



ISSN (E): 2277- 7695
ISSN (P): 2349-8242
NAAS Rating: 5.23
TPI 2021; SP-10(10): 824-829
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www.thepharmajournal.com
Received: 14-08-2021
Accepted: 29-09-2021

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Efficacy of neem (*Azadirachta indica*) leaf extracts in combination with camphor with respect to wound healing in animals

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Abstract

Current study deals with the efficacy of Neem (*Azadirachta indica*) in combination with camphor, both as aqueous and alcoholic extract in treatment of wound. The wound healing potential of aqueous and alcoholic extracts of Neem (*Azadirachta indica*) leaves with camphor was evaluated on the basis of clinical and histological studies. The formulations comprised of the aqueous and alcoholic extracts which were used alone and in combination with camphor in the ratio of 1:1. The formulations comprised of the aqueous and alcoholic extracts which were used alone and in combination with camphor in the ratio of 1:1. A total of thirty wounds of which fifteen infected and fifteen contaminated present on various body locations were treated. Before treatment clinical examination of all the wounds revealed presence of variable degrees of swelling around the wounds, itchiness, pain upon palpation and variable degree of discharge with foul smell. With increasing antibiotics resistance and in animals and human alike, the herbal medicament is the need of the hour. More research on the current topic with varying concentration of camphor and Neem in a defined age, sex and species of animal could help to provide clearer and in-depth knowledge about the efficacy of these two important herbal drugs.

Keywords: Granulations, epithelialization, alcoholic extract, aqueous extract, percentage healing, *Azadirachta indica*

Introduction

Wound is defined as a loss or break in the cellular, anatomic or functional continuity of living tissues (Gerald *et al.*, 1994; Bennet, 1998; Ayello, 2005) [9, 4, 2]. Skin wounds could be created by several means, the most important one is physical injury resulting in break of the skin continuity. The most common symptoms of wounds are bleeding, loss of feeling and/or function below the wound site, heat and redness around the wound, painful or throbbing sensation and swelling of the tissues in the area and exudate discharge (Rashed *et al.*, 2003) [25]. Herbal medicaments have been used in the treatment of wounds for many years as they are effective, produce little or no toxicity and are quite cheap and more easily available.

Neem tree has been referred to as “Sarva Rog Haro Nimbah” and is aptly called as the “village dispensary” of rural areas (Siddiqui and Mitra, 1945) [29]. Almost every part of the *Neem* tree has some medicinal use (Mitra, 1963) [19]. Pure organic *Neem* leaf, (no stems or bark) contains 20 percent fibre, 50 percent carbohydrates, 15 percent proteins, 5 percent fat, 8 percent ash, 2 percent calcium, small quantity of essential amino acids along with carotene and ascorbic acid (Sadekar, 1998) [26].

Cinnamomum camphora (camphor), an active, oriental herbal medicine has long been prescribed for the treatment of inflammation-related diseases (Choi, 1997) [7]. According to phytochemical studies, it contains a volatile oil comprising camphor (Miyazawa *et al.*, 2001) [20], safrole, eugenol and terpineol along with a Type 2 ribosome-inactivating proteins (cinnamomin and camphor) (Ling *et al.*, 1995; He and Liu, 2003) [17, 10]. The ingredients of *C. camphora* are thought to be responsible for the anti-inflammatory property. Lee *et al.*, (2006); Sekut and Connolly, (1996) [27]; Oh *et al.*, (2001) [21]; Bingham, (2002) [5] and Payne, (2003) [22] investigated the modulatory effect of *C. camphora* on macrophage-mediated inflammatory phenomena *viz.* cytokine production, nitric oxide release, PGE2 release, functional activation of adhesion molecules and oxidative stress and concluded that *C. camphora* exerted a significant immuno-modulatory effect on various inflammatory responses at the transcription level mediated by pharmacological selectivity.

Considering above facts, the present study was designed to study the wound healing property of different preparations of *Azadirachta indica* (Neem) leaves in combination with camphor in different types of wounds in animals.

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Materials and Method

The present study of wound healing was carried out on clinical cases of different types of wounds brought to the college teaching hospital or present in the Instructional Dairy Farm, Nagla of G.B. Pant University of Agriculture and

Technology, Pantnagar.

All animals irrespective of age, breed, sex and species suffering either with infected or contaminated wounds on anybody surface and at least of 15 days duration were included in the study.

