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Isolation, Structural Characterisation and Wound Healing Activity of Flavonoid and its Glycoside from *Euphorbia hirta* L. Methanolic Extract

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Abstract

Euphorbia hirta is a plant belonging to the Euphorbiaceae family that is widely used in traditional medicine to treat various dermal diseases in tropical and subtropical countries. It has been found to have anti-inflammatory and wound-healing effects, which may make it a promising natural treatment for different medical conditions. The aim of the present study focuses on the investigation of the main active compounds associated with the wound-healing activity of plants. The plant powder of aerial part was sequentially extracted with n-hexane, chloroform and methanol using the serial exhaustive extraction method. The extract with the best activities was further fractionated using various chromatographic techniques to isolate the active compounds responsible for wound healing. The isolated compounds were assessed for wound healing activity by measuring migration and proliferation against human dermal fibroblast cells. The structure elucidation of compounds was confirmed by IR, LC-MS, and NMR. These isolated compounds were subsequently identified as kaempferol and its glycoside, kaempferol-3-O-glucoside (astragalin). The findings also revealed that both isolated compounds were able to stimulate the cell population with no sign of toxicity and the highest cell migration ability shows after 48-hour treatment. This study provides new evidence to support the traditional use of *E. hirta* as a natural promising wound healer.

Keywords: Wound healing, *Euphorbia hirta*, Euphorbiaceae, Kaempferol, Astragalin.

عزل وتشخيص مركبات الفلافينويد ومشتقاته الكلايكوسايديه للمستخلص الميثانولي لنبات الفربيون ودراسة فعاليتها في التئام الجروح

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الخلاصة

نبات الفربيون هو نبات ينتمي إلى عائلة الفربيون الذي يستخدم على نطاق واسع في الطب التقليدي لعلاج الأمراض الجلدية المختلفة في البلدان الاستوائية وشبه الاستوائية. لقد وجد أن له تأثيرات عديدة منها مضادة للالتهابات والتئام الجروح ، مما قد يجعله علاجًا طبيعيًا واعدًا لمختلف الحالات الطبية. يركز الهدف من هذه الدراسة على التحقيق في المركبات النشطة الرئيسية للنبات والمرتبطة بنشاط التئام الجروح. تم استخلاص الجزء الهوائي للنبات بمذيبات الهكسين والكلوروفورم والميثانول والماء بالتتابع. ثم تم تجزئة المستخلص الفعال باستخدام تقنيات كروماتوغرافيا

مختلفة لعزل المركبات النشطة المسؤولة عن التئام الجروح. تم فحص التئام الجروح للمركبات المعزولة عن طريق قياس تكاثر وهجرة الخلايا (اختبار الخدش) للخلايا الجلدية البشرية الطبيعية. تم التأكيد من التركيب الكيميائي للمركبات بواسطة فحص مطيافية الاشعة تحت الحمراء(IR)
وفحص مقياس الطيف الكتلي اللوني السائل(LC-MS) وفحص الرنين المغناطيسي النووي(NMR). تم التعرف على هذه المركبات المعزولة بالكامفر kaempferol وkaempferol-3-O-glucosid. كشفت النتائج أيضًا أن كلا المركبين المعزولين كانا قادرين على تحفيز تكاثر الخلايا الجلدية وتحفيز هجرة الخلايا دون أي علامة على السمية. تقدم هذه الدراسة أدلة جديدة لدعم الاستخدام الشعبي لنبات الفربيون كمعالج طبيعي واعد لالتئام الجروح.

الكلمات المفتاحية : التئام الجروح، نبات الفروبين، عائلة الفروبين، الكامفرول، الاستراغالين.

Introduction

Euphorbia hirta L. (Euphorbiaceae) is a widely distributed annual plant found in tropical and subtropical regions. It has a long history of use in a traditional medicine as a remedy for wound healing and has been the subject of numerous studies investigating its pharmacological properties [(1)]. Previous studies have shown that *E. hirta* has a range of beneficial activities including antioxidant, antimicrobial, antiepileptic, sedative anxiolytic, anti-inflammatory, pain and fever relieving, antihistaminic, antidiabetic, anticancer, gastrointestinal, diuretic, antiparasitic, immunological, liver protective, galactogenic, angiotensin converting enzyme inhibiting, and anti-dipsogenic effects [(2, 3)]. These properties make *E. hirta* a potentially valuable natural source for developing new drugs for various medical conditions.

A wound is a physical, chemical, or thermal injury that results in an opening or disruption of the skin's integrity [(4)]. It can range from a simple breach of the skin's epithelial layer to deeper damage affecting subcutaneous tissue and potentially other structures such as tendons, vessels, nerves, muscles, parenchymal organs, and bone [(5)]. The process of normal wound healing is generally divided into four stages: coagulation and hemostasis, inflammation, proliferation, and remodelling [(5-7)]. Herbal medicines have a promising role in wound healing; the natural compounds contained in the herbs may promote wound healing and tissue regeneration through varied mechanisms [(8)]. *E. hirta* itself has been reported to possess anti-inflammatory and wound healing effects due to the flavonoid compounds present in the whole plant extract [(9, 10)]. Thus, the main aim of this present study focuses on the isolation and identification of the active compounds associated with wound healing activity of the plant.

Materials and Methods

Chemicals

Solvents used for the extraction and isolation works such as hexane, chloroform, methanol, acetonitrile, and acetic acid (analytical and HPLC grades) were obtained from Fisher Scientific (Fair Lawn, NJ, USA). Methanol-*d4* solvent was obtained from Merk (Germany) For cell culture experiments, Dulbecco's Modified Eagle Medium (DMEM), fetal bovine serum (FBS), penicillin/streptomycin (P/S), Trypsin-EDTA, phosphate-buffered saline (PBS), 3-(4, 5-dimethylthiazol - 2yl) - 2,5 - diphenyltetrazolium bromide (MTT) and dimethyl sulfoxide (DMSO) were obtained from Gibco (Invitrogen, USA).

Collection, identification, and preparation of plant materials

The aerial parts of *E. hirta* L. were collected from various locations in Penang and Kedah, Malaysia. It was authenticated at the Herbarium of School of Biological Sciences, Universiti Sains Malaysia, Penang, under the voucher specimen (No: 11858). After removing all debris, dust, and soils from the plant materials, they were dried in the shade at room temperature for three weeks and then milled into a coarse powder. The dried, ground, plant materials were stored in a clean and tightly closed container until further analysis.

Extraction, fractionation, isolation, and structure elucidation procedures

The air-dried powder of the plant (400 g) was extracted with hexane (1000 mL) using a Soxhlet apparatus for 24 hrs. until all the extractable material was obtained. The residues plant material was then air-dried, and the extraction process was continued with chloroform (1000 mL) and methanol (1000 mL), respectively for 72hr. Each extract was filtered to remove any plant material residue. The filtrate was concentrated using a rotary evaporator (Buchi, Switzerland) to produce three crude extracts, i.e., hexane, chloroform, and methanol extracts.

The extract with the best activities, the methanol extract, was further fractionated using gravity column chromatography. For the stationary phase, a sintered glass column (60 cm × 6 cm) with a 1-L reservoir was packed with silica gel (70-230 mesh) at a ratio of 25:1 silica to extract. The column was eluted with n-hexane – ethyl acetate – methanol using a gradient of increasing polarity eluent, starting with 100% n-hexane to 100% ethyl acetate and ending with 100% methanol. Five fractions (F1-F5) were obtained. Fractions 3 (100 mg) and 4 (300 mg) (F3 and F4, 20-40% n-hexane: ethyl acetate) were separately purified using another silica gel column chromatography (15 cm × 1.5 cm) eluting with a gradient mixture of chloroform: methanol (100% to 5٠%) to afford compound 1 (5 mg) from F3 and compound 2 (25 mg) from F4.

The structures of the isolated compounds were characterized using various spectroscopic and spectrometric techniques. Attenuated total reflectance-Fourier transform infrared (ATR-FTIR) spectroscopy along with 1D and 2D nuclear magnetic resonance (1D-NMR and 2D-NMR) spectral analyses were performed on a Bruker Advance spectrometer operating in 700 MHz for ^{1}H and 175 MHz for ^{13}C in methanol-*d4* solvent. Tandem mass spectrometry with multiple reaction monitoring (MRM) via electrospray ionization (ESI) in the negative ionization mode was also used to measure the molecular weight of the isolated compounds.

