IJSRM
INTERNATIONAL JOURNAL OF SCIENCE AND RESEARCH METHODOLOGY
An Official Publication of Human Journals

Human Journals
Research Article
September 2016 Vol.:4, Issue:3

Evaluation of Wound Healing Activity of Ethanolic Extract of Leaves of *Croton megalocarpus* Using Excision Wound Model on Wistar Albino Rats

IJSRM
INTERNATIONAL JOURNAL OF SCIENCE AND RESEARCH METHODOLOGY
An Official Publication of Human Journals

FRANCIS KAROMO WAMBUGU, WILSON RWAI WAWERU

School of health sciences, Mount Kenya University Kigali-Rwanda.

Submission: 5 September 2016
Accepted: 10 September 2016
Published: 25 September 2016

Keywords: Wound healing, Ethanolic extract, *Croton megalocarpus*, Histopathology

ABSTRACT

This study evaluated the wound healing potential of ethanolic extract of *Croton megalocarpus* leaves. Different doses (i.e. 50mg/ml, 100mg/ml and 150 mg/ml) of the extract were topically applied daily to excision wounds in Wistar albino rats and their healing potential was investigated while neomycin sulphate and normal saline were used as positive and negative control respectively. Percent wound contraction was determined at three-day intervals and a histopathology examination of wound tissue was also done on day 10 of post-application of the extract to evaluate the different stages of wound healing in the different treatment groups. Phytochemical screening of the plant extract was also done. Both the 100 mg/ml and the 150 mg/ml concentrations on day 9 showed no significant statistical difference ($p>0.05$) in percent wound contraction when compared to the positive control indicating that use of the extract at these concentrations was comparable to neomycin sulphate. However, there was no significant statistical difference ($p> 0.05$) between 50mg/ml and the negative control on day 9 indicating that the extract is not effective at this dose. Histopathology examination of section showed almost complete healing in the groups treated with 100 and 150mg/ml. There were signs of early stages of wound healing for the group treated with 50mg/ml. The phytochemical profile of the plant extract indicated presence of alkaloids, flavonoids and tannins and these are probably responsible for the wound healing potential. The current study showed that the ethanolic extract of *Croton megalocarpus* has wound healing potential.

INTRODUCTION:

The use of plants as a whole or extracts of certain parts of the plants to accelerate the process of wound-healing has been in use since ancient times (Fronza et al., 2009). With the increasing cost of living being experienced globally and especially among the developing countries, the search for alternative therapies and natural remedies for various health conditions is on the rise (Cragg *et al.,* 1997). Although many traditional healers have successfully used hundreds of medicinal plants to manage various forms of health conditions, the healing activity of most of this plants have not been proven scientifically (Peres et al., 1997). The genus *Croton* comprises about 1200 species and is a deciduous flowering tree native to East Africa. It is grown mostly for its attractive yellow flowers and for fence. The fruits of *C. megalocarpus* have a significant place in folk medicine (Orwa *et al*., 2009). Fruits are usually pounded and mixed with soot from copper kettle and then applied on inflammatory wounds.

Patients with unhealed wounds have an increased risk of infection and a subsequent amputation of limbs that are suffering from ulcers, on the same breath commercially available ointments for the management of wound are very expensive for the common man. Despite all medical advances in the management of chronic wounds, it remains an overriding problem in the society. Therefore there is a dire need to come up with an easily accessible, effective and yet cost effective remedy. Some healers used decoctions of the dried fruiting branches to bathe and heal, mostly in abscesses and wounds of kids, other healers used ash of the plant to make ointment in vegetable oil (Musa *et al*., 2011). Previous studies have indicated that this plant possesses healing properties of pneumonia, whooping cough and fever (Orwa *et al.,* 2009). The purpose of the present study was to investigate the wound healing effect of ethanolic leaves extract of *Croton megalocarpus*.

