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EVALUATION OF PHYTOCHEMICALS AND WOUND HEALING ACTIVITY OF HYDROALCOHOLIC EXTRACT OF *Cocus nucifera* L. ROOTS

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Abstract:

Herbal medicines are being used by about 80% of the world population for primary health care due to their efficacy, safety, cultural acceptability and less side effects. The present study was carried out to evaluate wound healing potential of hydro alcoholic extract of Cocus nucifera L., (HACN) in experimental animals by incision, excision and burn wound models. The HACN was administered topically in graded doses (5% & 10% w/w) for evaluating the wound healing property for fourteen days respectively. Neomycin sulphate ointment served as standard (0.5% w/w). Dose dependent activities resulted in both the wound models when compared to the standard and vehicle control. Topical application of HACN in excision & burn wound models significantly decreased the wound area by 15th day, as compared to vehicle control. In incision wound model, the breaking strength of wounds in animals treated with HACN was increased significantly from vehicle control. The HACN roots accelerated wound healing activity in rats and supporting its traditional use.

Keywords: *Wound healing, Incision, Excision, Burn wound, hydro alcoholic extract of Cocus nucifera L.*

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INTRODUCTION:

Wound healing is a complex process of restoring cellular structures and layers in damaged tissue together to its normal state and commencing in the fibroblastic stage where the area of the wound undergoes shrinkage [1]. It comprises of various phases such as contraction, granulation, epithelization and collagenation [2, 3]. The complete healing process can be discussed via three phase's viz. inflammatory phase, proliferative phase and maturational or remodeling phase. The inflammatory phase is characterized by haemostasis and inflammation. Proliferative phase is followed by epithelialization, angiogenesis and collagen deposition. In the maturation phase, the wound undergoes contraction resulting in a smaller amount of apparent scar tissue [4]. Granulation tissue which is formed in the final part of the proliferative phase is primarily made up of fibroblasts, collagen, edema and new small blood vessels.

A large number of plants are used by folklore traditions in India for treatment of cuts, wounds and burns. Various research data revealed that plants may worked as healing and regeneration of the tissue by multiple mechanisms. *Cocus nucifera* Linn. (Family: Palmae) is commonly referred to as Coconut or Nariel. The coconut palm is a long lived plant that may live as long as 100 years. It has a single trunk which can grow up to 20-30 meters tall. Its bark is smooth and grey, marked by ringed scars left by fallen leaf bases. Unlike some other plants, the palm tree does not have tap root hairs but has fibrous root system. The plant is native to tropical eastern regions. Today it is grown both over the Asian continent (India, Ceylon, Indonesia) and in Central and South America (Mexico, Brazil). In Africa, the largest producing countries are Mozambique, Tanzania and Ghana. The traditional uses of the roots include properties like astringent, dentifrice, decoction of root promotes flow of urine and is used in the diseases of the uterus, bronchitis and dysentery. Pharmacologically the root extract has been proved for anthelmintic activity and as anti bacterial agent, in treatment for urinary tract infections and also in some skin infection [5, 6].

Based on the information, the current study was aimed at evaluating wound healing activity of *Cocus nucifera* L. using *in vivo* animal models.

MATERIALS AND METHODS:

Identification, Authentication of plant materials:

The roots of *Cocus nucifera* L. were collected from surrounding areas of Tirupati rural and was authenticated (Voucher No.2547) by Dr. K. Madhava Chetty, Assistant Professor, Department of Botany, S.V. University, Tirupati, Andhra Pradesh, India.

Preparation of extract:

The roots were washed under running tap water, cut into small pieces of 2-3cm and shade dried (30^{o} C) for 15 days. The shade dried plant material was powdered using a dry grinder into coarse powder. The powder was stored in air tight container for further use. The shade dried and coarsely powdered root of *Cocus nucifera* Linn. was extracted with hydroalcoholic solvent (70%) water : methanol by cold maceration in a narrow mouthed bottle for seven days with occasional shaking. After completion of extraction, it was filtered and the solvent was removed by evaporation and then dried [7]. The residue was then weighed and yield was recorded. Preliminary phytochemical screening of HACG was carried out for the detection of the various bioactive constituents [8].