In vitro wound healing assay

Cell lines

Hs27 cells were cultured in DMEM high-glucose medium (d-glucose: 4500 mg/l) containing 10% (v/v) FBS, and 1% (v/v) P/S (100 units penicillin per 100 g streptomycin). The cells were maintained at 37ºC in a humidified CO2 incubator with 5% CO2 and the culture medium was changed every 48 hours. When the adhered cells reached 80% confluence, they were trypsinized using trypsin-EDTA, centrifuged at 800 rpm for five minutes, and passaged onto multi-well plates for assays.

Cell viability and cell proliferation Assay

The effects of isolated compounds on Hs27 fibroblasts cell viability and proliferation were evaluated using the MTT assay method (Alvarez et al., 2021). Serial concentrations of the isolated compounds (100, 50, 25, 12.5, 6.25 and 3.125 µg/mL) were prepared (Latif et al., 2019) and added to 96-well plates containing Hs27 cells that had been seeded overnight at a density of 2 x 10^3 cells/well. The growth control consisted of cells maintained in DMEM media with 10% v/v serum, while the blank consisted of medium with 10% v/v serum without cells. After 24 hours of treatment, 20 µL of MTT solution (5 mg/mL in PBS) was added to each well and the cells were incubated at 37°C for another 4 hours. The medium was then aspirated, and 100 µL of DMSO was added to dissolve the water-insoluble purple formazan crystals. The plates were then shaken using a microplate reader (Thermo Scientific Multiskan Spectrum, Pittsburgh, PA, USA) for 20 seconds and the optical density (OD) was measured at 570 nm. The percentage of cell viability was calculated as follows formula:

$$\%\ \boldsymbol{of\ cell\ viability} = \frac{\boldsymbol{Absorbance\ of\ sample - Absorbance\ of\ blank}}{\boldsymbol{Absorbance\ of\ control - Absorbance\ of\ blank}}\ \boldsymbol{x\ 100}$$

The effects of isolated compounds on Hs27 fibroblasts cells proliferation were evaluated at 24-, 48-, and 72-hours post-treatment for each different concentration. The results were compared with the growth of control consisted of cells maintained in DMEM media with 10% v/v serum [(11)].

Scratch-wound Assay

The scratch-wound assay was utilized to assess the ability of isolated compounds to stimulate cell migration, as described by Li *et al.* (2019). Hs27 cells (1 x 105 cells) were seeded in a 24-well cell culture plate and allowed to reach confluence. A horizontal scratch was made through the cell monolayer using a P100 pipette tip, and the resulting cellular debris was removed by washing with phosphate buffer saline (PBS). The cells were then incubated with the test compounds and observed for migration across the scratch wound. The cultured medium was then replaced with fresh medium containing plant extracts at a concentration of 25 µg/mL in triplicates. The chosen concentration of 25 µg/mL was based on the results of a preliminary cell viability study. Images of the cells were taken at three different time (0, 24 and 48 hr) and the data was further analysed to determine the distance moved by the cells, using imaging 1.42q (National Institutes for Health, US). The percentage of the closed area was measured and compared to the value obtained before treatment (zero time). The percentage of cell migration was calculated using the following formula:

$$\%\ of\ cell\ migration = \frac{(A_0 - A_n)}{A_0}\ x\ 100$$

Where, A_0 = Average of initial scratch area, and A_n = Average of scratch area at the measured time

An increase in the percentage of closed area indicated an increase in cell migration [(11)].

Statistical analysis

All the experiments were carried out in triplicates and the results are stated as mean ± standard deviation (SD) from three separate experiments. One-way analysis of variance (ANOVA) analysis was performed using the GraphPad Prism 8. A p-value greater than 0.05 was regarded as a statistically significant.

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Results and Discussion

Identification and structure elucidation of the isolated compounds

The methanol extract of *E. hirta* (10 g) was fractionated using silica gel to afford five fractions, where F3 and F4 were afforded pure compounds **1** and **2**, respectively.

Compound **1** was obtained as a yellow powder with a molecular weight of *m/z* 285.1684 [M-H]-, [calculated molecular mass $C_{15}H_{10}O_6$: 286.2378] (Figure 2). The FTIR spectrum of compound 1 showed absorption of O-H stretching at3307.92 cm^{-1} which was supported by the absorption of C-O stretching at 1170.70 cm^{-1}.(Figure1). There is a strong absorption at 1656.85 cm^{-1} which was a characteristic of C=O stretching absorption. The peaks at peak 3151.69 cm^{-1} indicated the stretching of C-H of an alkene. The presence of aromatic functional group was confirmed with the bending absorption at 817.82 cm^{-1}. These data are comparable to the spectral data for kaempferol reported earlier by Telange [(12)].

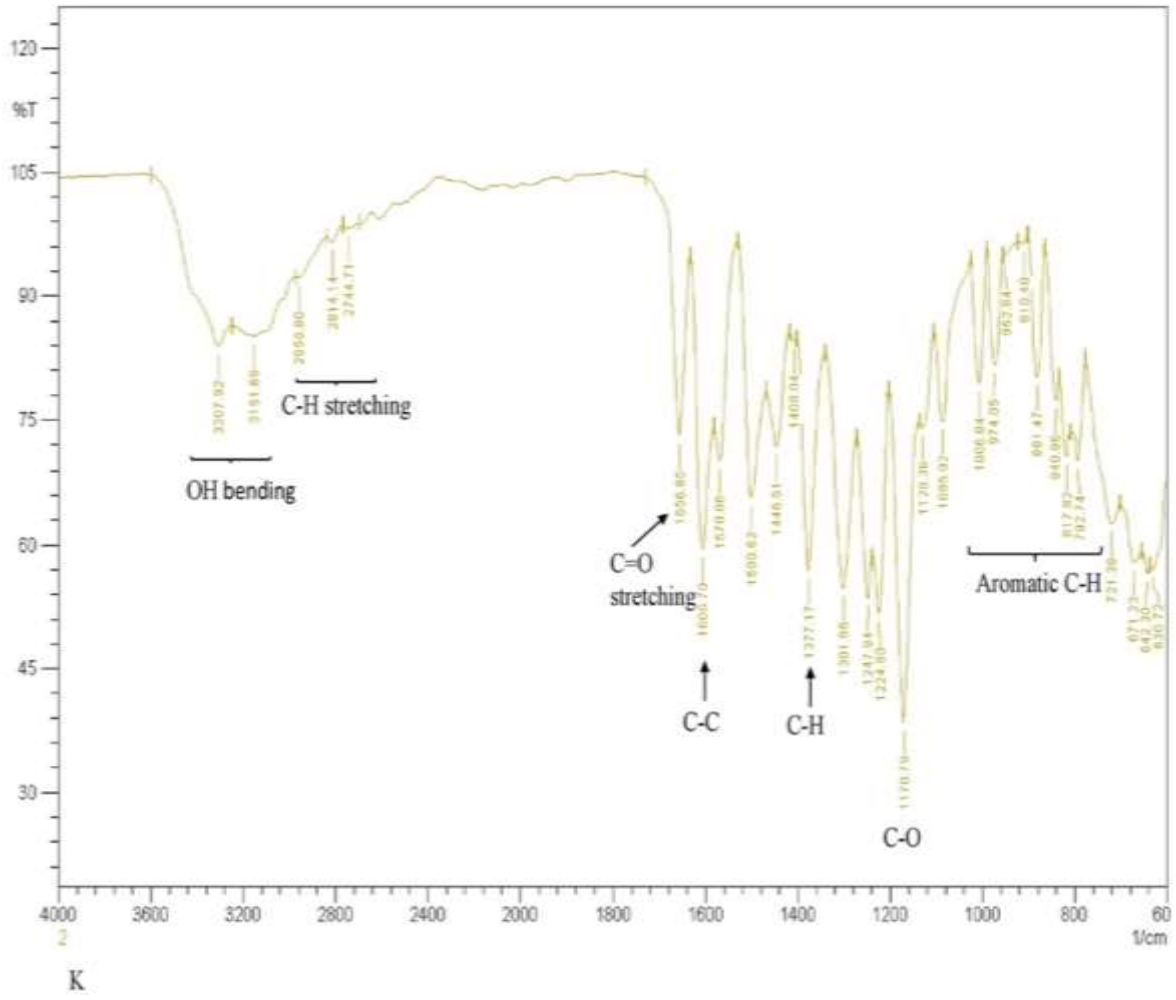


Figure 1. FTIR spectrum of Compound 1.

