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ACTIVITY GUIDED ISOLATION OF CHEMICAL CONSTITUENTS FROM THE BIOLOGICALLY ACTIVE METHANOL EXTRACT OF *EUPHORBIA SCHIMPERI* C. PRESL

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ABSTRACT. In this study we investigated the chemical constituents of bioactive methanol extract of *Euphorbia schimperi* C. Presl. For this the methanol extract was fractionated into 20, 40, 60, 80% MeOH in $CHCl_3$, and 100% MeOH fractions respectively by vacuum liquid chromatography. Excision wound surface of the animals were topically treated with these fractions at a dose of 100 mg/kg body weight for twenty days. Povidone-iodine ointment was used as a reference drug. Wound contraction measurement and period of epithelialization were used to assess the effect of fractions on wound repairing. The 100% MeOH fraction treated animals achieved significant ($p < 0.001$) value by showing 100% wound contraction and minimum period of epithelization (17.75±0.47) on the 20th day as compared to standard drug treated animals on the same day. The active 100% MeOH fraction was subjected to various chromatographic techniques led to the isolation of miquelianin (**1**), kaempferol 3-*O*-glucuronide (**2**) and quercitrin (**3**). Compounds (**1-3**) were isolated from this plant for the first time.

KEY WORDS: *Euphorpia schimperi* C. Presl, Wound healing, Chromatographic techniques, Chemical constituents

INTRODUCTION

Euphorbia is the largest genus in the plant family *Euphorbiaceae* with ca 2000 known species [1], and well recognized for chemical diversity of their isoprenoid constituents [2]. The latex of these plants contains many natural compounds, some of which are of therapeutic rank or of commercial usage. The latex usually defends these plants from browsing animals because of its unpleasant or poisonous nature [3]. The plants of the *Euphorbia* species are reported to have triterpene alcohols in their latex used as chemotaxonomic markers [4]. Additionally other constituents like cerebrosides, phloracetophenones, glycerols, sesquiterpenoids, steroids, and flavonoids are also reported [2].

Euphorpia schimperi C. Presl. (Euphorbiaceae) is available in Wadi Gama, Kingdom of Saudi Arabia [5]. Previously *E. schimperi* chloroform fraction had characterized cycloart-25-en-3β,24-diol, cycloart-23-en-3β,25-diol, α-amyrin, β-sitosterol-β-D-*O*-glucoside, scopoletin, luteolin and kaempferol [6]. It was also reported to possess anticancer, antimicrobial and antioxidant activities [6, 7].

Our previous work [8] on this plant demonstrated that methanol extract of the plant have significant wound healing activity. In continuation of our work we fractionated the methanol extract and tested for the wound healing activity. Furthermore the fraction that was showing highest wound healing activity was investigated for its chemical constituents that led to isolation of three compounds (**1-3**). Structure of these compounds was established on the basis of 1D, 2D NMR and mass spectral analysis and by comparison with reported values in literature. Compounds **1-3** were identified as miquelianin, kaempferol 3-*O*-glucuronide [9] and quercitrin [10], respectively (Figure 1).

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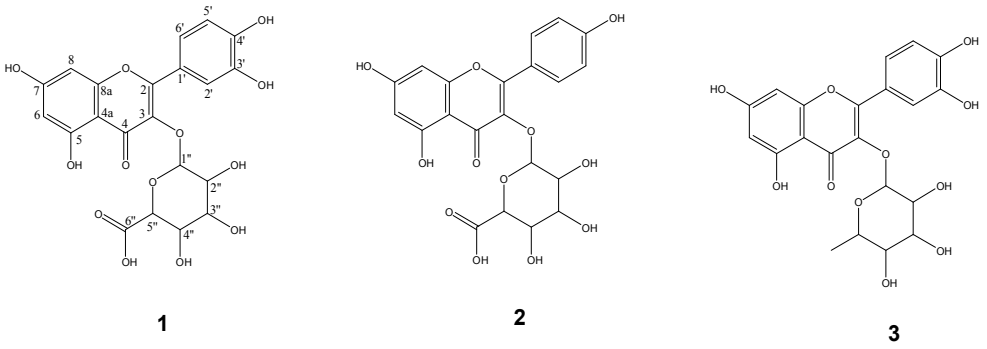

Figure 1. Structure of compounds **1-3**.