Animals:

Albino Wistar male rats weighing 150-200gms were procured and acclimatized to the experimental area having a temperature of 24 ± 2^{o} C, controlled humidity conditions and 12:12 hr light and dark cycle. Animals were kept in polypropylene cages and were fed with standard food pellets and water *ad libtium*. The study was approved by Institutional Animal Ethical Committee (IAEC), Seven Hills College of Pharmacy, Tirupati, Andhra Pradesh, India (Registered No: 1995/PO/Re/S/17/CPCSEA).

FTIR Analysis: Procedure for analysis of HACN:

A small quantity of HACN was mixed with potassium bromide (KBr) and pellets were prepared and this pellet was analyzed using FTIR spectroscopy. This was used to detect the characteristic peaks and their functional groups. A KBr pellet was prepared by grinding the solid potassium bromide and applying great pressure to the dry mixture. 2 mg of extract was taken with dry IR grade KBr at about 2% ratio in a motor. The grinding was performed until uniformly distributed throughout the KBr. Some amount of the mixture was transferred to the pellet making die and by applying some pressure to the die before pulling the vacuum. Then full pressure of 8000 pounds to 15000 pounds was applied to the die for 2 min. initial vacuum was released along with pressure. Then the vacuum was pulled for 1 to 2 min. The die set was disassembled by removing the base by twisting it off and releasing the U ring. Then the pellet was discharged by using the clear cylindrical pellet extractor located above the end of the bore and the plunger located between the assemblies. Usually background was first scanned by

using a blank potassium bromide pellet. Then sample was scanned. The spectrum was collected in the region of 650cm^{-1} to 4000 cm^{-1} with 8 cm^{-1} resolution [9].

Preparation of ointment by fusion method:
Preparation of Simple ointment: Wool fat - 2gm; White soft paraffin - 34gm; hard paraffin - 2 gm; Cetosteryl alcohol - 2gm. Each ingredient was mixed and heated gently with stirring and then cooled. The base was then packed in a wide mouth container [10].

Preparation of 5% test extract ointment: 1 gm of HACN was added slowly to the above melted ingredients and stirred thoroughly until the mass cools down and a homogeneous product is formed. The ointment was then packed in a wide mouth container.

Preparation of 10% test extract ointment: 2 gm of HACN was added slowly to the above melted ingredients and stirred thoroughly until the mass cools down and a homogeneous product is formed. The ointment was then packed in a wide mouth container.

Experimental Design:
Adult male Wistar albino rats (200-250 g) were divided into 4 groups, each group consists of six animals. The first group of rats fed with normal laboratory diet along with Simple ointment (IP) applied topically served as vehicle control. The second group of rats was applied topically with 0.5% w/w Neomycin sulphate ointment for 15 consecutive days served as standard control. The third and fourth groups of rats were applied topically with HACN (5% w/w & 10% w/w) root ointment respectively for 15 consecutive days served as test control [11].

Excision wound model:
Four groups of animals containing six rats in each group were anesthetized by open mask method with anesthetic ether. The rats were depilated on the back and a predetermined area of 500 mm^2 full thickness skins was excised in the dorsal interscapular region. Rats were left undressed to the open environment. The test formulation ointment of both doses and standard drug were applied daily for 15 days. In this model, wound contraction was measured as percent contraction in each 5 days after wound formation [12,13,14]. An excision wound margin was traced after wound creation by using transparent paper and area measured by graph paper. Wound contraction was measured in each 2 days interval, until complete wound healing and expressed in percentage of healed wound area [15]. The evaluated surface area was then employed to calculate the percentage of wound contraction, taking initial size of wound, 500 mm^2, as 100%, by using the following formula as:

% Wound contraction = [[initial wound size – specific day wound size] / initial wound size] × 100

