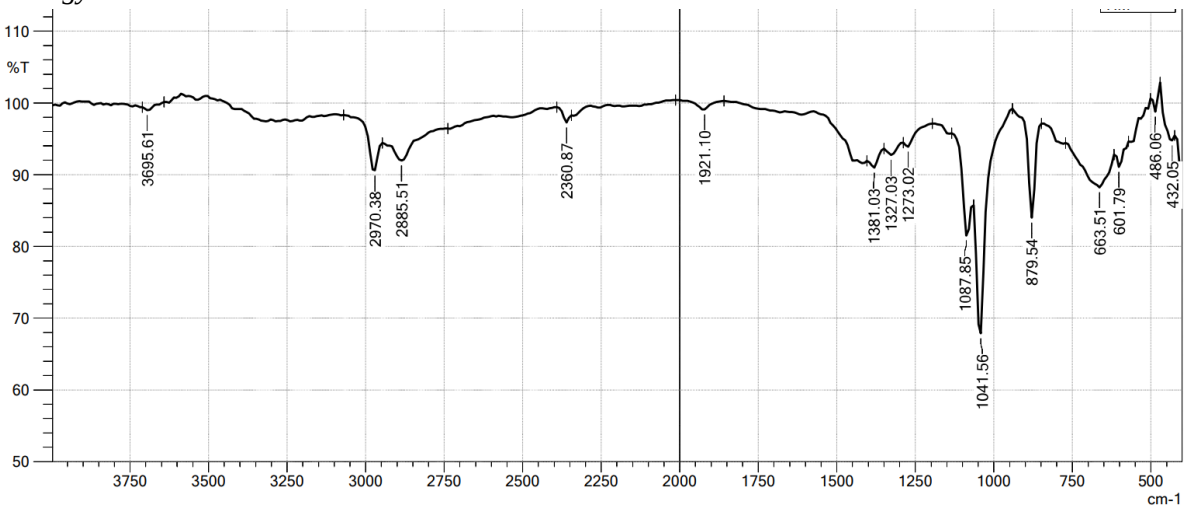


Figure 1: FTIR spectrum for C. monogyna ethanol fruit extract F9 fraction

GC-MS/MS Analysis

Identification and investigation on the antioxidant compound appeared in Fraction F9 of *C. monogyna* ethanol fruit extract was analysed by GC-MS-MS. A total of eleven (11) compounds were identified and listed in Table 5 by comparing the obtained compound's mass spectra with mass spectra available in NIST and Wiley mass spectral electronic libraries 2020.

Table 5: Antioxidant compounds in ethanolic extract of C. monogyna fruit fraction

Retention time (min)	Molecular formula	Name	Match Factor (%)	Peak area (%)

6.2424	$C_6H_8N_2$	1H-Imidazole, 4-(2-propenyl)-	99.3	6.41
6.4040	$C_9H_{11}NO_2$	Tyramine, N-formyl-	64.8	5.81
8.1506	$C_{10}H_{12}O_3$	Tyrosol, acetate	63.0	5.34
8.4737	$C_{12}H_{14}N_2O_3S$	Benzenesulfonamide, N-[[5-(aminomethyl)-2-furanyl]methyl]-	52.0	8.21
9.1401	$C_3H_6O_4S$	1,3,2-Dioxathiolane-4-methanol, 2-oxide	73.6	7.49
10.0891	$C_{15}H_{10}O_7$	2-(3,4 dihydroxy phenyl)-3,5,7-trihydroxychromen-4-one	89.2	19.46
13.4814	$C_6H_8N_2$	1-Cyclopentene-1-carbonitrile,2-amino-	72.8	7.12
17.4997	$C_{15}H_{24}O$	4-Nonylphenol	79.7	8.01
18.6002	$C_9H_{14}N_6$	1H-1,2,3,4-Tetrazol-5-amine,1-ethyl-N-[(1-methyl-1H-pyrrol-2-yl)methyl]-	52.5	11.24
21.0435	$C_{14}H_{22}O$	Phenol, 4-octyl-	57.4	6.98
28.9792	$C_{14}H_{18}O$	(2-Methylene-cyclohexyl)-phenyl-methanol	54.2	6.47

