Wound Healing Capacity, Antibacterial Activity, and GC-MS Analysis of Bienertia sinuspersici Leaves Extract

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Abstract. This study evaluates the wound healing efficacy of Bienertia sinuspersici leaves, a recently discovered species in the Arabian Gulf. A topical cream was formalized from the leaves and its wound healing activity was evaluated for incision, excision, and burn wound models. Bienertia sinuspersici extract was prepared and used for preliminary phytochemical screening. Gas Chromatography-MS analysis, antibacterial assay (disk diffusion method S. aureus, S. pyogenes, E. coli, and P. aeruginosa), antioxidant study, and to formulate a cream that was used for wound healing clinical trial. The presence of glycosides, saponins, and tannins is not confirmed; GC-MS analysis indicates the presence of 3-O-Methyl-d-glucose (a sugar, 44.29% concentration) and l-(+)-Ascorbic acid 2,6-dihexadecanoate (fat-soluble Ascorbic acid, 9.19% concentration). Average zone of inhibition for gram positive and gram negative bacteria were respectively 12-18 and 14-21 mm. Epithelization period for incisions wounds was 6-8 days (negative control: 12 days) with high keratin production and decreased scarring. Healing in excision wounds was steadier, faster and infection-less, contrary to controls. Bienertia sinuspersici leaves promote rapid and steady wound healing for both excision and incision wounds, also its antibacterial activity seems to induce a rapid, infection-less healing process.

Keywords: wound healing; GC-MS of Bienertia sinuspersici; antibacterial effects; traditional medicine; phytochemistry.

1. Introduction
Plants have the immense potential for the management and treatment of wounds. A large number of plants are used by tribal and folklore in many countries for the treatment of wounds and burns. These natural agents induce healing and regeneration of the lost tissue by multiple mechanisms. Bienertia sinuspersici is a flowering plant in the family Amaranthaceae, although it has once been considered to belong to the family Chenopodiaceae. The species is native to the Arabian/Persian Gulf countries (Iran, Iraq, the United Arab Emirates, Saudi Arabia, Qatar, and Kuwait) and the northern side of the Gulf of Oman [1]. Bienertia sinuspersici naturally grows in hot climates, and has the ability to survive extreme salinity and basicity. It has a unique C4 photosynthesis that allows CO2 fixation in these
habitats [1]. The plant flowers appear during September and November and the plant dries out in June and July [2]. Traditionally, the leaves are squeezed onto wounds caused by sickles in order to accelerate their healing and reduce the resulting scars. A wound is the loss of the anatomy and function of the skin due to certain damage that maybe due to physical forces, chemicals, extreme temperatures, or certain diseases. The wound healing process involves the recovery of the anatomical structure of the skin into epidermis and dermis layers, as well as its functions of protection, regulation, and sensation [3,4].

The first step was extraction. Ethanol was chosen for the extraction because it can extract the maximum amount of plant constituents, including alkaloids, steroids, triterpenes, glycosides, carbohydrates, proteins, flavonoids, saponins, phenols, and tannins [5, 6]. The extract was used to formalize a topical cream which wound healing activity was evaluated for incision and excision wound models in case control study on rats [7]. GC-MS analysis was used to identify some of the active substances in the leaves, and the antibacterial activity of the extract was reported on several microorganisms due to the observation that the wounds treated with the extract showed no signs of infections compared to others. No research has previously studied the wound healing nor the antibacterial activity of Bienertia sinuspersici. Similarly, the chemical composition of Bienertia sinuspersici plant has not been previously studied. Prior to investigating the wound healing activity of Bienertia sinuspersici on rats, preliminary phytochemical screening and Gas Chromatography-Mass Spectroscopy analysis were performed for the ethanol extract in order to identify some of the plant’s constituents. The antibacterial activity has also been reported on a several microorganisms. In this study, Ethanol was used for the extraction due to its ability to extract the maximum amount of plant constituent, particularly the seven major plant constituent tested in the preliminary phytochemical analysis. [6]

The aim of this study was to assess the wound healing capacity of Bienertia sinuspersici leaves and report the chemical composition and antibacterial activity that may contribute to that capacity. The study of the chemical composition is helpful to understanding the biological activity of any plant because the response seen is based on the interaction of the plant compounds with the body.