Incision wound model:
In incision wound model, 6 cm long paravertebral incision were made through the full thickness of the skin on either side of the vertebral column of the rats, after all the animals of each group were anesthetized under light ether anesthesia. No local or systemic antimicrobials were used throughout the experiment. All groups were treated same as in excision model, the both edges kept together and stitched with black silk surgical thread (no. 000) and a curved needle (no. 11) was used for stitching. The continuous threads on both wound edges were tightened for good closure of the wound. After stitching, wound was left undressed then ointment base, standard ointment and test extracts ointment were applied daily until 15 days; when wounds were cured thoroughly the sutures were removed on the day 16 and tensile strength of cured wound skin was measured using tensiometer [16,17]. The tensile strength of a wound represents the degree of wound healing indicating how much the repaired tissue resists to breaking under tension and may indicate in part the quality of repaired tissue. In this method, wound breaking strength was measured as the weight of water at the time of wound breaking per area of the specimen [18].

Burn wound model
Burn wounds were created on dorsal part of shaved rat's skin surface using concentrate sulfuric acid, exposed for 10 sec. After 24 hrs, dead tissues were excised using sterile surgical blade through a template designed to produce a third degree burn [19]. All groups were treated same as in excision model. In this model, wound contraction was measured as per procedure followed in excision wound model.

Statistical Analysis:
All the values were expressed as mean ± standard error mean. The data's were statistically analyzed by one-way ANOVA followed by Dunnet's *t*-test, and value $P < 0.05$ was considered to be significant.

RESULTS:

Phytochemical screening:

Preliminary phytochemical screening revealed the presence of flavonoids, glycosides, tannins, terpenoids, saponins, carbohydrates etc.

FTIR analysis & Interpretation of HACN

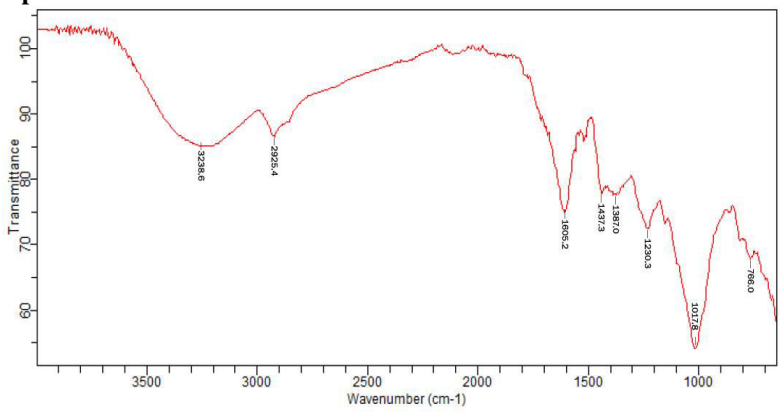


Figure 1: FTIR of HACN

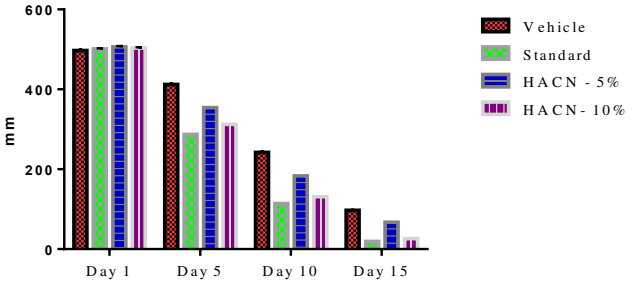
Table 1: FTIR interpretation of HACN

Peak	Bond	Group	Vibration
3238.6	OH	Alcoholic / Phenolic	Stretching
2925.4	OH	Carboxylic acids	Stretching
1605.2	C=C	Aromatic rings	Stretching
1437	C-H	Alkanes	Scissoring & Bending
1387	C-H	Alkanes	Scissoring & Bending
1230	C-O	Alcohols, Ethers, Carboxylic acids, Esters	Stretching
1017.8	C-O	Alcohols, Ethers, Carboxylic acids, Esters	Stretching
766	C-H	Alkenes	Bending