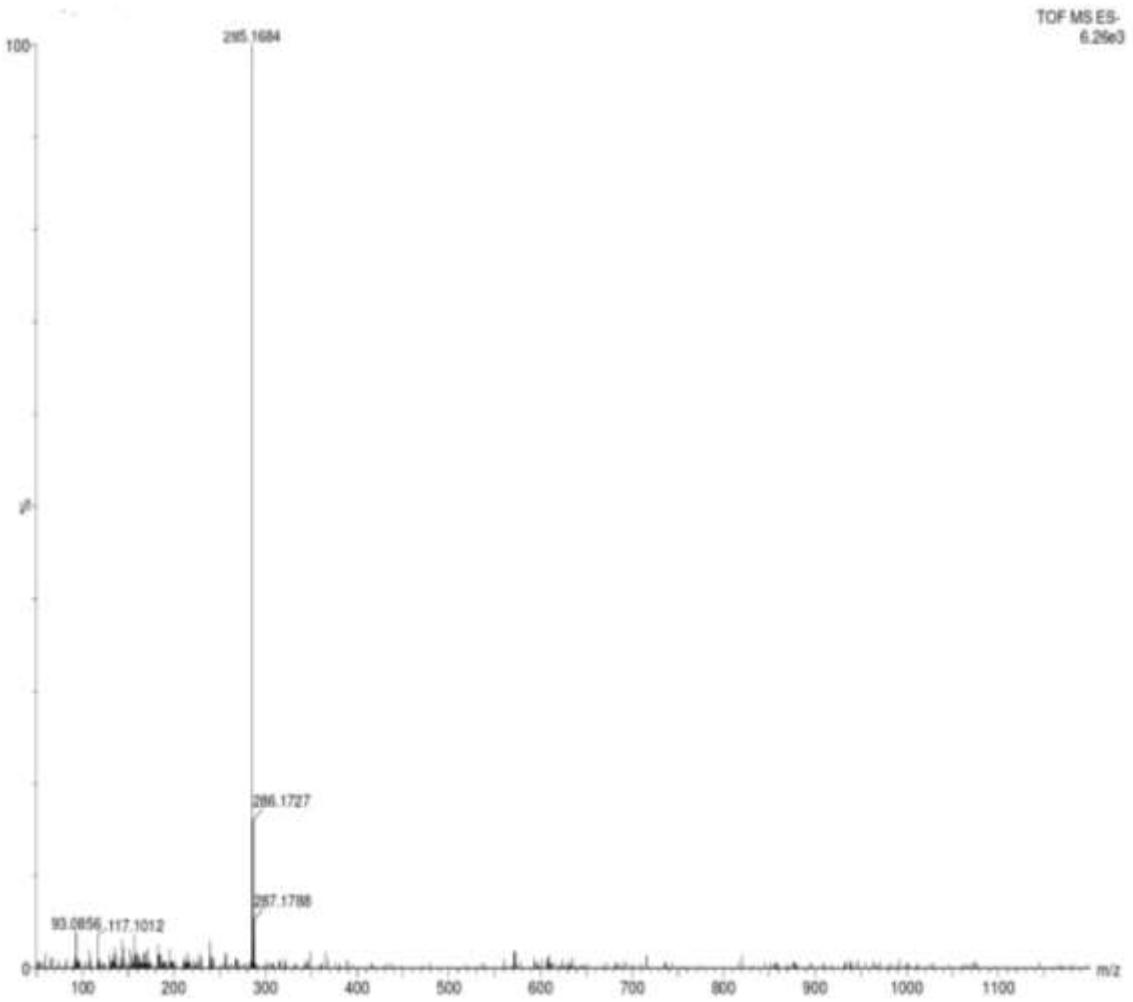


Figure 2. Mass spectrum of compound 1.

The inspection of ^{13}C-NMR spectrum of **1** displayed 15 distinct peaks, which were assigned to the various parts of the kaempferol skeleton and were observed in the range of 94.72 to 177.58 ppm. While the ^{1}H-NMR spectrum of compound **1** showed characteristic signals resonated in the aromatic area between 6 to 8 ppm. Four sets of aromatic protons for six protons; two set of singlet protons(δ_H/δ_C 6.18/99.8; 6.37/94.8;), two sets of doublet aromatic protons (δ_H/δ_C 6.87/116.5; 8.05/130.9) (Figure 3). Moreover, there is a characteristic peak of phenolic proton resonated at (δ_H/δ_C 12.24/162.2). Its ^{13}C-NMR spectrum, as shown in Table (1), showed the presence of 15 carbon signals which were typical of a flavanol skeleton (Figure 4). The correlation between carbons and protons atoms were further confirmed by Heteronuclear Single Bond Coherence (HSQC) and Heteronuclear multiple bond coherence (HMBC) correlations (Figure 5&6). Thus, based on the mass data, 1D and 2D-NMR spectral data, as well as comparison with literature data [13]. Compound **1** was identified as 3,4',5,7-tetrahydroxyflavone or kaempferol as shown in Figure (13).

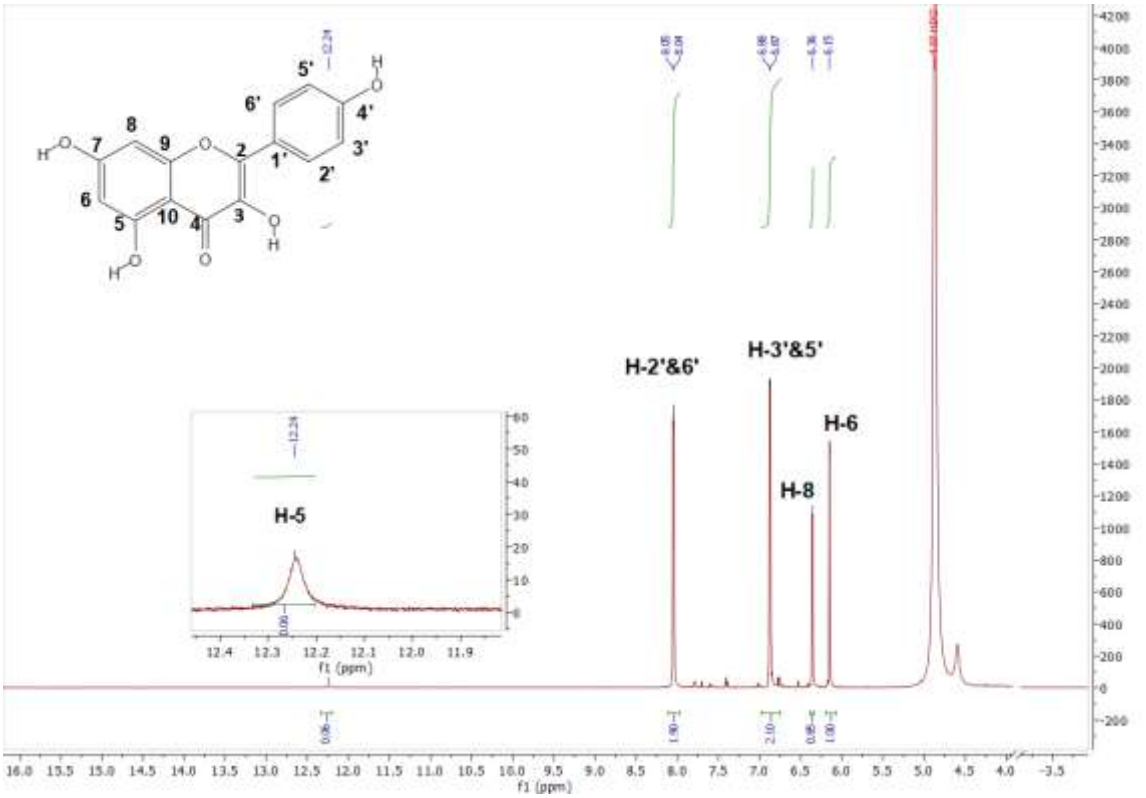


Figure 3 . ^{1}H NMR spectrum of Compound 1.

