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Antioxidant and Wound Healing Property of Polyherbal Ointment of Nepalese Medicinal Plants

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ABSTRACT

Methanolic extracts of *Aloe vera, Bauhinia variegate, Centella asiatica, Cuscuta reflex, Chromalaena adarata, Cynodon dactlon, Myrica esculenta, Nyctanthes arbor tritis, Psidium guajava, Rhododendron arboretum, Ficus lacorpysus, Pyrus pashia, Bombax ceiba* were evaluated for their total tannins and total phenolics. Among them, *Bauhinia variegata, Myrica esculenta, Rhododendron arboreum, Pyrus pashia* and *Psidium guajava* were found to have highest tannin content. These plants were further subjected to evaluate the antioxidant activity by DPPH assay. *Bauhinia variegata, Rhododendron arboreum,* and *Myrica esculenta* were formulated into 10% w/w ointment in the ratio of 1:1:2 respectively. In excision wound model, 9 days observation showed that wound was totally healed in herbal ointment treated rats while 2.72%, 4.5%, and 5.73% wound area was found remaining in Framycetin treated, blank and control group of rats, respectively. These traditional medicinal plants showed the significant wound healing activity in animal models, which justifies their use in traditional practice in Nepal.

Keywords: Medicinal plants, Plant Phenolics, Anti-Oxidant, Ointment, Wound Healing.

INTRODUCTION

Plants have been the basis for medical treatments through much of human history, and such traditional medicine is still widely practiced today. Plant extracts and their components are being used widely as peoples are interested on them for alternative health care. Indigenous cultures used herbs in their healing rituals while others developed traditional medical systems such as Ayurveda and Traditional Chinese Medicine in which herbal therapies were used systematically.

Wound healing is a natural biological process in human body. It is an intricate process in which the skin or other organ tissues repair itself after injury [1]. In normal skin, epidermis and dermis exists in equilibrium and forms the protective barrier against the external environment and in any case if the protective barrier is broken, the normal physiological process of wound healing sets in motion. Once bleeding is controlled, inflammatory cells migrate into the wound and promote the inflammatory phase which is characterized by the sequential infiltration of neutrophils, macrophages and lymphocytes [2]. A lot of research has been envisaged to develop the better healing agents. The effect of traditional herbal formulation was

screened on excision and incision wound models [3]. The rapidity of wound healing depends to a considerable extent on the contraction that begins a few days after injury and continues for several weeks [4].

The development of new wound healing ointment by using Nepalese traditional medicine provides important leads against various pharmacological targets. In present study for easy application on wound, ointment has been prepared and its feasibility was also checked by wound healing activity in animal model as compared to standard drug. This research was focused on preliminary phytochemical screening, total tannin and total phenolic content determination, evaluation of antioxidant property, formulation of ointment and comparison of wound healing activity of ointment against standard in albino rats.

MATERIAL AND METHODS

Plant Materials

Different parts of plants (Table 1) were collected from Chaukot jungle of Kavrepalanchok district of Nepal during March 2013. The location of research area is latitude 27°36'31.76''N and longitude 85°32'11' E, and 1439 m above the sea level. Voucher specimen was identified by Tirtha Maiya Shrestha and Dr. Rajendra Gyawali (Department of Pharmacy, Kathmandu University, Dhulikhel, Nepal). The collected plants were dried at room temperature.

Table 1 Plants collected based on the traditional use for the wound healing

Scientific name	Common name	Parts used
Aloe vera	Ghiu –kumara	Leaves
Bauhinia variegata	Koiralo	Bark
Bombax ceiba	Simal	Flower
Centella asiatica	Ghodtapre	Whole plant
Cuscuta reflex	Akashbeli	Creepers
Chromalaena adarata	Banmara	Leaves and stem
Cynodon dactlon	Dubo	Whole plant
Ficus lacorpysus	Kavro	Twig
Myrica esculenta	Kafal	Bark
Nyctanthes arbor tritis	Parijat	Leaves
Psidium guajava	Amba	Bark
Pyrus pashia	Mayal	Leaves
Rhododendron arboreum	Laliguras	Flower

Preparation of plant extract

Cold extraction was carried out using methanol. The whole dried plant sample was blended in home blender and powdered samples were initially soaked in methanol in a conical flask. The mouth of the flask was closed with aluminum foil to reduce the volatilization of the solvent. The flask was allowed to stand for 2 days with constant shaking in rotary shaker. After 2 days, the solvent along with soluble components were filtered. The traces of methanol from the extracts were removed by keeping the extracts on water bath at 40°C temperature. The extracts obtained were then weighed and percentage of yield was evaluated and kept aseptically until use.