2. Methodology

2.1. Collection of Plant Materials

Bienertia sinuspersici plant was harvested from Al Faw, Basra, Iraq on January 2018. The sample was identified by comparison with traits described in previous scientific literature [1]. Leaves were cut, washed, and refrigerated at 15°C prior to extraction. All chemical substances used in the research were obtained from Sigma Aldrich (St. Louis, MO, USA), unless otherwise stated.

2.2. Extraction Procedure

Bienertia sinuspersici leaves were immersed in ethanol absolute (99.9% concentration). The mixture was placed in a Philips HL mechanical grinder to obtain a thick juice, which was then placed in a lab-made water bath for 30 min at 60 ± 1 °C. Vacuum filtration was performed using a Whatman Grade 1 Filtration Paper using a VE115N vacuum pump. Liquid extract was stored in sterile, shaded, analytical grade containers at -18 °C for further use. To obtain the dry extract, the ethanol liquid extract was evaporated to dryness using a Rotavapor R-300 rotary evaporator. The yield of the dry extract was 12% (w/w).

2.3. Gas Chromatography- Mass Spectroscopy Analysis

Sample was send to GC-MS analysis in College of Agriculture– university of Basrah. Compounds were identified by their mass spectra and retention indices using the NIST Mass Spectral Library and the Retention Index Database. GC-MS Insight Software Package was used to process the data.

2.4. Preliminary Phytochemical Analysis

Preliminary qualitative chemical composition analysis was performed for the liquid ethanol extract using standard methods. Dragendorff’s reagent was used for alkaloids, Liberman Burchardt’s test for steroids/triterpenes, Legal’s test for Glycosides, Fehling test for Carbohydrates, Biuret’s test for...
proteins/amino acids, zinc-hydrochloric acid reduction test for flavonoids, and foam test for saponins [7].

2.5. Formulation of Cream for Tropical Administration

The extract was formulated for topical administration in the form of a cream. Oil phase and water phase ingredients of equal volume were separately heated at 75°C. The water phase was then slowly added into oil phase while stirring, resulting in an emulsified solution. The solution was cooled off prior to adding the extract to achieve 5% cream [8]. External properties (color, odor, grittiness, and homogeneity) were visually inspected. The cream was stored in 100-ml sterile bottle at 25 ± 5°C.

To evaluate thermal stability, the cream was observed for change in external properties at increasing temperatures of 20, 35, 50, and 65°C. To evaluate skin irritation, about 1 gram of the formulated cream was applied to the back of the hands of 5 human volunteers.

2.6. In vivo Wound Healing Study Sample

Eighteen male rats (2 months old, weighing 207±27 gram) were employed for the wound healing study following an acclimatization period of 8 days for incision and 14 days for excision. They were kept in ventilated cages bedded with shredded paper in the Animal Housing Unit at the College of Sciences/University of Basrah. Temperature was maintained at 25±5°C and relative humidity a 50%. Unrestricted access to food and drinking water was provided. The study was conducted in strict accordance with the recommendations of the Guide for the Care and Use of Laboratory Animals (NIH), and the experiment was approved by the Scientific Committee at the School for Gifted in Basra, Iraq.

2.7. Creation of Wounds

Animals were divided into two groups according to the type of wound, one with incision and the other with excision. Each of these groups was subdivided into three study groups with three animals each: Experimental Group, Negative Control Group, and Positive Control Group. The experimental group was treated with the 5% extract cream, the negative control group with simple cream base, and the positive control group with commercial silver sulfadiazine (0.01%) cream.

Prior to creating wounds, rats were anaesthetized by injection of 16.5 mg/Kg Xylazine and 112.5 mg/Kg Ketamine. The predetermined wound area was then sterilized with 10% povidone-iodine solution and depilated using a razor. To create the incision wound model, 3-cm long paravertebral incision were made through the full thickness of the skin on the side of the vertebral column of the rats. Both edges of the wound were kept together and stitched with black silk surgical thread (No. 000) using a curved needle (No. 11). The continuous threads on both wound edges were tightened for good closure of the wound. After stitching, wound was left undressed. For excision wounds, a predetermined area of 1 cm² full thickness skins was excised in the dorsal interscapular region using a biopsy punch. Similarly, excision wounds were left open to the air [9,10,11].