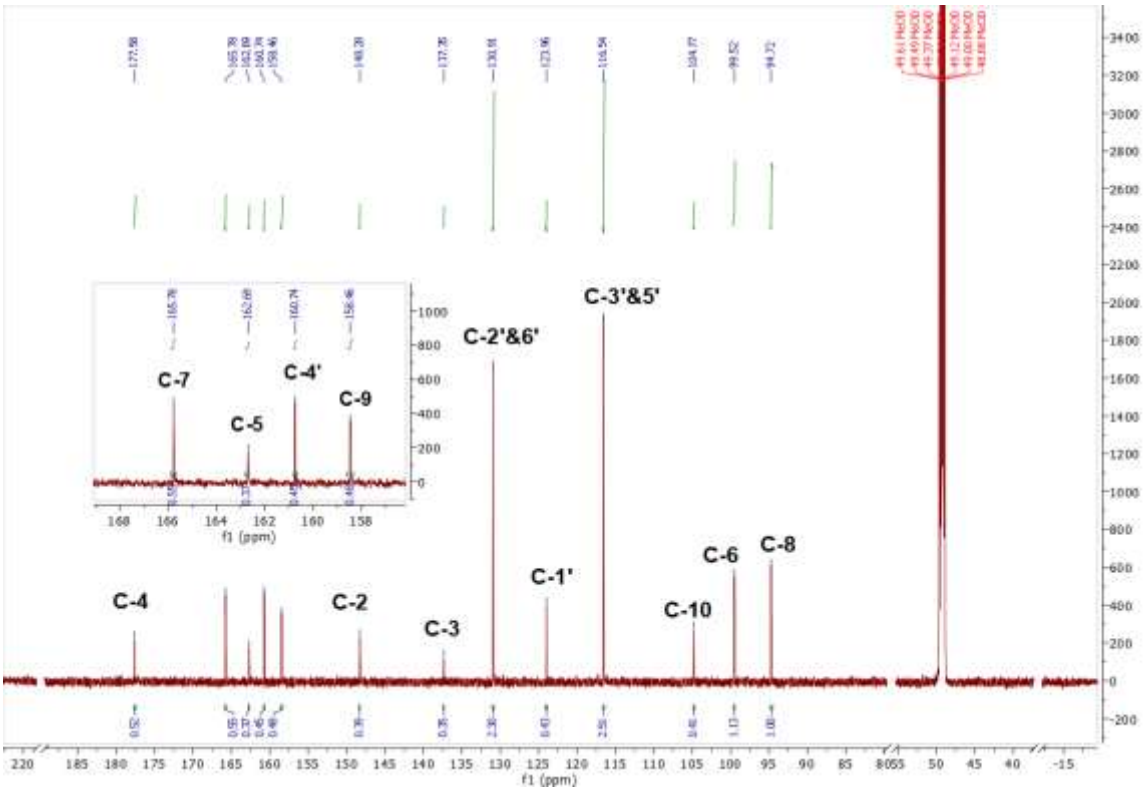


Figure 4. ^{13}C NMR spectrum of Compound 1.

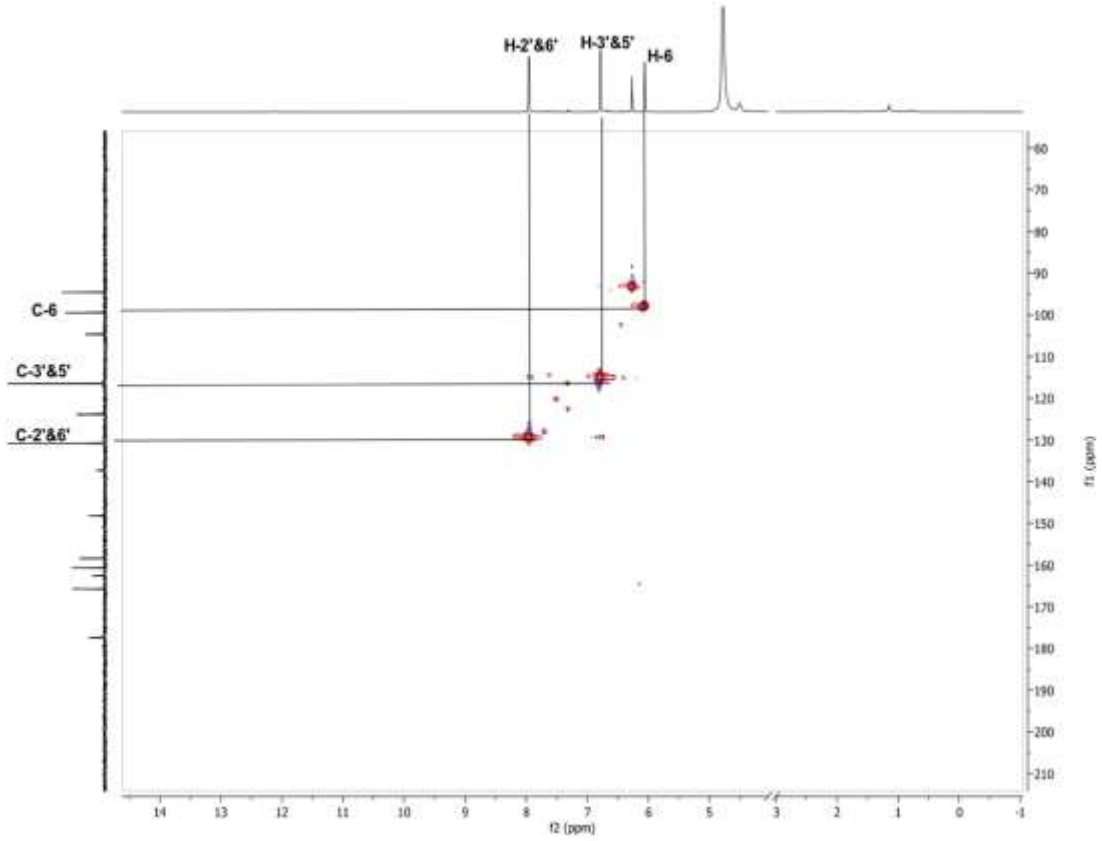


Figure 5. HSQC spectrum of Compound 1.

Figure 6. HMBC spectrum of Compound 1.

Compound **2** was afforded as a yellow amorphous solid powder with a molecular weight of m/z 447.0937 $[M-H]^-$ (calculated molecular weight, 448.1006 for $C_{21}H_{20}O_{11}$) (Figure 8). The FT-IR spectrum revealed the presence of hydroxyl groups indicated by a broad absorption at 3261.63 cm-1. The strong absorption at 1647.21 cm-1 - indicated a carbonyl group, 1552.70 while a medium absorption at 1469.76 cm-1 indicated an absorption for C=C stretching. Additionally, absorption at 1178.51 cm-1 indicated bending absorption for C-O (Figure 7). The obtained spectral data align with previously published data for compound **2** [14].

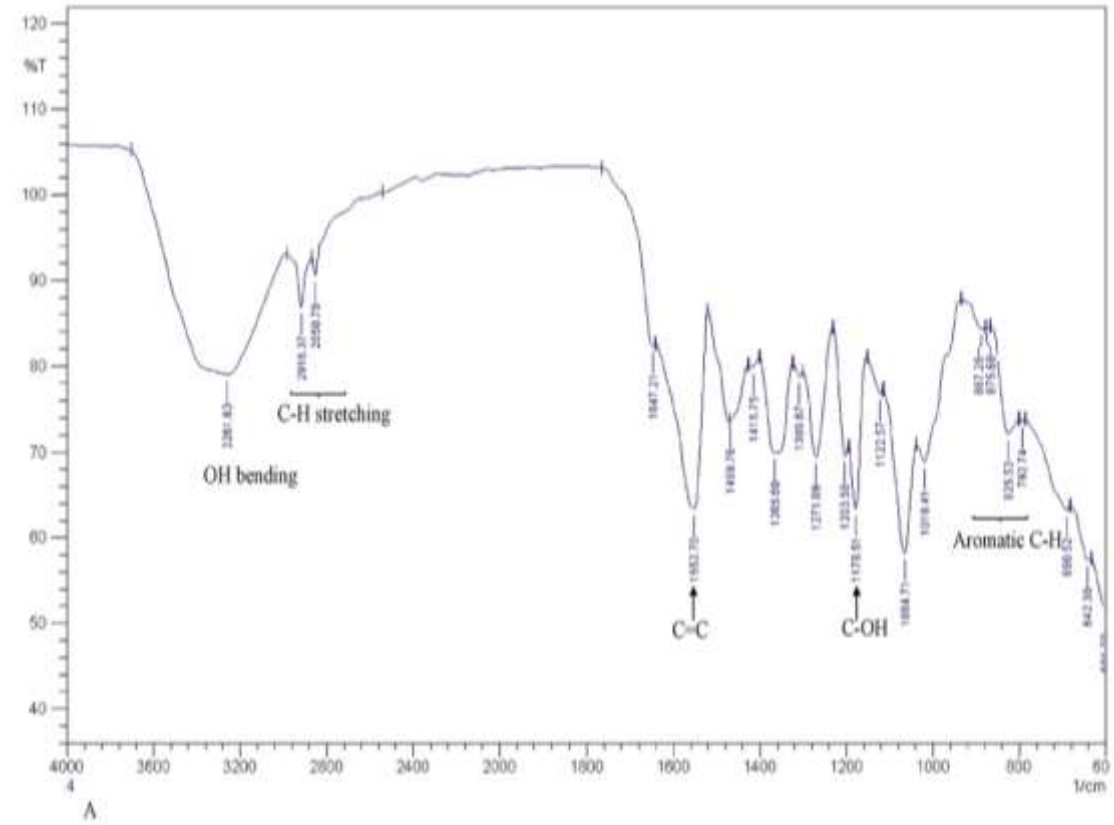


Figure 7. FTIR spectrum of Compound 2.