Table 1: Design of experiment

Groups	No. of Wounds		No. of contaminated wounds	Location of wounds on body surface	Total no. of animals	Treatment protocol
	No. of infected wounds	Location of wounds on body surface				
I	3	Base of neck, vulva, abdomen	3	Nasal bone, stifle region, vulva	6	Aqueous extract + Camphor
II	3	Tail, perineum, thigh	3	Pinna, nasal bone, thigh	6	Alcoholic extract + Camphor
III	3	Head, knee, hock	3	Abdomen, head	6	Control

Collection of Plant materials and preparation of extracts

Collection of Plant Materials

Neem leaves were collected from the surrounding areas and shade-dried till they became brittle. Thereafter they were grounded to fine powder and stored in airtight bottles for further use. Both aqueous and alcoholic extracts were prepared by cold extraction technique using 100 grams of shade-dried leaf powder. The powder was transferred in a 1 litre flask containing 250ml of either ethanol or water. The mixture was kept overnight with occasional shaking. Thereafter, the contents of the flask were filtered through 4 layers of muslin cloth. The filtrate was centrifuged at 2500 revolutions per minute for 15 minutes. Finally, the supernatant was concentrated at 37 deg C in an incubator. The solid extracts thus obtained were used for the preparation of ointment. The extracts being oily in nature, negated the incorporation of additional emollients. Therefore, the aqueous and ethanolic extracts were used in their pure forms. The camphor preparations were prepared by mixing camphor with the respective extract in the ratio of 1:1 (weight by weight). The clinical examination of the wounds was done daily to record the degree of inflammation, general appearance, extent of cicatrization and percent healing of the wound. Wound evaluation was done on weekly intervals and the colour of wound, type and quantity of exudates, peripheral swelling, warmth, pain, granulation tissue and scab formation were taken into consideration. The wounds were gently cleaned using sterile gauze pieces and the appropriate medicament applied once daily until complete healing occurred. No local or systemic antimicrobial was used throughout the experiment.

Percent Healing

The percent healing was observed by the method of Kumar and Tyagi (1972) [15] with slight modifications.

$$\text{Percent healing} = [(a - b)/A] \times 100$$

Where, 'a' is the area of wound at the beginning of a particular period, 'b' is the area of the wound at the end of that period and 'A' is the initial area of the wound (incidentally a = A).

Further, in-vitro antibacterial activities of aqueous and ethanolic extracts of Neem leaves and their combination with camphor in the stated ratio were tested against the most common bacterial population of wounds.

For histological studies, the biopsy specimens were collected from the margins of healthy skin and healing wounds on 3rd,

7th, 15th and 30th day and preserved in 10% buffered neutral formalin solution which were processed by routine paraffin embedding technique and longitudinal sections of 5µ thickness were cut. Finally staining of the cut sections was done using H&E and Masson's Trichrome stain as described by Carleton and Drury (1965) and Masson (1929) [18].

At the end the data were subjected to the analysis of variance as per the procedures outlined by Snedecor and Cochran (1967) [28].

Result and Discussion

The microbes were found resistant when tested against the in-vitro antibacterial activity of the extracts as evidenced by the absence of any inhibitory zone around the medicated discs impregnated with the various formulations of *A. indica* leaf extracts. The assessment of wound healing was done on the basis of clinical and histopathological observations as follows-

Clinical Observations

Group-1: Aqueous Neem extract mixed with camphor



Fig 1: Day of Presentation



Fig 2: Day One



Fig 3: Day 15



Fig 6: Day 30

Group II (Alcoholic extract mixed with camphor)



Fig 4: Day of Presentation



Fig 5: Day One

Table 2: Percent contraction in group I extract camphor at different durations (figures-1, 2, and 3)

Animal No.	Day of observation			
	3	7	15	30
1	5.00	21.66	60.00	93.33
2	6.45	19.35	61.29	96.77
3	13.39	32.14	55.35	86.60
4	16.66	50.00	88.88	100.00
5	10.00	30.00	70.00	90.00
6	20.00	40.00	74.50	83.66
Average	11.91a	32.19 b	68.33c	91.71d
SE	2.39	4.68	5.00	2.52

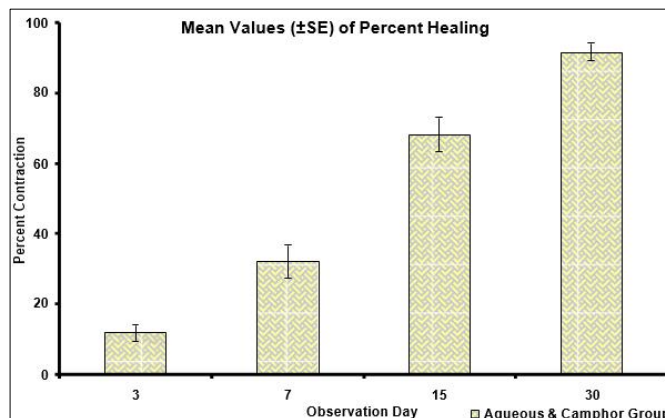


Fig 7: Means bearing different superscripts differ significantly ($P < 0.01$)