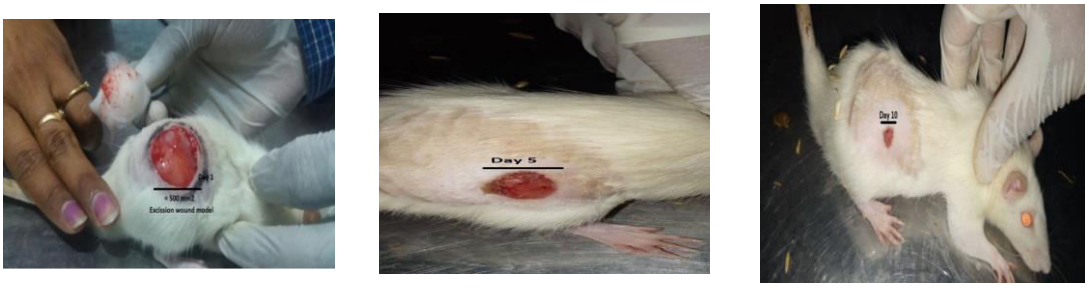
Table 2: Effect of HACN root on area of contraction in excision wound model

Treatment group	Area of contraction (Post wound day) (mm)			
	Day1	Day 5	Day 10	Day 15
Vehicle Control (Simple ointment)	497 ± 2.42	412 ± 2.89	242 ± 2.43	97 ± 1.21
Standard (Neomycin sulphate 0.5% w/w) ointment	501 ±1.82	287 ± 1.92***	114 ± 1.89***	19 ± 1.06***
5% w/w HACN ointment	506 ± 2.34	354 ± 1.67**	183 ±2.63**	67 ± 1.12**
10% w/w HACN ointment	503 ± 2.06	312 ± 1.21***	131 ± 2.14***	26 ± 0.93***

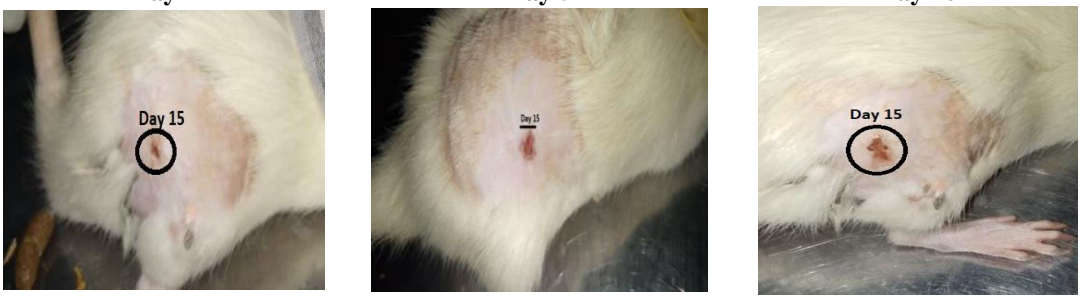
Values are expressed as Mean ± SEM values (n=6), Comparison is made by One Way Anova by Dunnett's Comparison Test at Significance value (P < 0.05), ***P<0.001, **P<0.001, *P<0.05, **ns**-non-significant when compared to vehicle Control.



Graph 1: Effect of HACN root on area of contraction in excision wound model



Day 1 **Day 5** **Day 10**



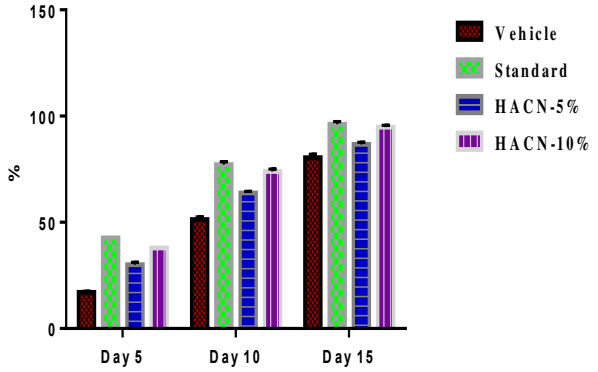
Day 15 – Standard (Neomycin sulphate 0.5% w/w) ointment **Day 15 - 5% w/w HACN ointment** **Day 15 - 10% w/w HACN ointment**