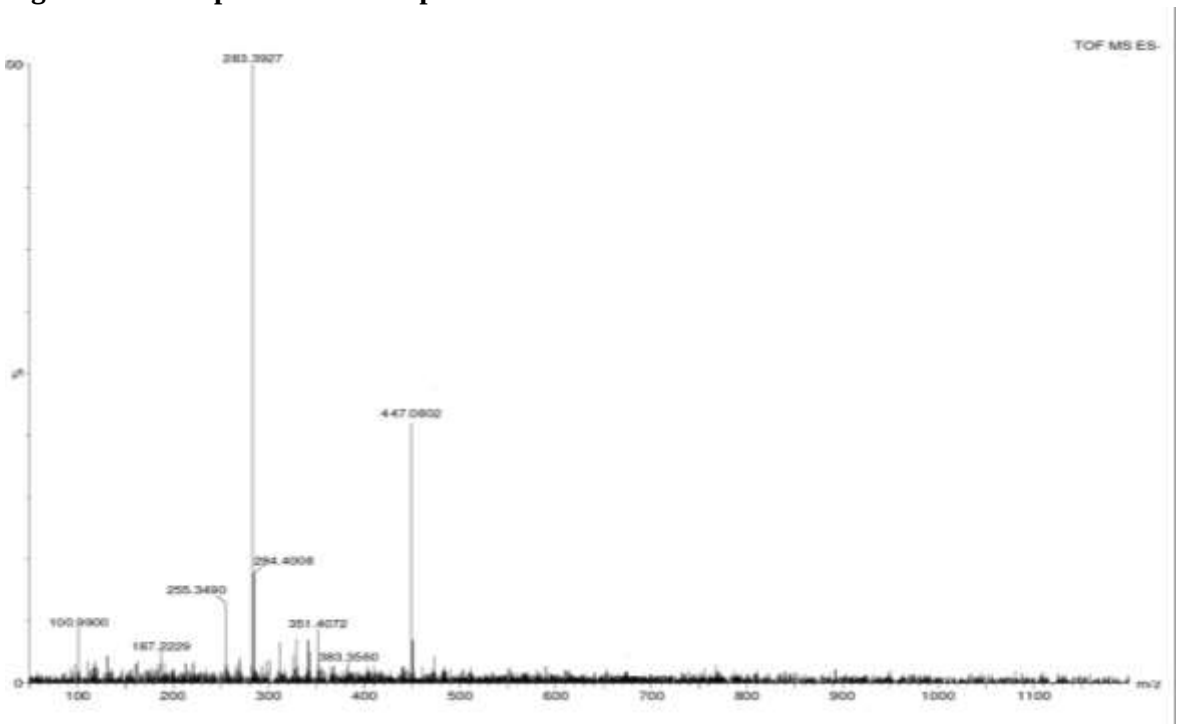


Figure 8. Mass spectrum of Compound 2.

The ^{13}C-NMR spectrum showed the presence of 21 carbon signals typical of a flavanol glycoside skeleton, Table (2). Where peaks resonated in the area of 50-80 ppm showed the characteristic peak of glycone part of **2**. On the other hand, inspection of ^{1}H-NMR spectrum showed the appearance of signals resonated between (δ_H 3-8 ppm), with a complex signal at δ 3.23-5.25 ppm are likely due to the oxymethine protons of a sugar group (Figure 9). Characteristic signals for a flavanol glycoside were also observed including: two set of singlet aromatic protons (δ_H/δ_C 6.37/94.7; 6.18/99.6), four sets of doublet aromatic protons (δ_H/δ_C 3.71/62.6; 5.26/105.7; 6.87/116.0; 8.05/132.2) (Figure 10). Moreover, there is a characteristic peak of phenolic proton resonated at (δ_H/δ_C 12.24/161.5). The correlation between carbon and proton atom where further confirmed by Heteronuclear Single Bond Coherence (HSQC) and Heteronuclear multiple bond coherence (HMBC) correlations (Figure 11&12). Thus, based on the 1D and 2D-NMR spectral data, as well as comparison with the literature [15, 16], the chemical structure of Compound **2** was defined as kaempeferol-3-O-glucoside (astragalin) as shown in Figure (14).

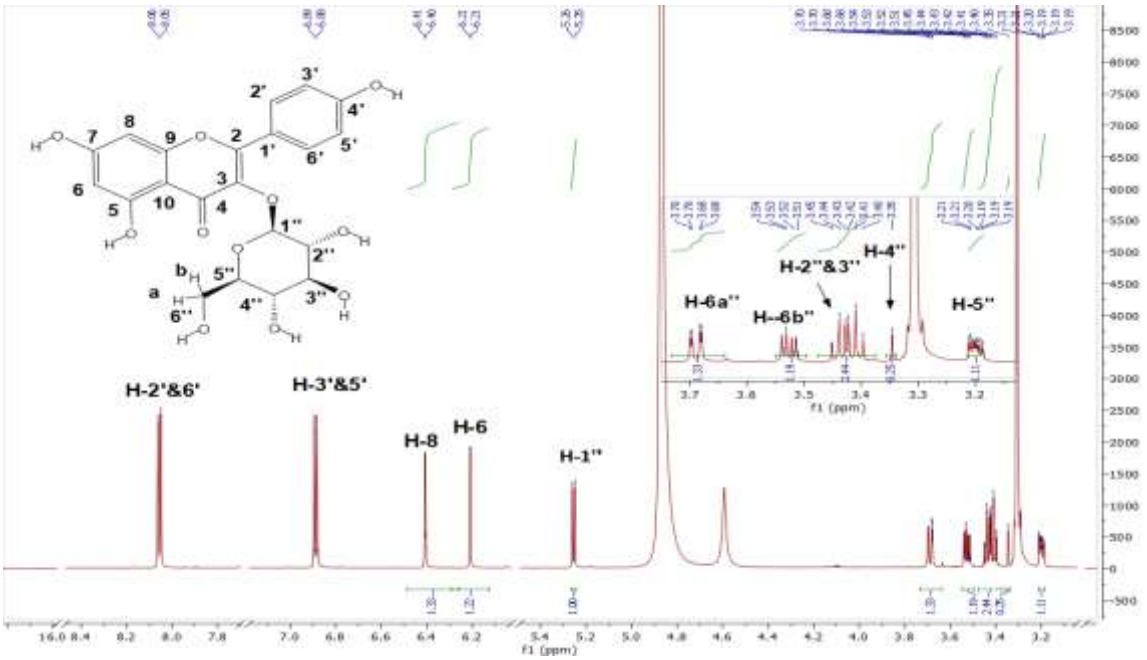


Figure 9.^{1}H NMR spectrum of Compound 2.

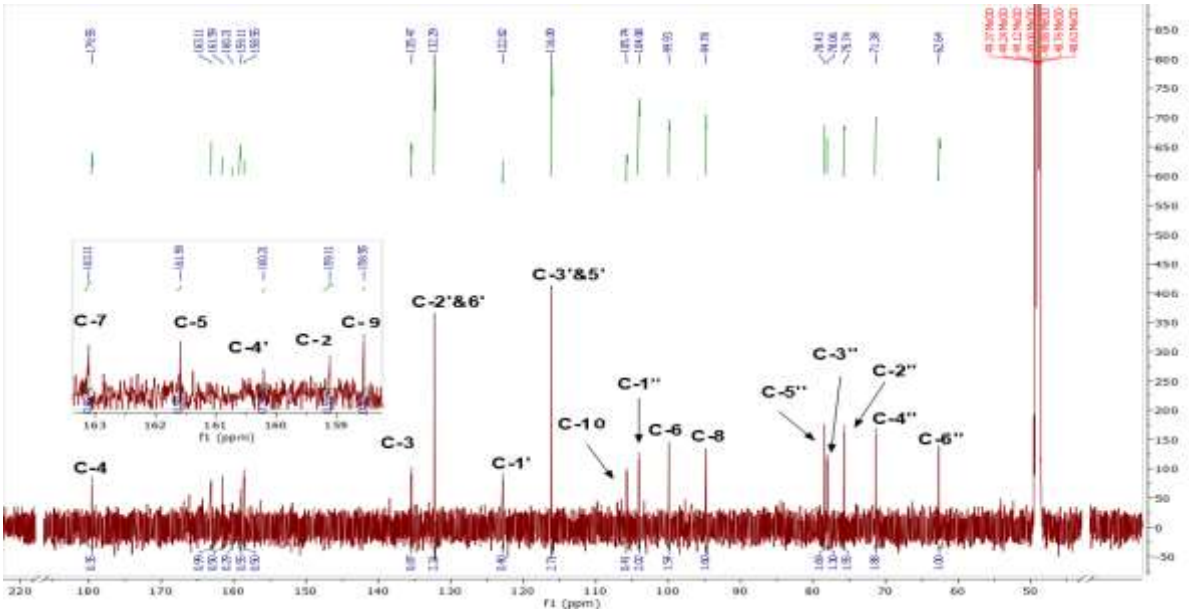


Figure 10. ^{13}C NMR spectrum of Compound 2.

