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EVALUATION OF THE EFFECT OF AQUEOUS AND ETHANOL EXTRACT OF *HYDROCOTYLE SIBTHORPIOIDES* LEAF ON WOUND HEALING

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Abstract
The objective of the present investigation was to evaluate the wound healing action of *Hydrocotyle sibthorpioides* leaf extract in rats. The extraction abilities of different solvents for recovering extractable components from leaves followed the order: ethanol >water>pet ether. The acute toxicity study suggests a LD_{50} of more than 2000 mg/Kg for both the ethanolic and aqueous extracts of the plant. The ethanolic and aqueous extracts of *Hydrocotyle sibthorpioides* leaves were evaluated for the *in vivo* wound healing effect by the excision model. The topical application of 5 % w/w of the *Hydrocotyle sibthorpioides* resulted in an enhanced and statistically significant ($p < 0.05$) wound healing activity *in vivo* when using the ethanolic extract. The wound healing by the aqueous extract was not significant in comparison to the control group. The ointment containing plant extract (ethanolic) exhibited 98.56 ± 3.61 % contraction of wound on the 21st day whereas only 63.99 ± 6.33 % contraction of wound was found in the control animals. The results revealed by aqueous extract were also significant as it could reduce the wound area by 88.89 ± 6.33 % on the 21st day.

Keywords: - Wound, excision, *Hydrocotyle sibthorioides*, extract, toxicity

Introduction

Plants have provided the basic supplies of life to human beings from the very beginning of human origins. Any physical injury might lead to disturbed integrity of the dermis and might create a wound.[1] Wounds have affected humans since prehistoric times and the treatment and healing of a wound is an art as old as humanity.[2] The acute inflammatory response during the early stages of injury generates factors that are essential for tissue growth and repair.[3] When prolonged, however, chronic inflammation can to be detrimental, preventing wound remodeling and matrix synthesis, leading to delay in wound closure and an increase in wound pain.[4] The production of free radicals at or around the wound may contribute to delay in wound healing through the destruction of lipids, proteins, collagen, proteoglycan and hyaluronic acid.[5]

Plants are known to be a source of potential anti-inflammatory, antioxidant and antimicrobial agents.[6] A survey of the literature related to pharmacological actions of *Hydrocotyle sibthorpioides* exhibited several properties like anti-inflammatory, antioxidant, neuroprotective, antimicrobial etc.[7] Hence in this investigation it was proposed to examine the effect of ethanolic and aqueous extracts of *Hydrocotyle sibthorpioides* leaf on healing of experimentally induced wound in rodent.

Materials

The chemical and reagent used in the present study were procured from various scientific suppliers and were used as obtained without any further purification. The instruments used were available in the laboratory of the institution and were used without calibration.

Preparation of the plant material for extraction

The leaves of the plant were washed with distilled water and dried in shade (preventing from direct sunlight). The dried leaf has been powdered using slow speed blender and is kept in closed airtight container.

Solvent extraction of phytoconstituents[8]

Powdered plant material (98.6 g) was evenly packed in the extractor of the soxhlet apparatus and extracted successively with various solvents of increasing polarity including petroleum ether and ethanol by hot continuous extraction process for about 15 h. The aqueous extraction was carried out by cold maceration process after completion of the solvent extraction process. The extracts were concentrated by distillation to reduce the volume and transferred to 100 mL beaker and the remaining solvents were evaporated on water bath. The extracts obtained collected and placed in desiccators to remove the excessive moisture**.** The dried extracts were stored in desiccators for further investigation.

Phytochemical screening of extract[9]

The extract was suitably treated to prepare the testing solution and the test solution was qualitatively interacted with various reagents to observe the presence or absence of the various classes of secondary metabolites.

Pharmacological Evaluation

Animals

Healthy male Wistar male rats weighing 180-250g were used for the study. The animals were housed in cages during the course of experimental periodadn maintained at 12 day and night schedule with a temperature [17-26°C] maintained at standard experimental condition. The animals were fed with standard rodent pellet feed and water *ad libitum*. The animals were fasted 12 hours before the experiment with free access to only water.

Acute Toxicity Study[10]

A total of three animals were used which received a single oral dose (2000mg/kg) of ethanolic and aqeuous extracts of *Hydrocotyle sibthorpioides*. Animals were observed individually at least once during the first 30 min after dosing, periodically during the first 24 h and daily thereafter for a period of 14 days. Once daily observations were made for changes in skin and fur, eyes and mucous membrane (nasal) and also respiratory rate, circulatory (heart rate and blood pressure), autonomic (salivation, perspiration, urinary incontinence, and defecation) and central nervous system (drowsiness, tremors and convulsion) changes. Mortality, if any, was also observed over the period of 2 weeks.