Major compounds in Fraction F9 were identified as 1. Benzenesulfonamide, N-[[5-(aminomethyl)-2-furanyl]methyl]- (2-Methoxyidazoxan); 2. 1,3,2-Dioxathiolane-4-methanol, 2-oxide (trimethylene sulfate); 3. 2-(3,4 dihydroxy phenyl)-3,5,7-trihydroxychromen-4-one (quercetin); 4. 4-Nonylphenol (nonylphenol) and 5. 1H-1,2,3,4-Tetrazol-5-amine,1-ethyl-N-[(1-methyl-1H-pyrrol-2-yl)methyl]-) (diallylmelamine). Minor compounds identified were 1. 1H-Imidazole, 4-(2-propenyl)- (2-Ethylsuccinonitrile); 2. Tyrosol, acetate (4-Phenoxybutyric acid); 3. Phenol, 4-octyl- (4-Octylphenol); 4. Tyramine, N-formyl- (phenylalanine); 5. 1-Cyclopentene-1-carbonitrile,2-amino-(2-Ethylsuccinonitrile) and 6. (2-Methylene-cyclohexyl)-phenyl-methanol (amyl cinnamaldehyde). From FTIR and GC-MS/MS analysis, majority of the compounds identified were phenolic compounds (Cha *et al.*, 2012).

H9c2 Rat Myoblast Cardiac Cell Line Study

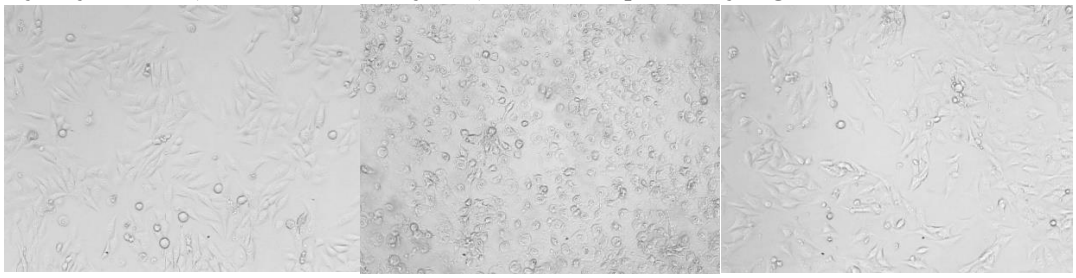
In the current study, four samples were evaluated to investigate the cardiac effect on the cell line (H9c2). The first sample was the blank sample. The culture medium used was Dulbecco's Modified Eagle Medium-high glucose (DMEM). The second sample was the control sample (untreated cell H9c2 cells). The third sample was the standard (10 ug/ml of Doxorubicin) and the fourth sample was the *C. monogyna* fruit ethanol, fraction F9 with five different concentrations (12.5, 25,50,100,200 ug/ml). The percentage of cell viability for all the samples was calculated and tabulated in Table 6.Table 6: The percentage of cell viability of the test fraction with different concentrations against the H9c2 cells

	Samples

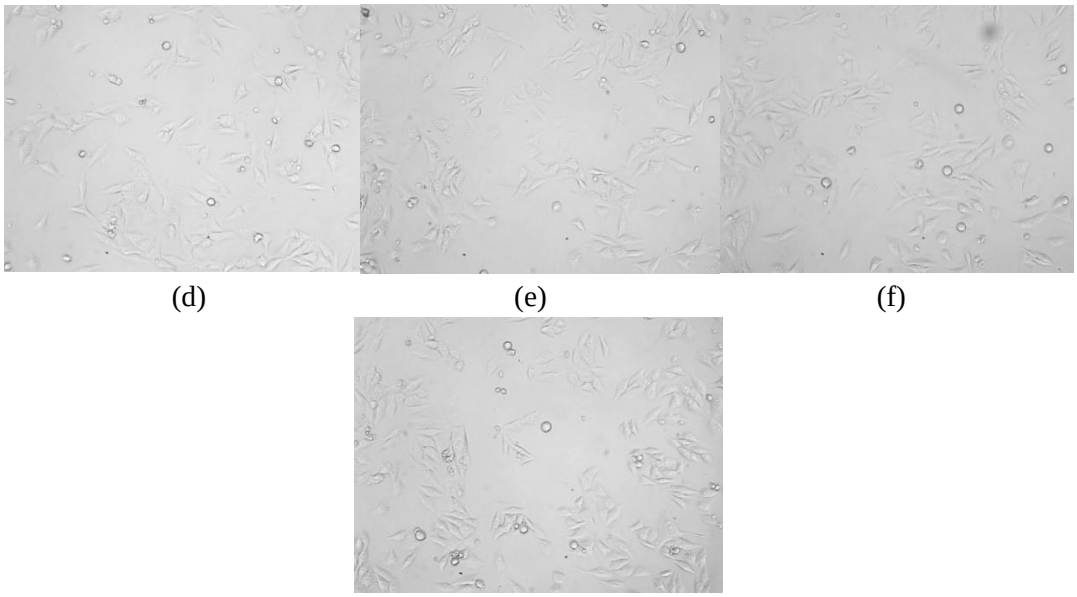
	Blank	Control	Standard	Five different concentrations of *C. monogyna* fruit ethanol, fraction F9				
				12.5 µg/ml	25 µg/ml	50 µg/ml	100 µg/ml	200 µg/ml
Mean *abs*	0.046 ± 0.005	0.931 ± 0.006	0.3645 ± 0.005	0.927 ± 0.003	0.918 ± 0.003	0.911 ± 0.004	0.899 ± 0.004	0.879 ± 0.005
Mean *abs* (sample-blank)		0.885 ± 0.006	0.3185 ± 0.005	0.881± 0.003	0.872 ± 0.003	0.865 ± 0.004	0.853 ± 0.004	0.833 ± 0.005
% Cell viability		100	35.9887	**99.5480**	98.5310	97.7401	96.3841	94.1242