Phytochemical screening

The extracts of thirteen plant samples were screened for alkaloids, tannins and flavonoids [5].

Total Tannin Content Determination

Total tannins were determined by Folin and Ciocalteu method. In this method, 0.1 ml of the sample extract was added with 7.5 ml of distilled water and 0.5 ml of Folin phenol reagent, 1 ml of 35 % sodium carbonate solution and diluted to 10 ml of distilled water. The mixture was shaken well, kept at room temperature for 30 minutes and absorbance was measured at 725 nm. A blank solution was prepared with water instead of sample. A set of standard solutions of Gallic acid were treated in the same manner as described earlier and

read against the blank. The results of tannins were expressed in terms of gallic acid mg/g of extract [6].

Total Phenolic Content Determination

Total phenolic content of plant extract is determined by using Folin Ciocalteu's reagent. Concentration of total phenolic compound in all plant extracts can be expressed as mg of tannic acid equivalent per gram dry weight of sample in linear equation. For preparation of calibration curve, stock solution was prepared by mixing 10 mg of Gallic acid in 100 ml of distilled water. Different volumes of stock solutions i.e. 200 µl, 400 µl, 600 µl, 800 µl, 1000 µl were mixed with 4 ml distilled water and 0.6 ml Folin-Ciocalteu's phenol reagent. After 5 minutes, 1.6 ml of 20% sodium carbonate was added. Finally volume was made up to 10 ml. After 30 minutes absorbance was measured at 765 nm using UV – spectrophotometer and a calibration curve was drawn [7].

DPPH (1, 1-diphenyl-2-picrylhydrazyl) assay

The DPPH assay was done by the method given by Brand-Williams *et al.* with some modifications [8]. Test samples were prepared by adding 750 µl of 0.1mM methanolic DPPH solution in 750µl methanolic plant extract solution of varying concentrations (5, 10, 50, 100, 250, 500 and 1000 µg/ml). Corresponding blank samples were prepared and L-Ascorbic acid (1, 2.5, 5, 10, 25, 50 and 100 µg/ml) was used as reference standard. Mixer of 750 µl methanol and 750 µl DPPH solutions was used as control. The decrease in absorbance (*A*) was measured at 517 nm after 30 minutes in dark using UV-Vis spectrophotomer. The inhibition % was calculated using the following formula:

Inhibition % = A (Control) – A (Test Sample)/A (Control) X 100

Brine Shrimp Lethality test

Dried cysts were incubated (50 mg cysts per 300 ml) in a hatcher 37°C with strong aeration, under a continuous light regimen for 24h. Approximately, after 24 h of hatching, the phototropic nauplius were collected with a pipette from the lighted side and concentrated in a small vial. Ten brine shrimp were transferred to each well using adequate pipette. Each test consisted of exposing groups of 10 *Artemia* aged 24h to various concentrations of the extracts. The toxicity was determined for 24 h and 48 h of exposure [9]. However, in cases where control deaths were detected, the percentage of mortality (% M) was calculated as: % M = percentage of survival in the control - percentage of survival in the treatment [10].

Formulation of ointment

For the formulation of ointment from methanolic extracts, three plants with high tannin content and with significant anti-oxidant property were used. PEG 400 and PEG 4000 were used as ointment base. The bases were melted at 70°C and plant extracts were dissolved in bases (Table 2).

Table 2. Formulation component of ointment

Materials required	Weight(g)
PEG 400	25.11
PEG 4000	11.05
Extracts	4.087

Animals

Animal testing was done using eight Albino rats, which were brought from Department of Plant Resources Babarmahal Kathmandu. Institutional Animal Ethics Committee permission was taken before the experiment.

Experiment protocol

The herbal extracts (10% ointment) were applied topically for 9 days once in a day and the area of wound was measured once in two days. Albino rats of either sex were distributed into 4 groups consisting of 2 rats per group; Group 1 is blank and consists of 2 rats, that were treated by plain ointment base daily, Group 2 is standard and is consisted of 2 rats, that were treated by 1%(w/w) Framycetin cream daily. Similarly, Rats of Group 3 were treated with 10%(w/w) combined methanolic extract of *Bauhinia variegate* (koiralo), *Rhododendron arboreum* (laliguras), *Myrica*

esculenta (kafal) were prepared in ratio of 1:1:2. Group 4 is a control and is consisted of 2 rats that were treated with liquid paraffin.