Each model group was subdivided into three groups with three animals each: Experimental Group 1: Negative Control treated with simple cream base, Group 2: Positive Control treated with commercial silver sulfadiazine (0.01%) cream, Group 3: the experimental group was treated with the 5% extract cream.

2.8. Drug Administration and Wound Healing Evaluation

Creams were applied daily to all wounds. Prior to each application, wounds were sterilized with 10% povidone-iodine solution using a soft cotton pad, and were observed for any infection or inflammation. No local or systemic antimicrobials were used throughout the experiment [9,10,11].

For excision wounds, wound healing was evaluated on 2-day intervals in terms of percent wound contraction, which is given by

\[
\text{% wound contraction on n}^{\text{th}} \text{ day} = \left(1 - \frac{\text{wound area on n}^{\text{th}} \text{ day}}{\text{wound area on 1}\text{st day}}\right) \times 100\%
\]

To measure the area of the wounds, rats were individually secured to the base of a cage and a ruler was used to measure the diameter of the wound from different angles, and area was approximated.

For incision wounds, the skin breaking strength after the incision healed was measured by excising a strip of the healed wound area and putting it in a lab-made tension apparatus. The apparatus was made
by vertically supporting an allice forceps and attaching the forceps to one end of the striped tissue; another forceps is attached the hanging tissue on which a hook is attached. Weights are then added gradually to the hook until the skin breaks. Epithelization period was also measured.

2.9. In vitro Antibacterial Activity Test
The antimicrobial activity of the extract was studied in different concentrations (5, 25, 50, 100, and 250 μg/ml) against four pathogenic bacterial strains. Disk diffusion method was used for the test. DMSO was used as a solvent, and the bacterial species used were two Gram-positive (Staphylococcus aureus and Streptococcus pyogenes) and two Gram-negative (Escherichia coli and Pseudomonas aeruginosa), which were cultured in Mueller-Hinton agar (MHA, Merck). Whatman Paper Discs of 5-mm diameter were immersed in the obtained solution and were placed on the surface of agar plates at equal distance with the control. The diameter of a zone of clearing (inhibition) around the disc was calculated after 48 h of incubation at 35 ± 2 °C. Negative control disks contained DMSO and positive control disks contained Ciprofloxacin.

3. Results
3.1 GC-MS Analysis
The Gas Chromatography - Mass Spectroscopy chromatogram contained 20 peaks corresponding to 20 different compounds, as shown in Figure 2. Also, Table 1 shows the identity and concentration of these compounds.

The concentration of identified compounds ranged between 0.44% (Thymine) and 44.29% (3-O-Methyl-d-glucose, a sugar). A fat-soluble form of ascorbic acid (l-(+)-Ascorbic acid 2,6-dihexadecanoate) was found with concentration of 9.19%. The only naturally-occurring cyclohexanetetrol, (1,2,3,5-Cyclohexanetetrol, (1 α,2 β,3 α,5 β)-) had a concentration of 7.22%. Octadecnoic acid and its derivative are common saturated fatty acids that made up 11.61% of the extract. γ-Sitosterol, an antioxidant common in plants and animals, was also present with concentration of 1.73%.

![Figure 1. GC-MS Chromatogram for the Extract.](image)

| Name                                                                 | RI* | RC (%)* |
|----------------------------------------------------------------------|-----|---------|
| 2-[4-Chloro-trans-styryl]-6-chloro-5-[4-chlorophenyl]-4-[3,5-bis[pyrrolidinomethyl]-4 | 0   | 5.98    |
| Palladium(I), (S)-O.N-bis(dicyclohexylphosphino)-2-pyrrolidinemethanol| dichloro | 0       | 1.36    |
| 1-Butanol, 2-amino-3-methyl-,(+/-)-                                   |     | 876     | 3.35    |
3.2 Preliminary Phytochemical Analysis

Results of the preliminary phytochemical analysis tests are summarized in Table 2.

| Phytoconstituent      | Test                          | Results |
|-----------------------|-------------------------------|---------|
| Alkaloids             | Dragendorff’s Reagent         | +       |
| Steroids/triterpenes  | Libermann-Burchardt’s test    | +       |
| Glycosides            | Legal’s Test                  | -       |
| Carbohydrates         | Fehling test                  | +       |
| Proteins/amino acids  | Biuret’s test                 | +       |
| Flavonoids            | Zinc-hydrochloric acid reduction test | + |
| Saponins              | Foam test                     | -       |
| Phenols/tannins       | Ferric chloride test          | +       |
| Tannins               | The gelatin test              | -       |