MATERIALS AND METHODS:

Plant collection, preparation and extraction

The leaves of *C. megalocarpus* were collected from Kanyanya, one of Kampala's suburbs. The specimen was authenticated by a botanist and a voucher specimen number deposited in Makerere University Herbarium. The leaves were dried under shade; free from wind currents after which

they were powdered and stored in a black polythene bag. The powder was extracted by cold maceration by soaking 500g of the powdered leaves in 1500ml of 70% ethanol for three days, with occasional shaking each day and then filtered. The extracts were then dried in an incubator set at 50°C for 48 hours. The yields of the extracts were 12.5%. The crude extract was stored in the refrigerator at a temperature of 2-4°C. The preparation of 150mg/ml concentration involved dissolving 15 grams of extract in 100ml of distilled water, for 100mg/ml concentration, 10grams were dissolved in100ml of distilled water and for 50mg/ml concentration, 5 grams were dissolved in 100ml of distilled water.

Phytochemical screening

The preliminary phytochemical analysis of the ethanolic leaves extracts was done using 100mg/ml of aqueous extract for each reaction. Tannins, alkaloids, flavonoids, saponins and triterpenes were among the phytoconstituents that were tested using different chemical tests (Rizk, 1982; Somolensk and Farnsworth, 1972; Kokate 1986).

Experimental animals

The study involved 30 normal albino rats of either sex weighing between 150g and 180 g and of 8 weeks old. The rats were obtained from the Pharmacology Laboratory, School of Veterinary Medicine, Makerere University. The animals were allowed to acclimatize for one week in the laboratory prior to the study as recommended by Komi-kuramochi *et al.,* (2005). The rats were housed in clean cages and maintained under standard laboratory conditions of 12 hours natural light and 12 hours darkness. They were maintained on standard pellet diet and water ad libitum was availed throughout the experiment. A minimum of six animals were used in each group.

Wound excisions

On the first day of the experiment, the rats were anaesthetized with intraperitoneal injection of ketamine 50mg/kg of body weight (Somashekar *et al.,* 2007) and xylazine 65mg/kg (Songqing *et al.,*1999). The mid back regions of the rats were shaved and disinfected using 70% ethanol. Circular wounds were created on the middorsal area of the rats each with diameter of approximately 2cm, using sterile curved scissors (Rajabi *et al.,* 2007). To ensure comfort of the rat in the immediate post-operative recovery period, analgesia was provided by oral

administration of diclofenac solution (100mg/kg of body weight). After the wound incisions longitudinal and transverse diameters of the wound were measured using a vernier caliper. The areas of each wound on every animal were calculated from the formula as determined by Komi-kuramochi *et al*., (2005).

$$\text{Area} = \frac{ab\pi}{4}$$

Where, a and b were the transverse and longitudinal diameter of the wound respectively.

Treatments

Parallel design was used where each group was subjected to only one treatment. Single-blind trial was applied where the data analyst and the pathologist were blinded and did not know the treatment group. The animals were randomly distributed to 5 study groups consisting of at least 6 animals each. Group 1 consisted of those receiving positive control (neomycin sulphate), groups 2, 3 and 4 received 50mg/ml, 100mg/ml and 150 mg/ml of extracts in water respectively while group 5 animals received normal saline i.e. negative control. The summary of the treatments is indicated in Table 1. The plant extracts, neomycin sulphate and normal saline were applied topically immediately on excision on the respective study group. The treatments were applied twice a day for 9 days.

Table 1: Treatment groups for the experimental animals

Group	Treatment
1	Neomycin sulphate (positive control)
2	50mg/ml of plant extract
3	100mg/ml of plant extract
4	150mg/ml of plant extract
5	Normal saline (negative control)

Determination of the rate of wound healing

The measurement of the wound was done every three days starting from the day of excisions were made i.e. on day 0, 3, 6 and 9. The areas of wounds after treatment were calculated and the percentage wound contraction was determined for the different treatment and control groups. Percent wound contraction was computed as:

$$\text{Percent wound contraction} = \frac{x-y}{x} \text{ X } 100$$

Where, x = original wound area y = wound area 3 days after previous measurement

Histopathology examination

On day 10, two animals were randomly selected from each study group and sacrificed using chloroform for histopathological examination. The specimen tissue sample was cut from the wound, fixed in formalin and taken for histopathological examination. The sample tissues fixed in 10% formalin were processed in a histokinette and embedded in paraffin wax. Serial sections (5 µm thickness) of paraffin-embedded tissues were cut and fixed on glass slides. The tissues were then stained with haematoxylin and eosin, which were then examined by a light microscope. Ulceration, necrosis and epithelisation were evaluated in the skin tissues. Congestion, edema, PMN, mononuclear cells, fibroblasts and vascularization were also qualitatively evaluated in the skin tissues.