Figure No.2: Area of contraction post wound day – Excision wound model

Table No. 3: Effect of HACN root on % of contraction in Excision wound model

Treatment group	% wound contraction		
	5th day	10th day	15th day
Vehicle Control (Simple ointment)	17.1 ± 0.5	51.3 ± 1.2	80.4 ± 1.6
Standard (Neomycin sulphate 0.5% w/w) ointment	42.7 ± 0.91**	77.2 ± 1.4***	96.2 ± 1.23**
5% w/w HACN ointment	30.0 ± 1.2*	63.8 ± 0.8*	86.7 ± 0.93ns
10% w/w HACN ointment	37.9 ± 0.62**	73.9 ± 1.13**	94.8 ± 0.87**

Values are expressed as Mean ± SEM values (n=6), Comparison is made by One Way Anova by Dunnett's Comparison Test at Significance value (P < 0.05), ***P<0.001, **P<0.001, *P<0.05, **ns**-non-significant when compared to vehicle Control.

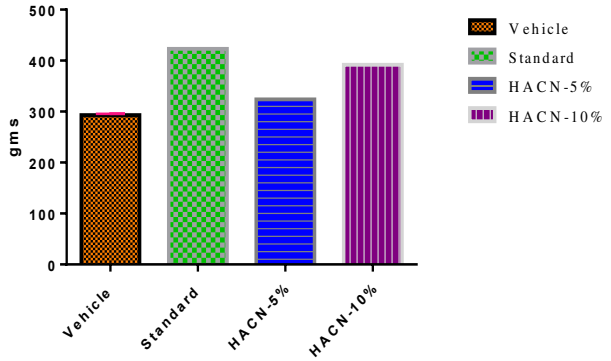


Graph 2: Effect of HACN root on % of contraction in Excision wound model

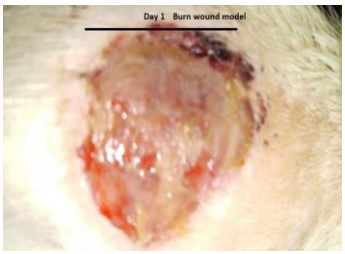
Table 4: Effect of HACN root on tensile strength in incision wound model

Treatment group	Tensile Strength (gms)
Vehicle Control (Simple ointment)	293 ± 2.43
Standard (Neomycin sulphate 0.5% w/w) ointment	423 ± 3.24***
5% w/w HACN ointment	324 ± 2.89*
10% w/w HACN ointment	392 ± 2.23***

Values are expressed as Mean ± SEM values (n=6), Comparison is made by One Way Anova by Dunnettes Comparison Test at Significance value (P < 0.05), ***P<0.001, **P<0.001, *P<0.05, when compared to vehicle Control.

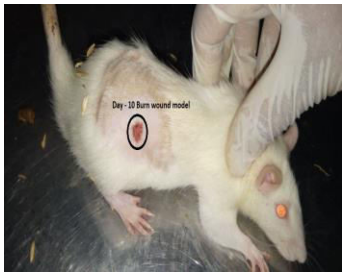


Graph 3: Effect of HACN root on tensile strength in incision wound model



Day 1

Day 5



Day 10

Day 15 – Standard (Neomycin sulphate 0.5% w/w) ointment

Day 15 - 5% w/w HACN ointment

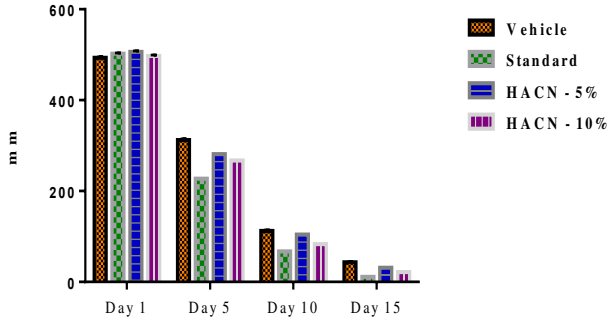
Day 15 - 10% w/w HACN ointment

Figure 3: Burn wound model – Area of contraction (mm)