Table 3: Percent contraction in group IV with Alcoholic extract having camphor at different durations (Figures 4, 5 and 6)

Animal No.	Day of observation			
	3	7	15	30
1	15.51	49.13	83.62	100.00
2	16.36	45.45	85.45	100.00
3	11.62	32.55	69.76	95.34
4	14.28	47.61	85.71	100.00
5	12.00	36.00	84.00	100.00
6	16.66	33.33	75.00	100.00
Average	14.40a	40.67b	80.59c	99.22d
SE	0.88	3.07	2.70	0.77

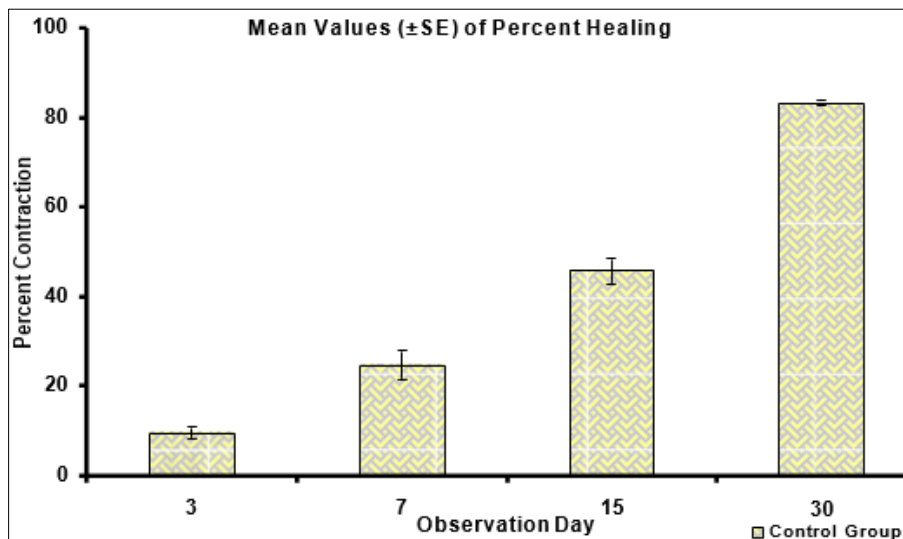


Fig 8: Percent contraction in group V (Control) at different durations

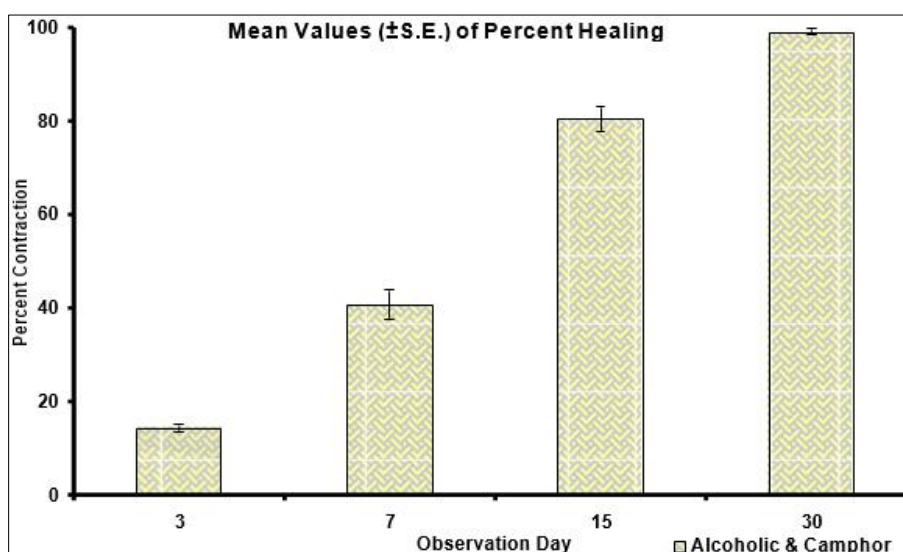


Fig 9: Show the different of observation day and alcoholic and camphor

In the control wounds, infiltration of neutrophils at the wound site was extensive and least was in alcoholic extract treated group (Fig. 10, 11). In wounds treated with aqueous mixed with camphor, infiltration of neutrophils was moderate, whereas wounds treated with alcoholic extract with camphor, it was comparatively more than aqueous extract with camphor treated wounds.