EXPERIMENTAL

General

The ^{1}H and ^{13}C NMR spectra were measured on a Bruker Avance spectrometer, operating at 700 MHz for ^{1}H and 175 MHz for ^{13}C. The ESI MS were obtained on an Agilent Mass spectrometer series 1100 SL. For analytical purposes TLC plates coated with UV_{254} fluorescence indicator (Merck) were used. Compounds were visualized under UV radiation in CAMAG UV cabinet dual wavelength, 254/366 nm and also by spraying with *p*-anisaldehyde/H_2SO_4 reagent to visualize spots. Silica gel 60 (230-400μm; Merck, Darmstadt) and Sephadex® LH-20, GE Healthcare, was utilized for column chromatography (CC). HPLC was performed on a Shimadzu system (Kyoto, Japan), consisting of two LC-6AD semi-preparative solvent delivery pumps, bus module CBM-20A, a multi wavelength photo-diode array detector (SPD-M20A), columns shim-pack PREP-ODS (H) Kit (A) 250 mm × 4.6 mm I.D. with 5 μm particles (B) 250 mm × 20 mm I.D. 5 μm.

Plant material

The plant was collected from Wadi Gama in February 2014, Kingdom of Saudi Arabia (KSA) and identified by taxonomist, Dr. M. Yousuf. A voucher specimen (16322) was deposited at the Herbarium of the College of Pharmacy, King Saud University, Riyadh, KSA.

Extraction and isolation

A 1.24 kg dried and ground material of plant was extracted with ethyl acetate followed by methanol at room temperature for 72 h (3×24 h). The extracts were filtered through Whatman No.1 filter paper and solvent was evaporated to dryness at 40 ^{o}C *in vacuo* using Buchi Rotavapor (Model R-215) yielded syrupy mass of ethyl acetate and methanol extracts. The methanol extract showed 100 % wound healing activity. Out of 90 g MeOH extract, 69 g was subjected to vacuum liquid chromatography and eluted in sequence with $CHCl_3$ and MeOH mixture gave five fractions and all fractions were tested for wound healing activity. The fraction obtained with 100% MeOH was found to possess significant activity and was considered for chemical investigation which led to the isolation of three compounds. The detailed isolation scheme is depicted in Figure 2.

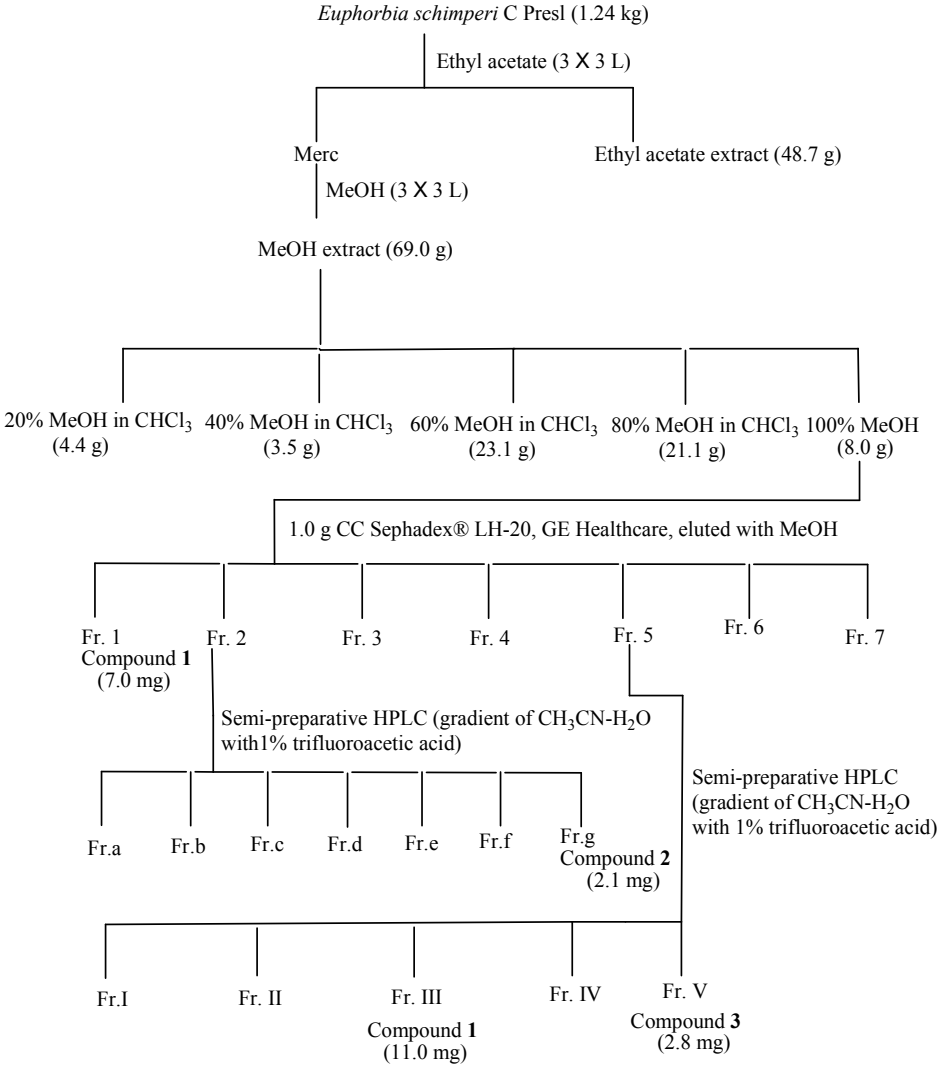

Figure 2. Isolation scheme.