Statistical analysis of the data

Data was analyzed using SPSS version 21. The students 't' test was used to assess for any statistically significant differences between the test and control groups. A p-value less than 0.05 were considered significant. The experimental results were expressed as the mean ± S.E.M (Woodson *et al.*, 1987). Histopathological data were considered to be non-parametric, therefore no statistical tests were performed.

Ethical considerations

The study was approved by the Research and Ethics Committee of Makerere University Faculty of Veterinary medicine Kampala. Study animals were handled in conformity with

guidelines for the care and handling of laboratory animals provided by the Institution and in accordance with guidelines for laboratory bio-safety Guidelines, 2004.

RESULTS:

Phytochemical profile

Preliminary phytochemical screening of ethanolic leaf extract of *Croton megalocarpus* contains high levels of alkaloids, moderate levels of tannins, flavonoids, saponins and steroids glycosides whereas triterpenes were absent. Results are summarized in Table 2.

Table 2: Phytochemical analysis of the ethanolic leaf extract of *Croton megalocarpus*

Phytochemical	**Intensity**
Tannins	++
Alkaloids	+++
Flavonoids	+
Saponins	+
Steroid glycosides	+
Triterpenes	-

+Weakly present, ++strongly present, +++Very strongly present, - absent

Wound contraction/ healing potential of the plant extract

Results of the progression of wound healing on application of the different concentrations of ethanolic extract shown that the percent wound contraction increased with increasing concentration of the extract as shown in Table 3. The group treated with 150mg/ml concentration of extract showed the highest percent wound contraction i.e. 79.31% on day 9. The mean percent wound contraction for this group was not significantly different ($p>0.05$) from that of the positive control group on day 9. Therefore, the extract at 150mg/ml concentration showed comparable wound healing potential as that of neomycin sulphate.

Table 3: Percent wound contraction (Mean ± SEM) at 3 day intervals after application of *Croton megalocarpus* ethanolic leaf extract using the excision wound model on Wistar rats

Group	Treatment	Day 3	Day 6	Day 9
1	Neomycin sulphate	37.01 ±2.56	62.25 ±1.83	84.3 ±1.82
2	50mg/ml Extract	28.13 ±5.73	48.92 ±2.99	63.15 ±5.2.09
3	100 mg/ml Extract	33.37 ±3.37	52.52 ±2.03	67.57 ±1.72
4	150 mg/ml Extract	35.12 ±2.57	57.57 ±3.06	79.31 ±2.62
5	Normal saline	25.67 ±3.65	45.64 ±3.22	60.13 ±1.76

On day 9 there was a highly significant statistical difference ($p<0.05$) in the mean percent wound contraction between the negative control and both the 100 mg/ml and 150mg/ml preparation of the extract showing that plant extracts accelerated wound healing.

On day 9, there was no significant statistical significance ($p>0.05$) between the negative control and 50mg/ml concentration of the extract, indicating that the extract should be used in higher concentration to be effective.

Histopathological examination

The histopathological examination revealed the early stage of wound healing in the negative control group, with ulceration containing fibrin and inflammatory-type cells at the tissue surface of the negative control group. Proliferating vascular structures with congestion, mixed inflammatory infiltration and a plump of fibroblastic cells were observed below the ulceration. It was also characterized with connective tissue still open and no epithelialization (Figure 1).