Preparation of test samples and standard drugs

The test samples were prepared by formulating the dried ethanolic and aqueous plant extract in simple ointment base (cetostearyl alcohol, wool fat, white paraffin, and hard paraffin) as a 5 % w/w ointment. Commercially available Povidone Iodine Ointment (5 %) was used as standard drug for comparison of action.

Preparation of simple ointment base[11]

Hard paraffin (5 g) and cetostearyl alcohol (5 g) were taken in a porcelain dish maintained on water-bath at 70°C. Wool fat (5 g) and white soft paraffin (85 g) are added to this mixture and stirred until

all the ingredients were in molten state and mixed. The mixture was stirred until cold and packed in suitable container.

Experimental procedure for wound healing by excision model

Experiment Design

The animals were divided in to 4 groups of 5 rat each and the experiment was designed as per table 1

Table 1 Experimental design for excision model

Group	Nomenclature	Treament
Group I	Control	Simple ointment base
Group III	Standard	Povidone iodine ointment (5% w/w)
Group IV	Test	*Hydrocotyle sibthorpioides* aqueous extract ointment (5% w/w)
Group V	Test	*Hydrocotyle sibthorpioides* ethanolic extract ointment (5% w/w)

All the test samples; vehicle and standard drug samples were applied topically on the wound of each of the animals daily, under sterile conditions.

Induction of wound[12]

On the day of inducing wound, each animal was anesthetized by the open mask method using short exposure to diethyl ether. The hair (fur) on the back of each rat was removed by shaving using an electric shaver. The area of the wound to be created was marked on the back of the animals with methylene blue using a circular stainless steel stencil. A full thickness of the excision wound of around 25 mm in width (circular area 490 mm^2) created along the markings using toothed forceps, a surgical blade and pointed scissors. The entire wound left open. All the surgical procedures were carried out under sterile condition. After 24 h of wound creation, the ointments were applied gently to cover the wounded area once daily until complete healing. Wound area and wound contraction, were monitored on each day.

Measurement of wound Contraction[12]

The progression of wound healing was judged by the periodic assessment of the contraction of excision wounds. Wound contraction was monitored by tracing the outline of the wound on tracing sheet and then using graph sheet to calculate the area of the wound size. All animals in each group were monitored until complete healing of wounds occurred and the day at which each wound healed was recorded.

$$\text{Percent wound contraction } = \frac{Healed\ area}{Total\ area}\ X\ 100$$

Results and Discussion

Extraction Yield and phytochemical analysis

The extraction abilities of different solvents for recovering extractable components from leaves followed the order: ethanol (13.9%) > water (8.7%) > petroleum ether (1.6%). The observations of qualitative testing of phytochemicals suggests the presence of alkaloids, saponin glycosides, phenolics, terpenoids, sterols, and flavonoids in the leaf of the plant.

Acute Toxicity Study

The acute toxicity test was performed by using the dried ethanolic and aqeuous extracts at concentration of 2000 mg/kg to the test animal, administered orally. No animal died and hence the dose of upto 2000 mg/Kg was considered to be safe. As none of the animals died, the LD_{50} was considered to be more than 2000 mg/Kg and any dose less than 2000 mg/Kg would be considered for evaluation of wound healing action (Table 2).

Table 2 Results of Acute Toxicity Study

Group	Extract	Number of Mice (n)	Dosage (mg/kg)	Deaths (n)	Survival (n)	Mortality rate (%)
1	Ethanolic	3	2000	0	3	0
2	Aqueous	3	2000	0	3	0

Wound Healing action

The ethanolic and aqeuous extracts of *Hydrocotyle sibthorpioides* leaves were tested to determine the *in vivo* wound healing effect by the excision model (n=5). The topical application of 5 % w/w of the *Hydrocotyle sibthorpioides* extract containing ointments on the wound resulted in an enhanced and statistically significant ($p < 0.05$) wound healing activity *in vivo*. The wound area measurements and the percent wound contraction results of the progressive healing of the excision wounds for the control; vehicle control; standard reference drug and plant extract are presented in table 6.4. From the results it can be clearly seen that the ethanolic extract of the plant had an excellent wound healing potential with almost complete closure of the wound of the animals by 21 days. The ointment containing plant extract (ethanolic) exhibited 98.56 ± 3.61 % contraction of wound on the 21st day whereas only 63.99 ± 6.33 % contraction of wound was found in the control animals. The results revealed by aqueous extract were also significant as it could reduce the wound area by 88.89 ± 6.33 % on the 21st day. The standard ointment was able to completely close the wound exhibiting 99.83 ± 1.066 % reduction in wound area.