Wound healing studies

Wistar albino rats of either sex used in these experiments were obtained from experimental animal house, College of Pharmacy, KSU, Riyadh. Animals used were of the same age group, weighing roughly about 180-200 g b.w. The experimental animals were accommodated under standard laboratory conditions, temperature (22 ± 2 ^{o}C), humidity (55%) and 12/12 hours light-dark environment. The animals were maintained on standard diet Purina chow and free access to drinking water *ad libitum*. The experimental protocol for animal work was approved (No. EACP7315) by Ethics Committee for Animal Experimentation, College of Pharmacy, KSU,

Riyadh, Saudi Arabia. All the experimental procedure exercised was according to the National Institutes of Health Guide for the Care and Use of Laboratory Animals, Institute for laboratory animals (NIH publication 8th edition, 2011).

The rats were anaesthetized with diethyl ether prior to and throughout the infliction of experimental wounds. The anesthetized rats inflicted with excision wounds as designed by Morton and Malone [11] and in our previous paper [8]. In short, the dorsal skin fur of the animals was shaved with an electric shaver, and the outline of the wound to be created was demarcated on the back of the animals with methylene blue using a circular stainless steel stencil. A full skin thickness of the excision wound of circular area of 254 mm^2 and 2 mm depth was formed along the markings with a surgical blade and pointed scissor.

The wounded animals were divided into seven groups of four animals in each group. Group 1 animals were left untreated (control group). Group 2 served as reference standard and treated topically with povidone-iodine (5%w/w), group 3 to 7 were treated topically with 20% MeOH in $CHCl_3$ fraction, 40% MeOH in $CHCl_3$ fraction, 60% MeOH in $CHCl_3$ fraction, 80% MeOH in $CHCl_3$ fraction and 100% MeOH fraction, respectively, at a dose of 100 mg/kg b.w. The fractions and standard drugs were applied topically once a day, starting from the day of wound creation, till complete epithelialization. The animals were housed individually in cages. The wounds were traced on mm^2 graph paper on the day of 4, 8, 12, 16 and 20 post wounding days. The percentage of wound closure (% contraction), and period of epithelialization were calculated.

Statistical analysis

Values are given as arithmetic means ± standard error of the mean (S.E.M.). Data was statistically analyzed by using one-way analysis of variance (ANOVA) followed by Student's t-test. The data were considered significant at $p < 0.001$.

RESULTS

Wound healing

The wound healing efficacy of the methanol extract fractions (20% MeOH in $CHCl_3$, 40% MeOH in $CHCl_3$, 60% MeOH in $CHCl_3$, 80% MeOH in $CHCl_3$ and 100% MeOH) used in the study is summarized in Table 1 and 2 and Figure 3. The parameters used in this excision wound model study were percentage wound closure and mean epithelialization time and these two parameters were measured on 0, 4, 8, 12, 16, and 20 days. During the course of study it was found that the wound contracting ability of 20% MeOH in $CHCl_3$ and 100% MeOH fractions treated group animals showed noticeable wound contraction and reduction in the epithelialization period from the fourth day onwards, when compared with that of standard drug treated group animals. On the fourth day the 20% MeOH in $CHCl_3$ fraction treated animals showed 14.66% of wound closure which was very near to that of standard drug treated animals 15.24% while 100% MeOH fraction showed higher 18.88% of wound closure compared to that of standard drug treated animals. Conversely on the 8th day the wound closure percentage was found 39.23% and 39.39% in 20% MeOH in $CHCl_3$ and 100% MeOH treated animals as compared to standard drug treated group animals (40.48%). While on 12th day of study 20% MeOH in $CHCl_3$ fraction treated animals showing 70.31% of wound closure which is slightly higher than 100% MeOH fraction treated animals (69.18%) as well as standard drug treated animal (68.51%). On the 16thday, 96.14 % wound shrinkage was found in 100% MeOH fraction treated animals compared to that of standard drug treated animals (96.39%) while on the same day 20% MeOH in $CHCl_3$ fraction treated animals showing 95.66% wound closure. On the 20th day of experiment only 100% MeOH fraction treated animals achieved significant value ($p <$

0.001) and showed 100% wound closure and minimum period of epithelialization which was the same as that of standard drug treated animals.