Excision Wound Healing Model

Excision wound was inflicted on the rats according to earlier described method with slight modification under light ether anesthesia [11]. The dorsal fur of the animals was cut with a scissor. Full skin thickness was excised from the marked area to get a wound measuring about 150 mm^2 by using toothed forceps, surgical blade and pointed scissors [12].

Wound Evaluation

The wound contraction was assessed by tracing the wound area first on transparent paper and subsequently transferring to a graph paper and squares counted excluding the day of the wounding on alternate day [13]. The evaluated surface area was plotted against the day of observation.

RESULT AND DISCUSSION

The phytochemical screening tests for methanolic extracts of *Centella asiatica*, *Rhododendron arboreum*, *Cuscuta reflexa* and *Psidium guajava* were tested positive for total tannin, total flavonoids and total alkaloid content. *Bauhinia variegata*, *Bombax ceiba*, *Aloe vera*, *Ficus lacorpysus*, *Myrica esculenta*, *Pyrus pashia*, *Nyctanthes arbortrius* and *Chromolaena odorata* were found with Tannin and Flavonoids. Similarly, *Cyanodon dactyalon* was found positive for Alkaloid. *Nyctanthes arbor tritis* showed the highest yield of 29.61% and *Bombax ceiba* showed the lowest yield of 3.42%.

Among the total phenolic calculation of methanolic extracts of thirteen different plants the highest phenolic content was found in *Bauhinia variegate* 276.6mg/gm of tannic acid and the lowest phenolic content was found in *Centella asiatica* 22.62mg/gm of tannic acid (**Fig 1**). Phenolic hydroxyl groups are good H-donating antioxidants, which scavenge reactive oxygen species and breaks the cycle of generation of new radicals. Hence, plants with higher Phenolic content act as antioxidants by inhibiting enzymes involved in radical generation and can be used in wound healing.

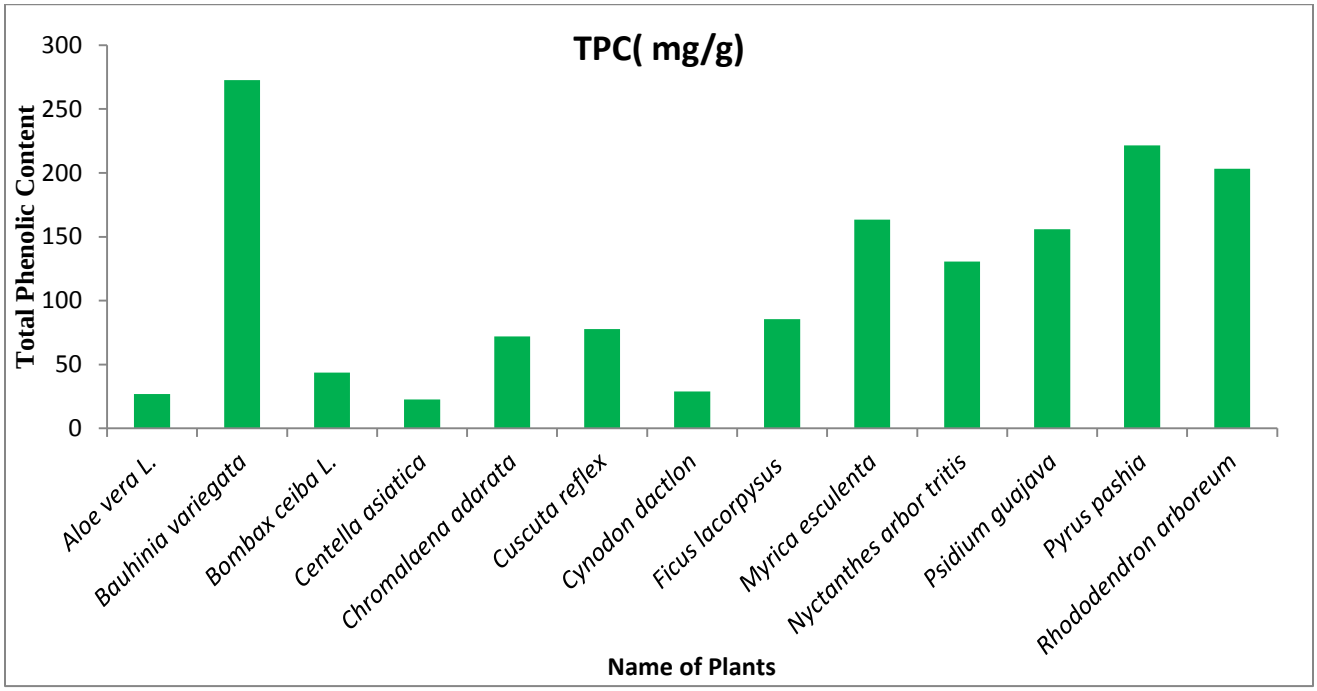


Figure 1. Total Phenolic Content of methanolic extract of different plant samples