Table 3 Area of wound and % contraction of wound by ethanolic and aqueous extract of *Hydrocotyle sibthorpioides (HS)*

Group	Control	Standard	*HS ethanolic extract*	*HS aqueous extract*
Day	**Area mm^2 (% contraction)**			
Day 0	490.63 ± 96.08	452.16 ± 61.2	452.16 ± 59.85	490.63 ± 87.44
	-	-	-	-
Day 7	379.94 ± 96.22	153.86 ± 59.61	283.39 ± 77.45	226.86 ± 89.51
	(12.0298 ± 4.68)	(47.1902 ± 3.89)	(36.7693 ± 6.18)	(27.4472 ± 3.011)
Day 14	254.34 ± 74.11	50.24 ± 16.9	153.86 ± 44.75	94.99 ± 23.65
	(24.2542 ± 7.18)	(75.288 ± 7.32)	(60.0484 ± 8.23)	(36.9481 ± 5.177)
Day 21	176.63 ± 17.5	0.785 ± 0.003	50.24 ± 15.8	7.07 ± 1.033
	(63.99 ± 6.33)	(99.83 ± 1.066)	(88.89 ± 6.33)	(98.56 ± 3.614)

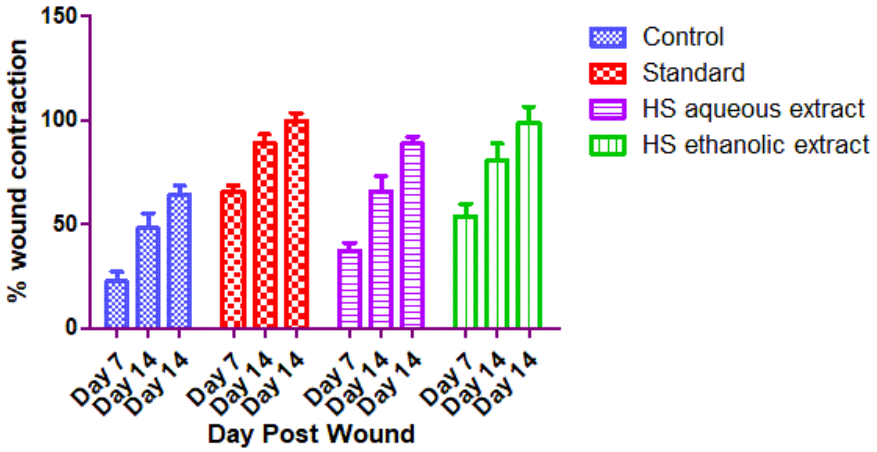

Figure 1 Wound healing efficacy of ethanolic extract of *Hydrocotyle sibthorpioides* by *in vivo* excision model

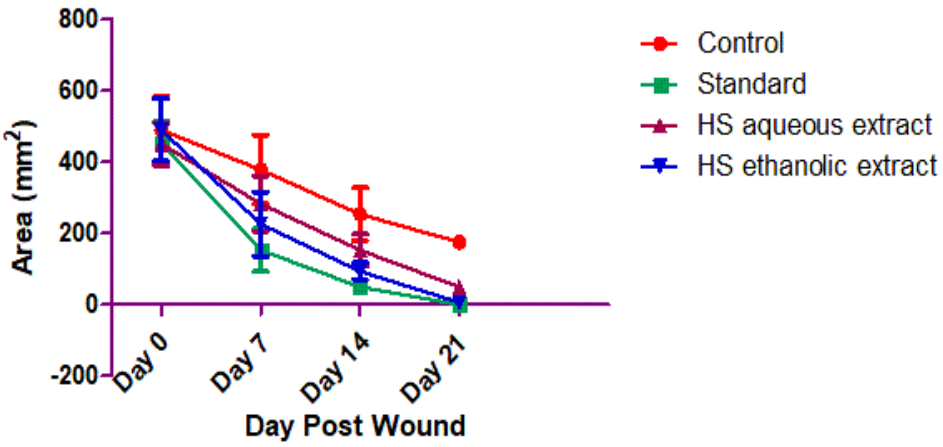

Figure 2 % contraction of wound exhibited by extracts of *Hydrocotyle sibthorpioides* by *in vivo* excision model

The occurrence of tannins in the ethanolic extract could be attributed for the effective wound healing action by the ethanolic extract. Previous studies on wound healing action of the plant extracts have also linked the presence of tannins in the extract to its wound healing property.

Conclusion

The present investigation had thrown light on the remarkable potential of commonly available plant *Hydrocotyle sibthorpioides* in terms of its pharmacological benefits it offers. The ethanolic extract of the leaves of *Hydrocotyle sibthorpioides* was found to be effective in the functional recovery of the wound. The result may be attributed to the phytoconstituents such as flavonoids, tannins and phenolics present in the extract which may be due to their individual or cumulative effect that enhanced wound healing.

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