The percentage of wound closure of 20% MeOH in $CHCl_3$ fraction treated animals increased gradually from 4th to 20th day and reached to 99.43%. This may be attributed due to the presence of other chemical constituents which are not present in 100% MeOH fraction because the TLC profile of 20% MeOH in $CHCl_3$ fraction was totally different from 100% MeOH fraction. On the other hand, the animals treated with 40% MeOH in $CHCl_3$, 60% MeOH in $CHCl_3$ and 80% MeOH in $CHCl_3$ fractions were not showing considerable activity. Since the wound healing effects of 100% MeOH fraction was found highest (100% wound contraction) encouraged us to investigate this fraction for its chemical constituents.

Table 1. Effect of extracts on excision wound healing potential in rats (wound area mm^2)

Groups	Dose mg/kg	Wound Contraction (%)					
		0 Days	4th	8th	12th	16th	20th
1		00	3.16	17.12	42.96	71.22	80.96
2	Topical	00	15.24	40.48	68.51	96.39	100
3	100	00	14.66	39.23	70.31	95.66	99.43
4	100	00	8.17	15.60	55.67	76.51	91.70
5	100	00	5.08	16.99	55.26	80.62	91.64
6	100	00	9.02	22.42	61.96	87.93	98.27
7	100	00	18.88	39.39	69.18	96.14	100

Data are mean of 4 male in each group ± SD; *p < 0.05, **p < 0.01, ***p < 0.001 student's t-test. Group 1 Untreated animals. Group 2 Animals treated with povidone-iodine. Group 3 Animals treated with 20% MeOH in $CHCl_3$ fraction. Group 4 Animals treated with 40% MeOH in $CHCl_3$ fraction. Group 5 Animals treated with 60% MeOH in $CHCl_3$ fraction. Group 6 Animals treated with 80% MeOH in $CHCl_3$ fraction. Group 7 Animals treated with 100% MeOH fraction.

Table 2. Effect of extracts on percent wound contraction in excision wound in rat.

Groups	Dose mg/kg	0 Days	4th Day	8th Day	12th Day	16th Day	20th Day	Period of epithelization (days)
1		333.25 ±2.97	322.80 ±2.44*	276.27 ±5.47***	190.12 ±8.36***	95.92 ±7.24***	63.45 ±6.84***	24.50±0.64
2	Topical	335.25 ±1.96	284.05 ±8.37***	199.45 ±6.31***	105.42 ±3.30***	12.07 ±0.93***	-	17.50±0.28***
3	100	334.62 ±3.35	285.55 ±7.42***	203.32 ±1.23***	99.35 ±4.93***	14.50 ±1.79***	1.87 ±0.67***	21.00±0.40**
4	100	329.45 ±2.69	302.52 ±5.34**	278.02 ±4.71***	146.02 ±6.24***	77.37 ±6.85***	27.32 ±1.99***	22.50±0.64
5	100	327.40 ±1.86	310.75 ±4.60*	271.75 ±3.47***	146.45 ±5.15***	63.42 ±4.90***	27.35 ±1.97***	21.25±0.8**
6	100	327.47 ±0.83	297.92 ±5.88**	254.02 ±4.38***	124.55 ±3.58***	39.50 ±1.38***	5.655 ±1.07***	21.50±0.28**
7	100	336.30 ±2.23	272.80 ±3.23***	203.80 ±1.16***	103.62 ±2.93***	12.95 ±0.82***	-	17.75±0.47***

Data are mean of 4 male in each group ± SD; *p < 0.05, **p < 0.01, ***p < 0.001 student's t-test. Group 1 Untreated animals. Group 2 Animals treated with povidone-iodine. Group 3 Animals treated with 20% MeOH in $CHCl_3$ fraction. Group 4 Animals treated with 40% MeOH in $CHCl_3$ fraction. Group 5 Animals treated with 60% MeOH in $CHCl_3$ fraction. Group 6 Animals treated with 80% MeOH in $CHCl_3$ fraction. Group 7 Animals treated with 100% MeOH fraction.