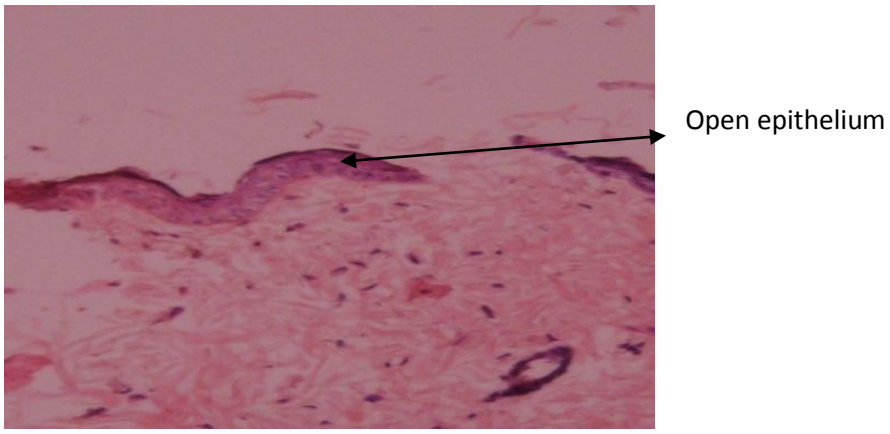

Figure 1: Histological section of an excision wound on day 10 after daily application of normal saline

The figure shows that the epithelium is still open; while below the wound, the fibrous mesh is formed (the section was prepared with hematoxylin and eosin (H & E) stain at 40×magnification).

The histopathological examination of the wounds in rats where 50 mg/ml of the extract was applied (group 2) revealed all elements of wound healing with more inflammatory cells and intense angiogenesis (Figure 2). Therefore the use of 50 mg/ml extracts at this concentration did not accelerate wound healing.

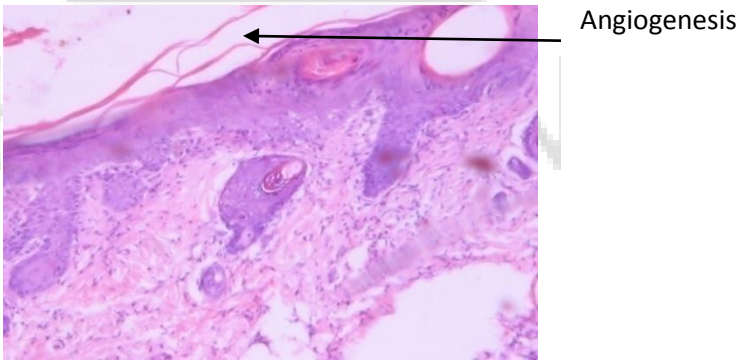

Figure 2: Histological section of an excision wound on day 10 after daily application of 50mg/ml of plant extract. The section shows intense angiogenesis (H&E 40× magnification)

The animals in the group treated with 100mg/ml concentration of the extract demonstrated an almost similar histopathological picture as those treated with 150mg/ml of extract with no

ulceration, no cell infiltration and complete epithelialization (Figure 3). At this concentration, the ethanolic leaf extract of *Croton megalocarpus* accelerated wound healing.

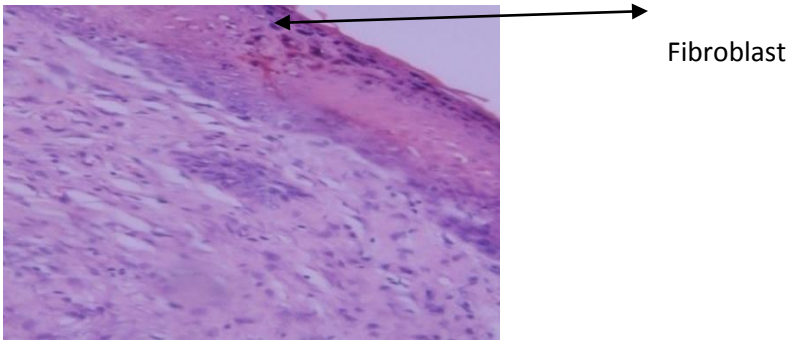

Figure 3: Histological section of an excision wound on day 10 after daily application of 100mg/ml of plant extract. The section shows complete epithelialization with no inflammatory cells (H&E 40× magnification).