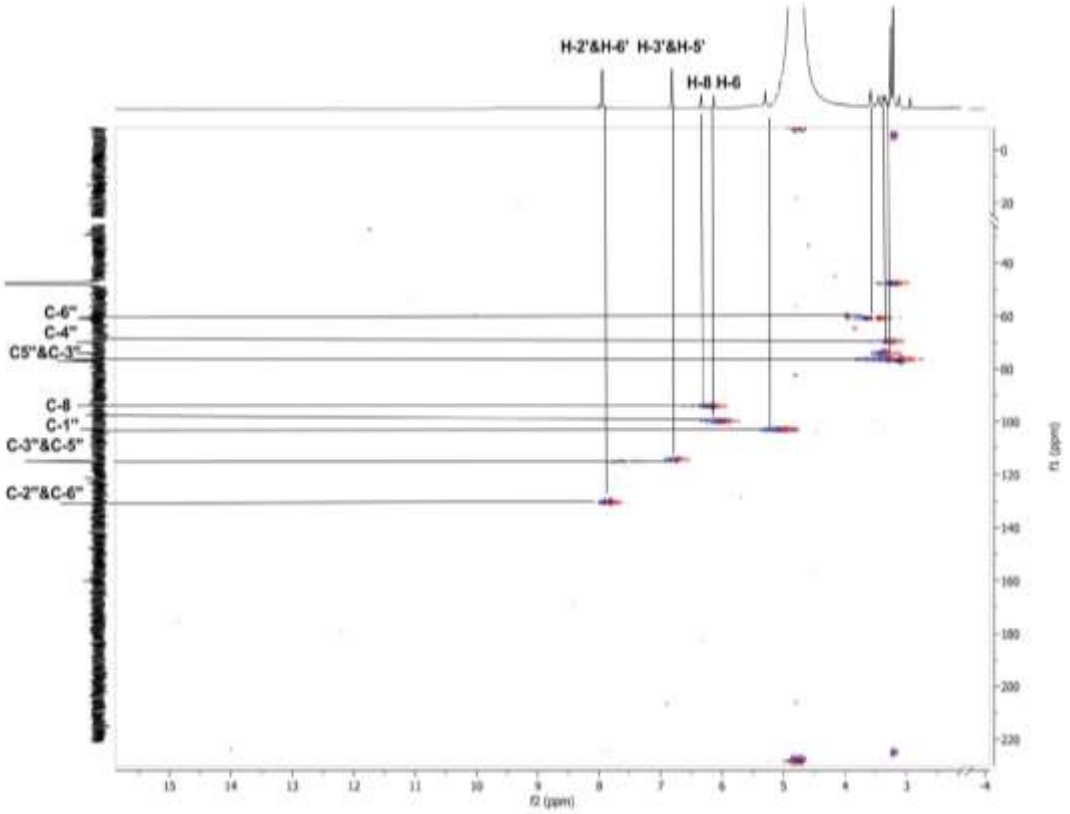


Figure 11. HSQC spectrum of Compound 2.

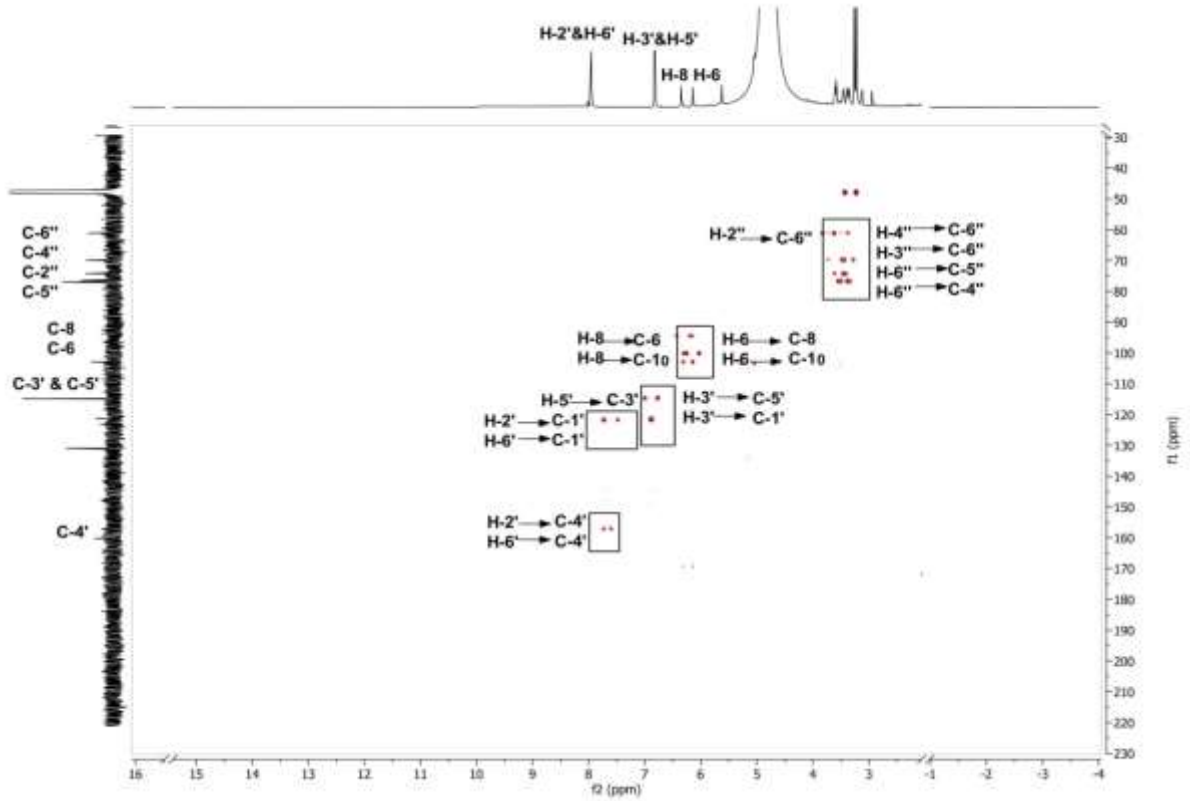


Figure 12. HMBC spectrum of Compound 2.

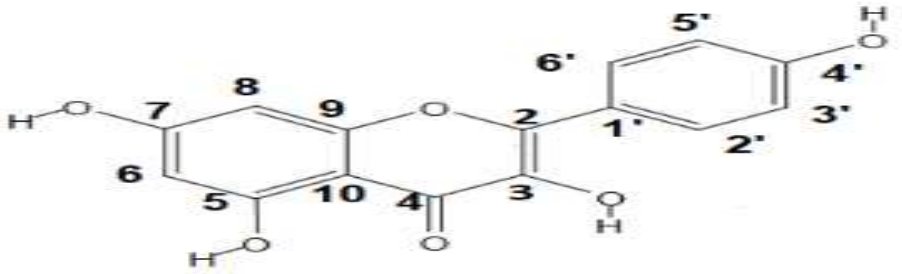


Figure 13. Structure of Compound 1; 4',5,7-tetrahydroxyflavone (kaempferol).