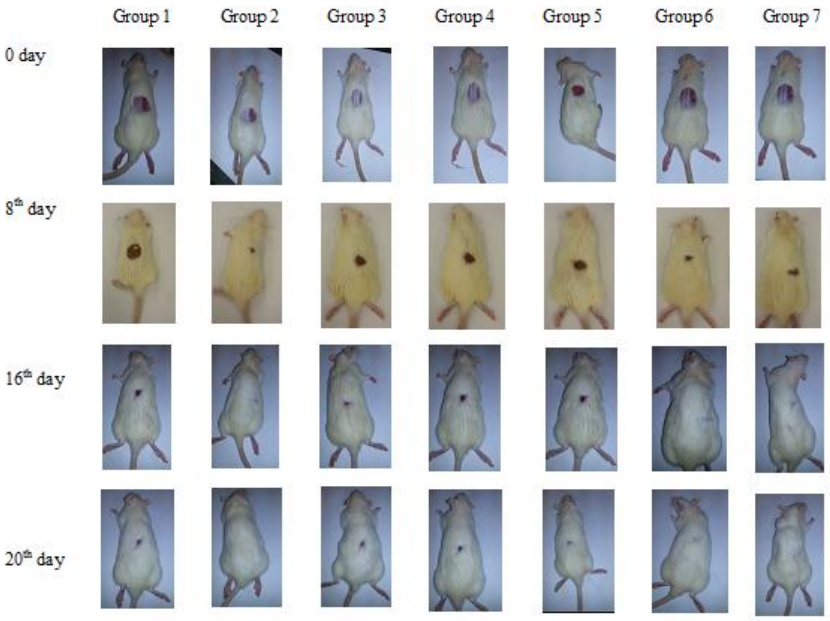

Figure 3. Effect of topical application of methanol extract fractions on 8th, 16th and 20th day on wound healing. Group 1 Untreated animals, Group 2 Animals treated with povidone-iodine, Group 3Animals treated with 20% MeOH in $CHCl_3$ fraction, Group 4 Animals treated with 40% MeOH in $CHCl_3$ fraction, Group 5 Animals treated with 60% MeOH in $CHCl_3$ fraction, Group 6 Animals treated with 80% MeOH in $CHCl_3$ fraction, Group 7 Animals treated with 100% MeOH fraction.

DISCUSSSION

The process of wound healing involved tissues restoration and re-establishment of damaged skin and tissues [12]. It includes an organized movement of actions i.e. inflammation, angiogenesis, proliferation and synthesis of collagen for final healing [13]. The signs associated with wounds are discharge of blood, redness and painful swelling around the wound, puss and water discharge accumulation beneath the skin or drainage and obscene odor from the wounds [14]. Although, wound healing is a natural process and have the capacity to its own healing to restore the integrity. To avoid severe injury to the body; rapid wound healing is required [15].

The results of the present study demonstrated excellent rate of wound healing through topical application of 100% MeOH fraction which showed 100% wound closure and healing on experimentally induced excision wounds, comparable to that of standard drug treated groups and control animals.

The role of antioxidant compounds from plant extractives in wound healing has been reported extensively [16]. Flavonoids, the main constituents present in many plant extracts, act as powerful free radical scavengers [17]. Many studies demonstrated positive correlation between antioxidant activity and phenolic content of plant extracts [18]. The free radical scavenging activity of plant flavonoids help in healing of wounds [19]. The antioxidant defense mechanism of the body comprises enzymes like superoxide dismutase, catalase, and glutatione

peroxidase conversely also have non enzymatic parts such as glutathione, ascorbic acid, and α-tocopherol. The enhancement in production of reactive oxygen species during injury results in consumption and reduction of the endogenous scavenging compounds. Flavonoids may have an additive effect to the endogenous scavenging compounds [20].

Since miquelianin possessing the structure of quercetin 3-*O*-glucuronide has been reported to have anti-depressant activity [21], anti-stress activity [22], cytotoxic and anti-oxidant activity [23], anti-psoriatic activity [24], anti-inflammatory and anti-viral activity [25]. Another isolated compound, kaempferol 3-*O*-glucuronide also possessed cytotoxic and anti-oxidant activity [23]. Quercitrin, a well-recognized flavonoid glycoside has anti-diarrheic activity [26], sedative activity [27], anti-inflammatory effect [28-30], anti-fungal activity [31] and anti-oxidant activity [32].

In view of the pharmacological properties related to flavonoids in general and particularly with isolated compound(s), possibly, the wound healing property of *E. schimperi* methanol extract may be attributed due the presence of compounds (miquelianin, kaempferol 3-*O*-glucuronide and quercitrin) which may be either due to their individual or additive effect that increase the wound healing process.

CONCLUSION

This study demonstrated significant wound healing activity of 100% MeOH fraction by increasing rate of epithelization and wound contraction. The chemical constituents of this methanol fraction have been identified. The methanol fraction and the isolated compounds therefore may be a lead in the drug discovery of new wound healing agents.

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