In the group where 150 mg/ml concentration of the extract was applied, the histopathological examination was characterized with increased presence of fibroblasts. There were few inflammatory cells below the epithelium indicating that the healing process was already advanced, and the epithelialization was complete. However, there was no keratinization. Generally, the healing process was almost nearing completion as illustrated in Figure 4.Therefore extracts at this concentration accelerated wound healing.

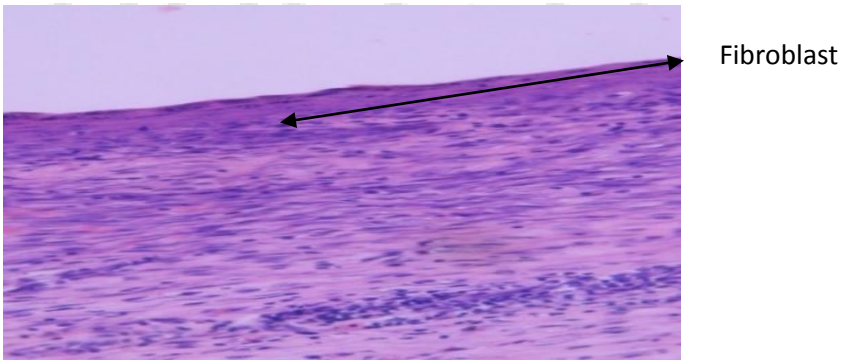

Figure 4: Histological section of an excision wound on day 10 after daily application of 150mg/ml of plant extract. Section showing collagen deposited on the wound, fibroblast as well as complete epithelialization an indication of advanced healing process (H&E 40× magnification).

For wounds treated with neomycin sulphate, a parallel histopathological examination demonstrated complete re-epithelialization without any ulceration. Keratinization of the epidermis, mature dermal layers and hair follicle were observed. A few mononuclear cells were present at the base of hair follicles. The histopathological picture does not differ much from that observed for the group where the 150 mg/ml concentration of the extract was applied. This is consonance with the results of the percent wound contraction.

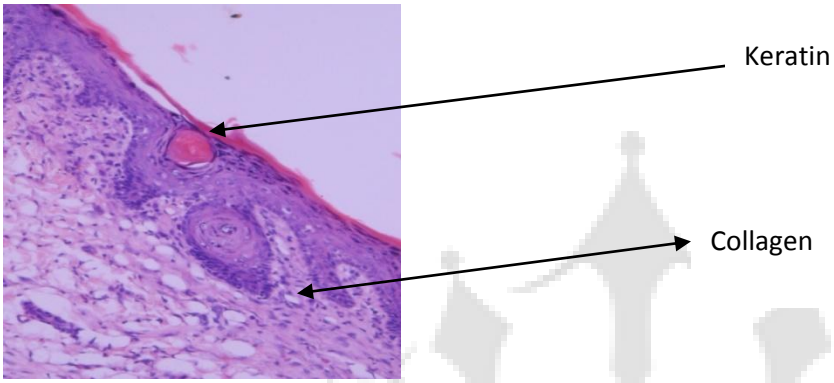

Figure 5: Histological section of an excision wound on day 10 after daily application of neomycin sulphate.

The section shows a well formed thickened granular layer. The underlying dermis contains deposited collagen fibre with few inflammatory cells. Epithelialization is complete and keratinization is almost complete (H&E 40× magnification).

DISCUSSION:

Wound healing is a complex and dynamic process of restoring cellular structures and tissue layers in damaged tissue as closely as possible to its normal state. Wound contracture is a process that occurs throughout the healing process, commencing in the fibroblastic stage whereby the area of the wound undergoes shrinkage (Quinn, 1998). This study majorly aimed at determining whether daily applications of different concentrations of the ethanolic leaf extract of *Croton megalocarpus* has any effect on the rate of wound healing (percent wound contraction) in excision wounds in a Wistar albino rat animal model.