3.3 Antioxidant Study

The measured absorbance was converted to Ascorbic Acid equivalents, indicating a 40-mg Ascorbic Acid equivalent antioxidant value (Figure 3).
3.4 Formulation of Cream for Topical Administration
The resulting cream was dark green, gritty, sweet-smelling, and homogeneous. No change in properties was observed while increasing temperature until 50 °C, which resulted in mild discoloration. The cream is thus said to be thermally-stable until 50 °C. Volunteers indicated no inflammation within 24 hours of cream application.

3.5 Wound Healing Study
For incision wounds, the epithelization period for extract, positive control, and negative control groups were 6-8, 8-10, and 10-12 days, respectively. A satisfactory skin breaking strength of >500 grams was reported for Experimental and Negative Control groups, while tissue of one rat in the Positive Control group suffered breakage before reaching 500 N. Lower elasticity was reported for the experimental group (figure 4, 5; Table 3).

![Figure 3. Percent wound contraction for excision wounds.](image)

**Table 3.** Epithelization period, elasticity, and breaking strength for incision-wounded group.

| Group             | Epithelization Period | Elasticity (cm) | Breaking Strength (N) |
|-------------------|-----------------------|-----------------|-----------------------|
| Experimental Group| 6-8                   | 2               | >500                  |
| Positive Control  | 8-10                  | 2.5 (1 Breakage)| 450                   |
| Negative Control  | 10-12                 | 2.3             | >500                  |

![Image 1](image)
In excision wounds, the results show the wound contraction percent for the extract group was on average significantly higher than both the negative and positive controls groups (Figure 6). In addition, visual inspection of excision wounds revealed the presence of edema in the wounds by day 4 in one standard-treated and two control animals, while in extract-treated animals didn’t appeared where was dryer and more clear.

| days | Experimental Group | Positive Control Group | Negative Control Group |
|------|--------------------|------------------------|------------------------|
| 0    | ![Image](image1)    | ![Image](image2)       | ![Image](image3)       |
| 2    | ![Image](image4)    | ![Image](image5)       | ![Image](image6)       |
| 4    | ![Image](image7)    | ![Image](image8)       | ![Image](image9)       |
| 6    | ![Image](image10)   | ![Image](image11)      | ![Image](image12)      |
| 8    | ![Image](image13)   | ![Image](image14)      | ![Image](image15)      |
Figure (5) Photographs of excision-wounded sample animals on 2-day intervals
3.5. Antibacterial Activity
Results for the antibacterial test are presented in Table 4. Zero zone of clearing (inhibition) was observed for control.

Table 4. Antibacterial Activity of *Bienertia sinuspersici* Extract and Ciprofloxacin Against Test Organisms.

| Microorganism          | Zone of Inhibition (mm) | 5 μg/ml | 25 μg/ml | 50 μg/ml | 100 μg/ml | 250 μg/ml |
|------------------------|-------------------------|---------|----------|----------|-----------|-----------|
|                        |                         | Extrac  | Standar  | Extrac  | Standar  | Extrac  | Standar  |
| *Staphylococcus aureus*| -                       | 17      | 10       | 19       | 15        | 21       | 17        | 22       | 19        | 22       |
| *Streptococcus pyogenes*| -                       | 17      | 12       | 19       | 14        | 21       | 17        | 22       | 18        | 23       |
| *Escherichia coli*     | -                       | 20      | 14       | 23       | 16        | 28       | 18        | 28       | 19        | 28       |
| *Pseudomonas aeruginosa*| -                       | 20      | 14       | 23       | 18        | 24       | 20        | 26       | 22        | 27       |

4. Discussion
The most notable compound identified with GC-MS was 1-(+)-Ascorbic acid 2,6-dihexadecanoate, a fat-soluble form of ascorbic acid that had a concentration of 9.19% Ascorbic acid (or Vitamin C) which is required for the synthesis of collagen, a substance necessary for the healing of wounds. It is also a highly effective antioxidant protecting cells from damage by free radicals. Studies have shown that the vitamin can help speed the healing process of wounds [13]. Another two compounds with strong antioxidant activity are γ-Sitosterol and chlorophyll, which are both present in significant amounts. Sugars and various kinds of saturated fats were found in the extract. A dioxin compound was also present, suggesting the possible pollution in the farms from which the plant sample is obtained (Al Faw, Basra, Iraq) [14].