* Mean *abs* (sample-blank) = Abs of treated and untreated cells

C. monogyna ethanol fruit, fraction F9 revealed the highest percentage of cell viability for all concentrations (12.5, 25, 50, 100, 200 ug/ml). The percentage of cell viability ranged from 94.12 % to 99.54 % and the highest percentage of cell viability (99.54 %) was recorded in fraction F9 with a concentration of 12.5 µg/ml, indicating *C. monogyna* fruit ethanol extract fraction F9 possessed the highest therapeutic role in treating H9c2 rat myoblast cell line. The presence of major and minor compounds Benzenesulfonamide, N-[[5-(aminomethyl)-2-furanyl]methyl]- (2-Methoxyidazoxan); 1,3,2-Dioxathiolane-4-methanol, 2-oxide (trimethylene sulfate); 2-(3,4 dihydroxy phenyl)-3,5,7-trihydroxychromen-4-one (quercetin); 4-Nonylphenol (nonylphenol); 1H-1,2,3,4-Tetrazol-5-amine,1-ethyl-N-[(1-methyl-1H-pyrrol-2-yl)methyl]-) (diallylmelamine); 1H-Imidazole, 4-(2-propenyl)- (2-Ethylsuccinonitrile); Tyrosol, acetate (4-Phenoxybutyric acid); Phenol, 4-octyl- (4-Octylphenol); Tyramine, N-formyl-(phenylalanine); 1-Cyclopentene-1-carbonitrile,2-amino-(2-Ethylsuccinonitrile) and (2-Methylene-cyclohexyl)-phenyl-methanol (amyl cinnamaldehyde). identified from GC-MS/MS demonstrated high phase of recovery in H9c2 cardiac cell line. The observations in statistical data of MTT cell cardiology study by ELISA reader evidently show *C. monogyna* ethanol fruit F9 fraction possesses significant cell viability potential (R^2 0.9550) properties against H9c2 cells. Direct microscopic observations of drug-treated images of cell lines captured with the magnification of 10X were enclosed in Figure 2. Figure 2(a) shows the untreated H9c2 cells with less toxic effect. Figure 2(b) shows the H9c2 cells treated with doxorubicin, indicating high cell degradation (% of cell viability is very low) resulting in more dead cells. In Figure 2 (c-g) show the F9 fraction with different concentrations added to H9c2 cells, demonstrating high proliferation and hence, the percentage of cell viability was comparatively higher.



(a) (b) (c)



(d) (e) (f)

(g)

Figure 2: (a) H9c2 untreated, (b) H9c2 toxic, (c) H9c2 with 12.5 µg/ml test fraction, (d) H9c2 with 25 µg/ml test fraction, (e) H9c2 with 50 µg/ml test fraction, (f) H9c2 with 100 µg/ml test fraction, (g) H9c2 with 200 µg/ml test fraction

Conclusion

The present study concluded *C. monogyna* fruit extracted using ethanol solvent had better extraction yield and antioxidant activity. The antioxidant-rich fraction (F9) from *C. monogyna* fruit was obtained and the major compounds contributing to the highest antioxidant activity of *C. monogyna* ethanol fruit were fractionated via open, TLC and column chromatographic techniques and identified via FTIR analysis and GC-MS-MS analysis. *C. monogyna* ethanol fruit fraction, fraction F9 shows effective cell viability potency on H9c2 cells on a dose-dependent manner after the treatment period of 24 hours of incubation at 37°C, indicating fraction F9 from the *C. monogyna* ethanol fruit extract possesses therapeutic potential against rat myoblast cells. Further studies need to be performed to evaluate the molecular mechanism of action behind the cell and its viability potential in in-vitro conditions. The results also suggest that *C. monogyna* ethanol fruit extract is an excellent source of natural antioxidants with the presence of phenols. Further purification of the antioxidant-rich fraction can be performed in future studies to pave way for the emergence of the targeted pure antioxidant compound to cure and treat various diseases which could be positively implemented on large scale in the pharmaceutical and nutraceutical industries.

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