Position	Type of Carbon	Experimental ^{13}C-NMR	Experimental ^{1}H NMR	^{13}C NMR Ref (13).	^{1}H NMR Ref (13).
2	QC	147.0		148.2	
3	-C(OH)	137.1		137.7	
4	C=O	177.3		177.8	
5	-C(OH)	162.2	12.24(s,1H,OH)	162.6	12.47(s,1H)
6	CH(=)	99.8	6.18 (s,1H)	99.4	6.18 (d,1H)
7	-C(OH)	165.8		165.7	
8	CH(=)	94.8	6.37 (s,1H)	94.3	6.42 (s,1H)
9	QC	158.2		158.4	
10	QC	104.0		104.7	
1'	QC	123.7		123.8	
2'	CH(=)	130.9	8.05 (d,2H)	130.6	8.05 (d,2H)
3'	CH(=)	116.5	6.87 (d,2H)	116.4	6.93 (d,2H)
4'	-C(OH)	160.5		160.4	
5'	CH(=)	116.5	6.87 (d,2H)	116.4	6.93 (d,2H)
6'	CH(=)	130.9	8.05 (d,2H)	130.6	8.05 (d,2H)

Table 1. ^{13}C-NMR and ^{1}H- NMR data of Compound 1.

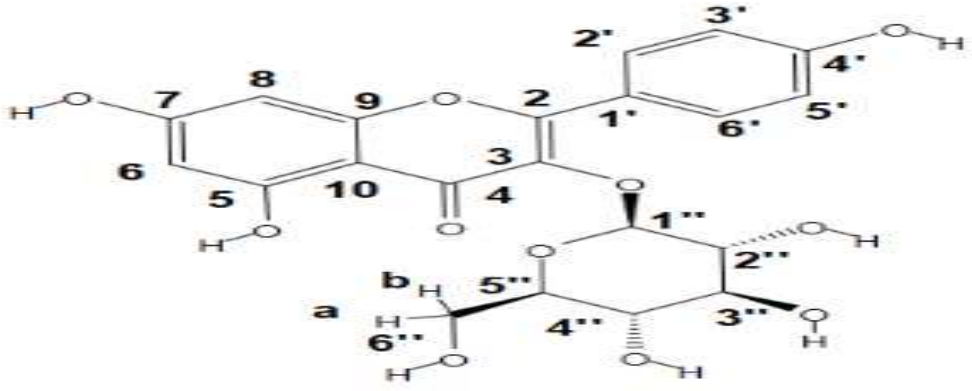


Figure 14. Structure of compound 2; kaempeferol-3-O-glucoside (astragalin).

Table 2. 13C-NMR and 1H- NMR data of Compound 2.

Position	Type of Carbon	Experimental ^{13}C-NMR	Experimental ^{1}H- NMR	^{13}C NMR Ref (16)	^{1}H NMR Ref (16)
2	QC	159.9		162.8	
3	-C(OH)	135.4		135.6	
4	C=O	179.7		179.5	
5	-C(OH)	161.5	12.24(s,1H)	161.4	
6	CH(=)	99.6	6.18 (s,1H)	99.3	6.22 (s,1H)
7	-C(OH)	163.5		165.8	
8	CH(=)	94.7	6.37 (s,1H)	94.8	6.41 (s,1H)

9	QC	158.5		159.2	
10	QC	104.0		105.6	
1'	QC	122.8		122.5	
2'	CH(=)	132.2	8.05 (d,2H)	132.3	6.91 (d,2H)
3'	CH(=)	116.0	6.87 (d,2H)	116.1	8.07 (d,2H)
4'	-C(OH)	160.2		158.4	
5'	CH(=)	116.0	6.87 (d,2H)	116.1	8.07 (d,2H)
6'	CH(=)	132.2	8.05 (d,2H)	132.3	6.91 (d,2H)
1''		105.7	5.26 (d,1H)	103.8	
2''		75.7	3.45(m,1H)	75.6	3.46(dd,1H)
3''		78.0	3.41(m,1H)	77.9	3.37(m,1H)
4''		71.3	3.35(m,1H)	71.3	3.24(m,1H)
5''		78.4	3.21(m,1H)	78.4	3.56(m,1H)
6''		62.6	3.71(dd,1H)	62.6	3.70(d,2H)

In vitro wound healing assay

Cell viability and proliferation assay

Cell migration and proliferation play a essential function in skin regeneration and wound healing and they have important role in the study and discovery of drugs. Previous studies revealed that the isolated compounds, which are the major flavonoids components of *E. hirta* methanolic extracts, were responsible for wound healing activity healing [3, 17]. Our study showed that both isolated compounds (3.12, 6.25, 12.5, 25, 50 and 100 µg/mL) were able to stimulate cell population in a dose-dependent manner with no sign of any toxicity, Figure (15). The number of viable Hs27 cells after 24 hours of treatment were almost similar in isolated compounds (kaempferol and astragalin) treated cells at the concentration of 25, 50 and 100 µg/ml, which is comparable with control culture. Furthermore, after 48 and 72 hours, an increase in the number of viable Hs27 cells was observed with increasing culture time and at all culture concentrations. These results revealed that the isolated compounds at a concentration of ≤50 µg/mL have no negative effect on the viability or proliferative ability of Hs27 cells during in vitro culture. However, at higher concentration of 100 µg/mL, the isolated compounds had an anti-proliferative effect on cultured Hs27 cells. Moreover, kaempferol showed the highest proliferation percentage at a concentration of 25 µg/mL, with 173% (post-24 hours) and 248% (post-48 hours) before decreasing to 175% after 72 hours, as shown in Figure (16).

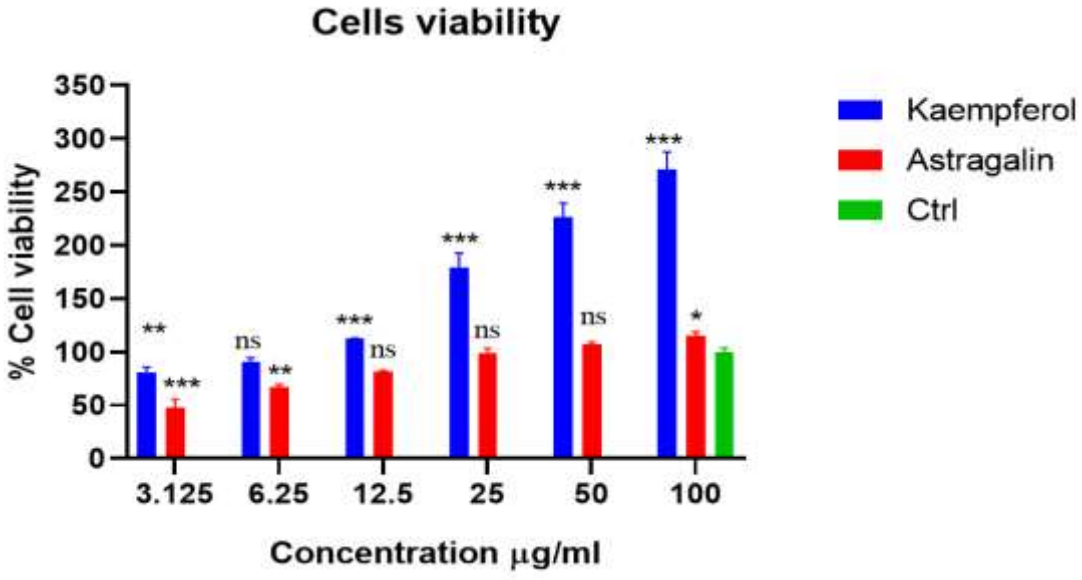
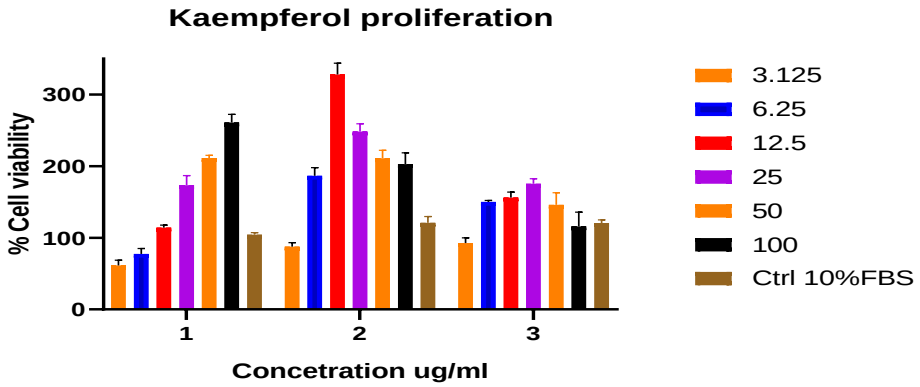


Figure 15. Percentage of cell viability of Hs27 cells after treatment with the isolated compounds kaempferol and astragalin in comparison to control (untreated cells). Each value is mean ± S.E.M (n = 3). (*) indicates statistically significant difference from control group using ANOVA, followed by Dunnett's

multiple comparison test (*p <0.01) (*p < 0.0001), (ns) indicates statistically no significant difference from control group (p > 0.05).**

a)



b)