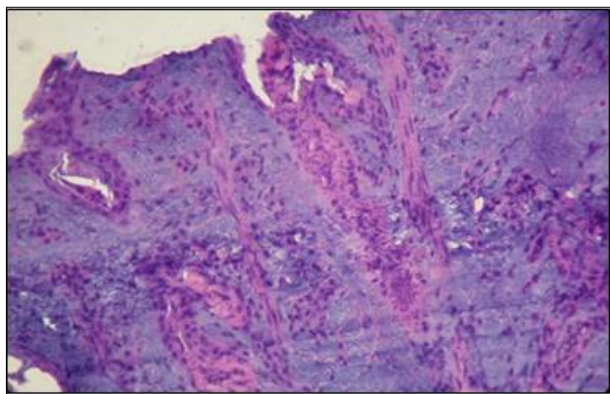


Fig 10: Photomicrograph of alcoholic extract treated wound, day 7th, note maximum angioblastic and fibroblastic proliferation. Stain H & E

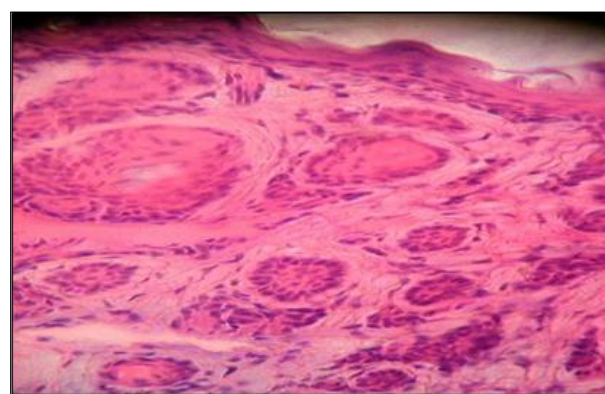


Fig 11: Photomicrograph of alcoholic extract treated wound, day 30th, note transversely cut bundles of collagen fibrils. Stain H & E

Early regression of inflammation could be due to the anti-inflammatory effects of nimbin and nimbidin found in Neem (Kraus, 1995) [14]. When aqueous or alcoholic extracts were mixed with camphor, their anti-inflammatory effect is reduced by the irritant nature of camphor. As a result, inflammation persisted for slightly longer duration as evidenced by histological observations of this study. Absence of exudates by the 5th day in all treated wounds is a further indication of

anti-inflammatory action of Neem leaf extract as compared to control group of animals. Besides, the alcoholic extract based formulations may contain more potent alcohol soluble ingredients of Neem i.e., limonoids (Aliero, 2003) ^[1]. Contrary to this the aqueous extract of Neem leaves contained only the water soluble compounds of Neem i.e., “neem bitters” or tetranotriterpenoids (Baral and Chattopadhyay, 2004) ^[3] which may not be as effective as that of the alcoholic extract. Prolongation of the inflammatory phase in control wounds (Group V) was in accordance with the observations made by Bhargava *et al.* (1988) ^[11] and Jhirwal *et al.* (2004). This can be attributed to tissue hypoxia developed due to presence of microbial toxins, foreign bodies and fragments of necrotic tissue as they are powerful local stimuli causing influx of neutrophils and macrophages for longer duration (Singer and Clark, 1999; Eming *et al.*, 2002) ^[30, 21]. Probst and Bright (1985) ^[24] have also stated that the presence of foreign material and bacterial toxins can greatly prolong the inflammatory phase and delay tissue repair. Wound contraction results in the reduction of wound size by the inward (centripetal) movement of surrounding skin (Peacock, 1986) ^[23]. In the present study, fast contraction was observed in both the groups following treatment. Beyond day 10, faster rate of contraction was observed in the alcoholic extract groups compared to the aqueous ones. This clearly indicated their greater efficacy in causing wound closure. Similarly, according to findings of Bwala *et al.* (2011) ^[6] A single application of the crude neem oil to the sutured wound resulted in a complete healing after seven days without systemic or topical application of antibiotics. Tran *et al.* 2015 ^[31], reported that camphor induced the expression of collagen IA, collagen IIIA, collagen IVA, and elastin in human primary dermal fibroblasts. In addition, post-treatment with 26 and 52 mM camphor for 2 weeks led to a significant reduction in the expression of MMP1 but increases in the expression of collagen IA, IIIA, and elastin in mouse skin exposed to UV for 4 weeks.

Summary and Conclusion

On the basis of clinical and histo-morphological studies, it was revealed that both the aqueous and alcoholic extract ointment of *A. indica* with camphor are an ideal preparation for accelerating infected and contaminated wounds under field conditions even when unaided with any antibiotics therapy (topical or oral) although with varying efficacy over the control group. The alcoholic group was found to have better efficacy over aqueous group. Current research if continued with more number of animals of similar age, sex, species or breed could help unravel more research findings related to wound healing properties of neem and camphor, hitherto unexplored.

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