The results of this study showed that daily topical application of *Croton megalocarpus* ethanolic extract at 100mg/ml and 150 mg/ml significantly accelerated the rate of wound healing and histopathological sections of the healing wounds showed that collagen, fibroblasts, and blood capillaries were contained in granulation tissue but without inflammatory cells. The wound healing effects observed could be due to the regulation of collagen I expression (Bonte *et al.*, 1994) and an increase in tensile strength of the wounds (Suguna *et al*., 1996). Enhanced healing activity was due to increased collagen formation and angiogenesis as shown in the histological sections. Angiogenesis in granulation tissues improves blood supplementation to the wound site, thus providing nutrients and oxygen essential for the healing process (Szabo *et al*., 1995).

The results of this study are similar to those obtained in investigations of the wound healing potential of other plants belonging to the Euphorbiaceae family, to which *Croton megalocarpus* belongs. Omale and Friday (2010) found high wound healing potential in excision wounds of experimental rats topically administered with aqueous and ethanolic extracts of *Euphorbia heterophylla*. The ethanol extracts exhibited high percentage of wound closure. The wound-healing property observed in *Croton megalocarpus* could be attributed to the phytoconstituents of the plant extract and the faster process of wound-healing could be a function of either the individual or additive effects of the phytoconstituents. It is conceivable that the *C. megalocarpus* extracts exert their wound healing activity through phytochemicals especially the flavonoids, which though present in low quantities as revealed by the results of phytochemical screening of this study, are reported to improve wound healing and protect tissues from oxidative damage (Saurez *et al*., 1996). The increase in neutrophil cells infiltrating the granulation tissue normally delays the wound healing process (Shimizu *et al*., 2000).

Neutrophils mediate lipid peroxidation through the production of superoxide anions which delays wound healing. However, this may be counteracted by the antioxidant activity of the flavonoids as reported by Zimmerman *et al.*, (1997). The rate of wound healing significantly accelerated with increasing concentrations of the extracts. This dose-dependent acceleration of wound healing may be attributable to an increase in the amount of phytochemicals (especially flavonoids) present with increasing concentration of the extract.

The effect on wound healing could also be attributed to antibacterial activity of this plant. Plants belonging to the same family as *C. megalocarpus* such as *Croton bonplandianum* and *Euphorbia*

hirta have been shown to possess antibacterial activity (Singh *et al.*, 2011). Phytochemicals such as alkaloids are known to have antibacterial activity as reported by Tsuchiya *et al*., (1996). In the present study, alkaloids were strongly present in *C. megalocarpus* extract on phytochemical screening. Wound healing can be delayed when microorganisms are present in large enough numbers (Rijswki *et al*., 2000). Therefore, reducing the bacterial load of a wound may be necessary to facilitate wound healing, as well as reduce local inflammation and tissue destruction (Faoagali, 1999). The *C. megalocarpus* extract possibly caused wound healing by preventing infection through destroying pathogens or stimulating immune activity. On the other hand, the wound healing activity observed could have been due to a combination of the antibacterial activity of the plant extract and the antioxidant activity of the flavonoids present in the extract.

In conclusion, the ethanolic extract of *C. megalocarpus* possesses considerable wound healing potential that validates its use in traditional communities as a wound healing remedy. Further investigations using pure fractions of this extract could possibly yield even better results. Finally, identification and elucidation of the active molecules responsible for the wound healing activity of this plant could lead to its utilization by the pharmaceutical companies as a source of modern drugs not only for wound healing but also other medicinal effects.

CONCLUSIONS

Topical application of the ethanolic leaf extract of *Croton megalocarpus* has wound healing potential which increases with the concentration of the extract. Therefore, this validates folkloric use of the plant in wound management.

The plant contains several Phytochemicals including flavonoids which may be responsible for its wound healing activity.

Acknowledgment:

The authors are grateful to the management and staff of Makerere University Herbarium for the technical support offered to this research work. We are also grateful to the Department of Pharmacology and Therapeutics, Makerere University for material and technical support.