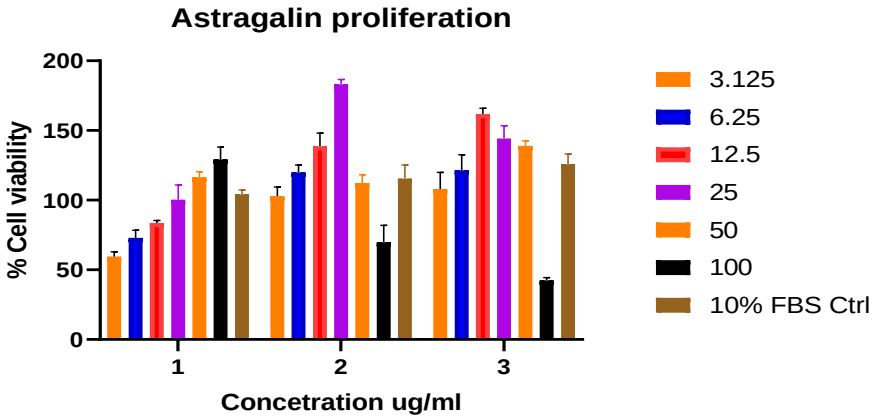


Figure 16. Proliferation percentage of Hs27 cells after treatment with the isolated compounds (a)kaempferol and (b) astragalin. Each value is mean ± S.E.M (n = 3).

Scratch-wound assay

Fibroblasts are crucial in wound healing, with their migration ability serving as a predictor in *in vitro* wound healing assays and being necessary for the regeneration and restoration of injured skin tissue. Upon injury, fibroblast migration and proliferation are triggered as a repair mechanism [18]. The concentration for the current study was determined based on the results of the cell viability study. The results showed that the isolated compounds significantly induced cell populations at test concentration ranging from 12.5 to 50 µg/mL as showed in Figure (15 and 16). At a result, 25 µg/mL was selected as the concentration for all extracts for the cell migration study. Treated cell groups with kaempferol and astragalin resulted in significant increase of the cell migration rates compared to untreated cells group control after 24 and 48 hours of treatment. The signs of cell migration were seen as early as 24 hours when the migration had reached nearly 50% and by 48 hours, complete wound closures were attained. Figure (17) shows the cell migration into the wound area of isolated compounds. Figure (18) show the migration percentage (% wound closure) resulting from treatment with the isolated compounds.

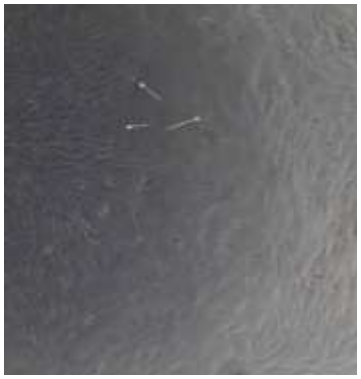
Time	(a) Control	(b) Kaempferol	(c) Astragalin
0 hours			
24 hours			
48 hours			

Figure 17. Cell migration of Hs27 fibroblast cells: (a) control (untreated cells) (b) kaempferol-treated cells and (c) astragalin-treated cells. Magnification 100× .

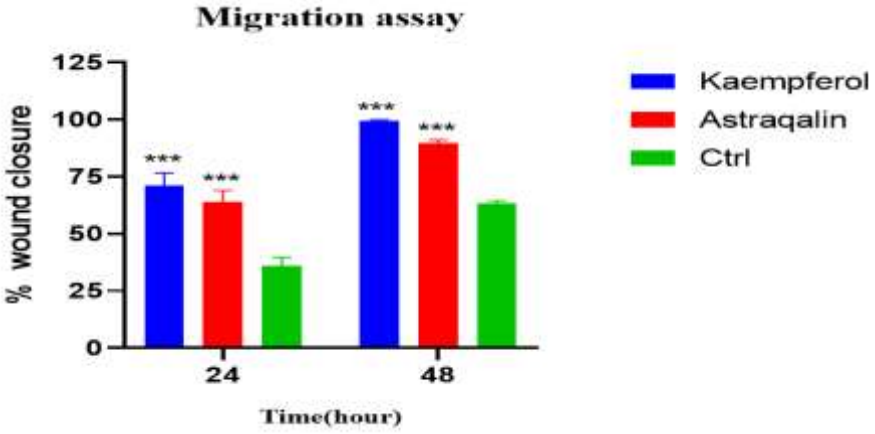


Figure 18. Cell migration percentage of Hs27 cells after treatment with the isolated compounds (a) kaempferol and (b) astragalin. Each value is mean ± S.E.M (n = 3). (*) indicates statistically significant difference from control group using ANOVA, followed by Dunnett's multiple comparison test (*p <0.01) (*p < 0.0001), (ns) indicates statistically no significant difference from control group (p > 0.05).**

Upon comparing the migration percentage of cells treated with these compounds, we found that after 24 hours of treatment, the astragalin treated cells had the highest migration percentage at 80.36%. However, after 48 hours of treatment, the kaempferol treated cells showed the highest migration percentage at 100%, while the astragalin treated cells had a migration percentage of 86.52%. These results can be seen in Figure (18). These findings suggest that the effect of the isolated compounds on cell migration may vary over time. Further research is needed to fully understand the mechanisms behind these differences and the potential clinical implications of these findings.

Flavonoids are a diverse group of natural compounds that have been shown to have various health benefits, including the ability to accelerate wound healing. This property may be effective in preventing scar formation and slowing down the aging process of the skin [19]. In addition, the antioxidant, anti-inflammatory and antimicrobial properties could be due to the flavonoids work that work to heal the wounds [20]. Kaempferol, a flavanol compound found in medicinal plants, has been shown to possess various pharmacological properties such as anti-inflammatory, antibacterial, and antioxidant activities [21]. Multiple studies have also shown that kaempferol exhibits wound healing properties in both diabetic and non-diabetic rats [22, 23]. Study by Ozay proved the healing effects of kaempferol on diabetic and nondiabetic rats by incisional and excisional wounds model. These properties are thought to be mediated by increasing the amount of hydroxyproline and collagen in the wound, improving wound resistance, and facilitating re-epithelialization. Based on these findings, kaempferol may have potential as a drug for the treatment of chronic wounds, including diabetic wounds and surgical wounds [23].

Flavanol glycosides, including astragalin, have also been shown to stimulate fibroblast proliferation and collagen synthesis, increase extracellular matrix (ECM) formation and accumulation, and induce keratinocyte re-epithelialization [19]. Astragalin (kaempferol-3-O-β-D-glucoside), a bioactive natural flavonoid glycoside found in a variety of plants [16], has a wide range of pharmacological properties including anticancer, anti-inflammatory, and antioxidant properties, which have been shown to promote wound healing [24]. These findings align with previous research indicating that flavonoids can promote wound healing through their antioxidant, anti-inflammatory, and antimicrobial properties. Previous study also found an evidence to support these activities as being beneficial for wound healing through their antioxidant, anti-inflammatory and antimicrobial activities [17]. These findings suggest the potential of flavonoids as a natural and effective means of promoting wound healing and underscore the need for further

research to fully understand the mechanisms behind these effects and the potential clinical applications of these compounds [19].

Conclusion

In conclusion, the study has led to the isolation of two active compounds, kaempferol and astragalin, from *E. hirta* methanolic extract. These compounds showed a significant increase in wound healing activity by enhancing the migration, proliferation, and viability of human fibroblast cells in an *in vitro* model. This confirms the traditional use of the E. hirta plant for wound healing and suggests that it may be a promising source for the development of natural wound healing agents. However, further studies are needed to fully understand the pharmacodynamics and pharmacokinetics of these isolated compounds, as well as the molecular mechanisms and pathways involved in their wound-healing effects. In addition, further *in vivo* studies using animal models will be necessary to verify the role of *E. hirta* in wound healing.

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Conflicts of Interest

The authors declare no conflict of interest.

Author Contribution

The authors confirm contribution to the paper as follows: study conception and design: N.H.K.A., D.F.A., M.B.A.; data collection: D.F.A., M.B.A., A.Y.; analysis and interpretation of results: D.F.A., M.B.A., R.D.; draft manuscript preparation: D.F.A., M.B.A., R.D, N.H.K.A., Admin, technical or material support: S.R.Y., N.A.H, A.Y. All authors reviewed the results and approved the final version of the manuscript.

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