Table No 5: Effect of HACN root on area of contraction in burn wound model

Treatment	Area of contraction (post wound day) (mm)			
	Day1	Day 5	Day 10	Day 15
Vehicle Control (Simple ointment)	493 ± 2.32	312 ± 2.69	112 ± 2.43	43 ± 1.26
Standard (Neomycin sulphate 0.5% w/w) ointment	502 ±1.72	227 ± 1.82***	67 ± 1.79***	11.3 ± 1.03***
5% w/w HACN ointment	506 ± 2.22	281 ± 1.77***	104 ± 2.63***	31.3 ± 1.14***
10% w/w HACN ointment	497 ± 2.16	267 ± 1.32***	83 ± 2.04***	21.8 ± 0.95***

Values are expressed as Mean ± SEM values (n=6), Comparison is made by One Way Anova by Dunnett's Comparison Test at Significance value (P < 0.05), ***P<0.001, **P<0.001, *P<0.05, when compared to vehicle Control.

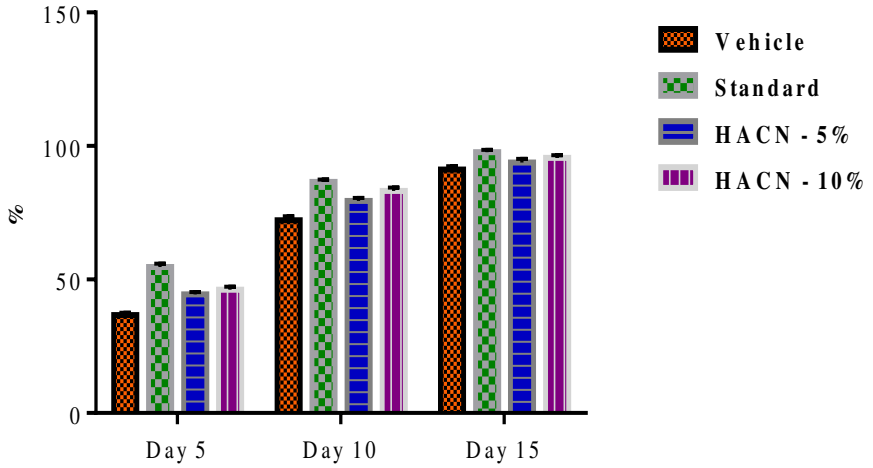


Graph 4: Effect of HACN root on area of contraction in burn wound model

Table No 6: Effect of HACN root on % of contraction in Burn wound model

Treatment	% wound contraction on days		
	5th day	10th day	15th day
Vehicle Control (Simple ointment)	36.7± 0.82	72.2 ± 1.43	91.2 ± 1.23
Standard (Neomycin sulphate 0.5% w/w) ointment	54.7 ± 1.21***	86.6 ± 0.86***	97.7 ± 0.82***
5% w/w HACN ointment	44.4 ± 0.94*	79.4 ± 1.17**	93.8 ± 1.41**
10% w/w HACN ointment	46.2 ± 1.12**	83.2 ± 1.29***	95.6 ± 0.93***

Values are expressed as Mean ± SEM values (n=6), Comparison is made by One Way Anova by Dunnett's Comparison Test at Significance value (P < 0.05), ***P<0.001, **P<0.001, *P<0.05, when compared to vehicle Control.



Graph 5: Effect of HACN root on % of contraction in Burn wound model

DISCUSSION:

Wounds are still a major problem in developing countries, often having severe complications and involving high costs for therapy. Healing is a complex process that involves a series of biochemical and cellular reactions initiated in response to an injury that restores the function and integrity of damaged tissue [1,2,3]. It is consented that reactive oxygen species (ROS) are deleterious to wound healing process due to the harmful effects on cells and tissues [20]. Free radical scavenging enzymes (FRSE) are a cytoprotective enzymal group that has an essential role in the reduction, deactivation, and removal of ROS, as well as regulating the wound healing process. Wound related non-phagocytic cells also generate free radicals by involving non-phagocytic oxidase mechanism [21]. Imbalance in free radical generations and antioxidants caused delayed wound healing. Therefore, elimination of ROS could be an important factor in wound healing [22].