The preliminary phytochemical screening has indicated the presence of alkaloids, steroids/triterpenes, carbohydrates, proteins, flavonoids, and phenols; glycosides, saponins, and tannins were not detected. These results are insufficient for the confirmation of the chemical composition of the plant; therefore, more identification techniques are necessary to confirm the chemical composition of *Bienertia sinuspersici*, notably HPLC (injecting with standards) and LC-NMR. *Bienertia sinuspersici* extract is found to be active against both Gram-positive (*Staphylococcus aureus* and *Streptococcus pyogenes*) and Gram-negative (*Escherichia coli* and *Pseudomonas aeruginosa*) bacteria; very significant zone of inhibition was observed. Based on these observations, it is evident that *Bienertia sinuspersici* leaves have potent antibacterial activity. Due to exposure with outside environment, wounded skin is prone to infection, and the antibacterial activity can help prevent skin infections. Wound infections may slow the healing process, promote scarring, produce pus, and cause pain and skin-itchiness [15].

For the antioxidant activity, a concentration of 40 mg Ascorbic Acid equivalent per ml was measured. However, TAC reagent is an aqueous solution in which fat-soluble forms (including L-(+)-Ascorbic acid 2,6-dihexadecanoate) of vitamins are only sparingly soluble, which means that their total antioxidant activity may not be detected properly using the regular TAC reagent and other methods are necessary. Alternative TAC reagents that might be useful for cheaply (and more effectively) determining antioxidant activities might use alcoholic solvents that have the potential to dissolve both polar and non-polar compounds instead of water; max absorption should then be recorded and the method be modified.

Based on the results of the wound healing clinical trial, *Bienertia sinuspersici* leaves possess notable wound healing capacity both for excision and incision wounds. The activity of the extract in healing incision wounds was expected because information from local farmers indicate that it is used to treat
wounds caused by sickles, which typically are incision wounds. The formulized Bienertia sinuspersici provided faster epithelization, less scarring, and higher skin breaking strength. The higher skin breaking strength indicates that more collagen fibers formed in the skin of the animals, which might be attributed to the high amount of Vitamin C in the extract, a substance that promotes collagen formation. The extract provided much faster epithelization and lesser scarring of tissue, and had a satisfactory breaking strength. The elasticity of the resulting tissue indicates that higher ratio of keratin produced as compared to the positive control and negative control groups, indicating a superior quality of healed tissue. The effects of this extra keratin production are generally favorable but the resulting healed tissue should be further investigated to determine the cases in which Bienertia sinuspersici provides the most favorable results.

In excision wounds, Bienertia sinuspersici cream provided faster and much steadier wound contraction compared to both negative and positive control. Unlike control groups, wounded cream with the formulized cream were not infected, which is attributed to the antibacterial activity of the leaves extract. Due to the dark green in color of the cream, the healed area had mild pigmentation. Thus, bleaching the cream in the future might be necessary to avoid unfavorable skin coloration.

Based on the observation that Bienertia sinuspersici cream provides superior wound healing capacity ad has the potential to reduce scarring and infection, it is suggested that more studies are conducted to indicate the efficiency and side of using the cream for humans. Because of the recumbence of their skin to human skin, pigs can be used for future studies [16,17].

It is worth mentioning that Bienertia sinuspersici is a wild species that is commercially unexploited, and that is often removed from agricultural land to provide more space for farming, which means that it is quite cheap to obtain and is abundantly available [5]. In addition, the extreme conditions that the plant can survive makes it fairly easy to cultivate. Amongst others, these factors suggest that the plant has the potential to provide some commercial benefit.

5. Conclusions
Bienertia sinuspersici leaves contain a wide array of bioactive compounds that might be beneficial for humans, and that are active in the wound healing processes. Bienertia sinuspersici extract is active against bacteria and possesses some antioxidant activity. Its formulated cream is able to treat incision and excision wounds effectively –better than commercial creams. More studies are needed to fully understand the chemical composition of the plant and to enhance the formulated cream for human-consumption purposes.

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