REFERENCES:

1. Bonte F., Dumas M., Chaudagne C., & Meybeck A. Influence of asiatic acid, madecassic acid, and Asiaticoside on human collagen I synthesis. *Planta Med.* 1994; 60: 133-135.
2. Fronza M, Heinzmann B, Hamburger M, Laufer S, Merfort I. Determination of the wound healing effect of Calendula extracts using the scratch assay with 3T3 fibroblasts. *Journal of Ethnopharmacology*. 2009;126(3):463–467
3. Komi-Kuramochi A., Yuko O., Asada M., Suzuki M. Expression of fibrobrast growth factors and their receptors during full thickness skin wound healing in young aged mice. *J Endocrinology* 2005; 186:273-289.
4. Musa S., Abdelrasool F., Elsheikh A., Ahmed M., Mahmoud E., Yagil M. Ethnobotanical study of medicinal plants in the Blue Nile State, South-eastern Sudan. *Journal of Medicinal Plants Research*, 2011; 5: 4287–4297
5. Omale J. & Friday T. Phytochemical composition, bioactivity and wound healing potential of *Euphorbia heterophylla* (euphorbiaceae) leaf extract. *International Journal on Pharmaceutical and Biomedical Research*. 2010; 1: 54-63.
6. Peres M, Delle Monache F, Cruz AB, Pizzolatti MG, Yunes RA. Chemical composition and antimicrobial activity of *Croton urucurana* Baillon (Euphorbiaceae) *Journal of Ethnopharmacology*.1997;56 (3):223–226.
7. Quinn P. Tissue Adhesives in Wound Care. *Journal of Ethnopharmacology*. 2001; 90: 115- 143.
8. Rajabi M. & Fatemeh R. The effect of estrogen on wound healing in rats. *Pakistan journal of medical scinces*. 2007; 23:1681-1715.
9. Rizk A. Constituents of plants growing in qatar: A chemical survey of sixty plants. *Fitoterapia*, 1982; 52:35-44.
10. Kokate CK. *In: Practical Pharmacognosy, Preliminary Phytochemical Screening*, first ed., VallabhPrakashan, New Delhi, 1986; 111.
11. Saurez J, Herreta MD, Marhuenda E. Hesperidine and neohesperidine dihydrochalcone on different experimental models of induced gastric ulcer. *Phytotherapy Research* 1996; 10: 61-618.
12. Shimizu N, Watanabe T, Arakawa T. Pentoxifylline accelerates gastric ulcer healing in rats: roles of tumor necrosis factor alpha and neutrophils during the early phase of ulcer healing. *Digestion*; 2000; 61: 157-164.
13. Somashekar, S., Sarswati, U. & Laxinarayana, U. Evaluation of antioxidant and wound healing effect of alcoholic and aqueous extracts of *Ocium sanctum. J Ethnopharmacol.* 2007; 6:19–36
14. Somolenski, J. & Farnsworth R. Alkaloid screening; *Lloydia*, 1972; 35: 1-34.
15. Songqing H, Carl Atkinson, Fei Qiao, Xiaoping Chen, and Stephen Tomlinson. Ketamine–Xylazine–Acepromazine Compared with Isoflurane for Anesthesia during Liver Transplantation in Rodents. *J Am Assoc Lab Anim Sci*; 2010; 49(1): 45–51.
16. Suguna L, Sivakumar P, Chandrakasan G. Effects of Centella asiatica extract on dermal wound healing in rats. *Indian J Exp Biol*; 1996; 34: 1208-1211
17. Szabo S, Kusstatscher S, Sakoulas G.Growth factors: new 'endogenous drugs' for ulcer healing. *Sc and J Gastroenterol Suppl*; 1995; 210: 15-18.
18. Woodson, R. Statistical *methods for the analysis of biomedical data, Willey series in probability and mathematical statistics* Willey, New York, 1987.315.
19. Zimmerman J., Ciesielski W., Lewandoski J. Neutrophil-mediated phospholipid peroxidation assessed by gas chromatography-mass spectroscopy. *Am J Physiol*; 1997; 273: C653-661.