The extraction by maceration method for 50 gms of powder yielded 8.8%. The preliminary phytochemical screening revealed the presence of flavonoids, tannins, carbohydrates, glycosides, steroids and saponins. Based on the previous data of acute oral toxicity studies, the extract was found safe up to a dose of 2000 mg/kg body weight [23]**.** The dried extract was powdered and used for the preparation of ointment by fusion method in the doses of 5 & 10% w/w. An ointment base classically contains 80% oil and 20% water, and therefore is more occlusive and will drive the medication into the skin more rapidly than a solution or cream base; thus ointments are an optimal delivery method for topical antibiotics [24]**.**

In excision wound model, HACN showed faster healing compared with vehicle control group. The faster wound contraction by HACN may be due to stimulation of interleukin-8, an inflammatory α-chemokine which affects the function and recruitment of various inflammatory cells, fibroblasts and keratinocytes, and may increase the gap junctional intracellular communication in fibroblasts, and induces a more rapid maturation of granulation tissue [25] as compared to the vehicle control rats.

The wound breaking strength is determined by the rate of collagen synthesis and maturation process, wherein there is binding of collagen fibers through inter- and intra-molecular cross-linking [26]. The granulation tissue of the wound is primarily composed of edema, fibroblast, collagen and new blood vessels. The mesenchymal cells of the wound adjust themselves into fibroblast and then begin migrating into the wound gap together with fibrin strands. Collagen is a principal component of connective tissue, plays a key role in the healing of wounds, and provides a structural framework for the regenerating tissue [27]. In the present study, HACN was found to increase wound breaking strength in incision wound model which may indicate increased collagen content, cross-linking collagen and maturation by HACN compared to the vehicle control treated with the simple ointment.

In Burn wound model, the centripetal movement of wound margin is believed to be due to the activity of myofibroblast. Granulation, collagen maturation and scar formation are some of the many phases of wound healing which run concurrently, but independent of each other [28]. Since HACN enhanced wound contraction, it might have either enhanced contractile property of myofibroblasts or increased the number of myofibroblasts recruited into the wound area when compared to the vehicle control group.

The wound healing action of HACN may probably be due to the phytoconstituents present in the plant or could be a function of either the individual or the additive effects of the phytoconstituents. The present results also indicate a significant decrease in wound area from day 5 onward indicates early healing. In incision wound, an increase in tensile strength of treated wounds was observed and this may be due to the increase in collagen concentration and stabilization of the fibers [29].

Since flavonoids are reported to improve wound healing and protect tissues from oxidative damage [30]. Increase in neutrophils cells infiltrating the granulation tissue delays the wound healing process [31] where by neutrophils mediate lipid peroxidation through the production of superoxide anions [32]. Hence, any drug that inhibits lipid peroxidation is believed to increase the viability of collagen fibrils by increasing the strength of collagen fibres, increasing the circulation, preventing the cell damage and by promoting the DNA synthesis [33]. Studies revealed that flavonoids are known to promote the wound healing process mainly due to their astringent and antimicrobial properties responsible for the wound healing and increased rate of epithelialisation [34]. Tannins are also the main components of many plant extracts and they act as free radical scavengers [35,36].

A large number of plants are used by folklore traditions in India for treatment of cuts, wounds and

burns. Various research data revealed that plants may worked as healing and regeneration of the tissue by multiple mechanisms. There are several reports stating that the extracts of several plants, used for wound healing properties [37-40].

CONCLUSION:

Despite the discovery of various antibacterial and antiseptic agents, burn wound healing still has remained a challenge to modern medicine. Plants, with a valuable traditional support, have been considered as potential agents for prevention and treatment of disorders in recent years. However, modern scientific methods should be applied to validate the claims about the therapeutic effects of the herbal products. The present study has demonstrated that the hydro alcoholic extract of HACN roots have properties that render them capable of promoting accelerated wound-healing activity compared to vehicle control treated with simple ointment. Wound contraction and increased tensile strength support further evaluation of HACN in the topical treatment and management of wounds. Therefore, we may interpret that the presence of such response may be due to the presence of various phytoconstituents which were found to be present in the roots of *Cocus nucifera* L. However further studies can be performed to isolate the particular component responsible for generating wound healing activity.

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