Among total tannin calculation of methanolic extracts of fourteen different plants, the highest tannin content was found in *Rhododendron arboretum* (288.2 mg/gm of tannic acid) and

minimum content was found in *Cyanodon dactyalon* (19.54, g/gm of tannic acid) (**Fig 2**). Since, tannins have character of tightening, reducing irritation and inflammation and creating a barrier against infection, plants containing high tannin content are helpful to treat wounds and burns.

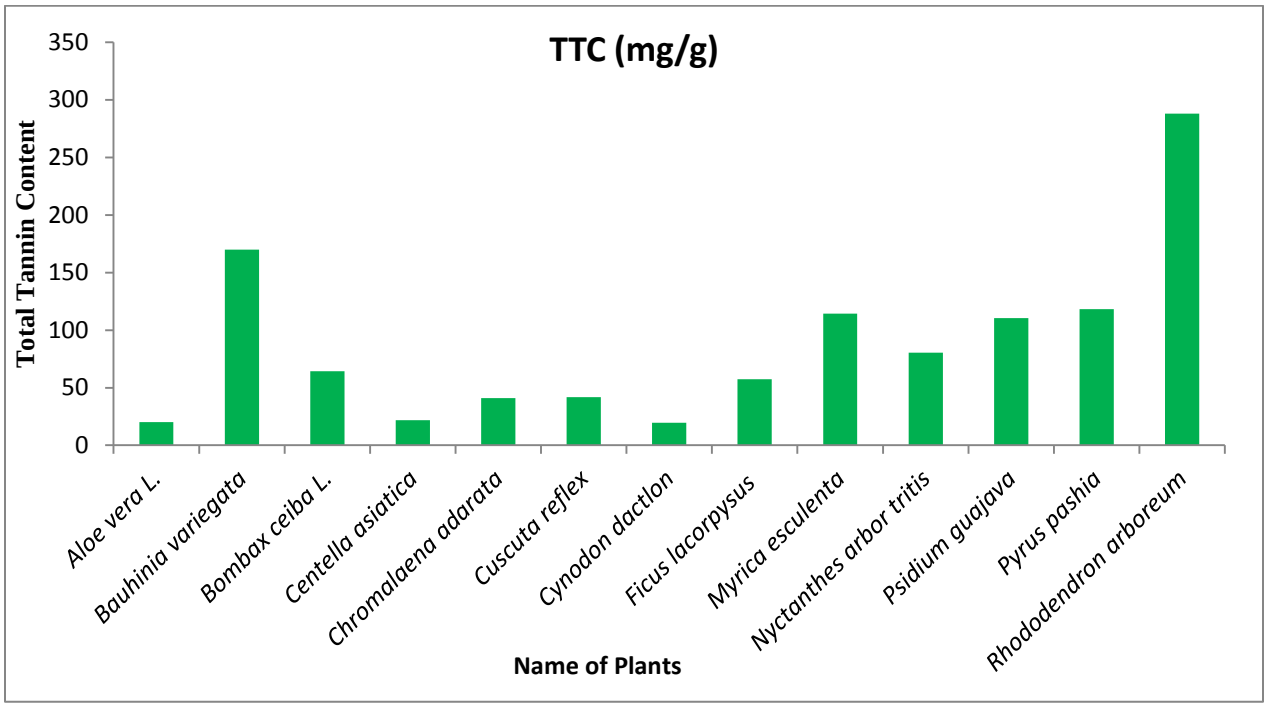


Figure 2. Total Tannin Content of methanolic extract of plant samples

Percentage inhibition of Ascorbic acid standard was found to be 4.43µg/ml. Among five different plants i.e. *Bauhinia variegata, Myrica esculenta, Rhododendron arboreum, Pyrus pashia and Psidium guajava,* selected for assay the maximum percentage inhibition was found in *Pyrus pashia* (13.46 µg/ml) and minimum percentage inhibition was found in *Myrica esculenta* (3.38µg/ml). plants *Myrica esculenta, Bauhinia variegate and Rhododendron arboreum* showed the highest antioxidant property and therefore chosen for the ointment formulation purpose.

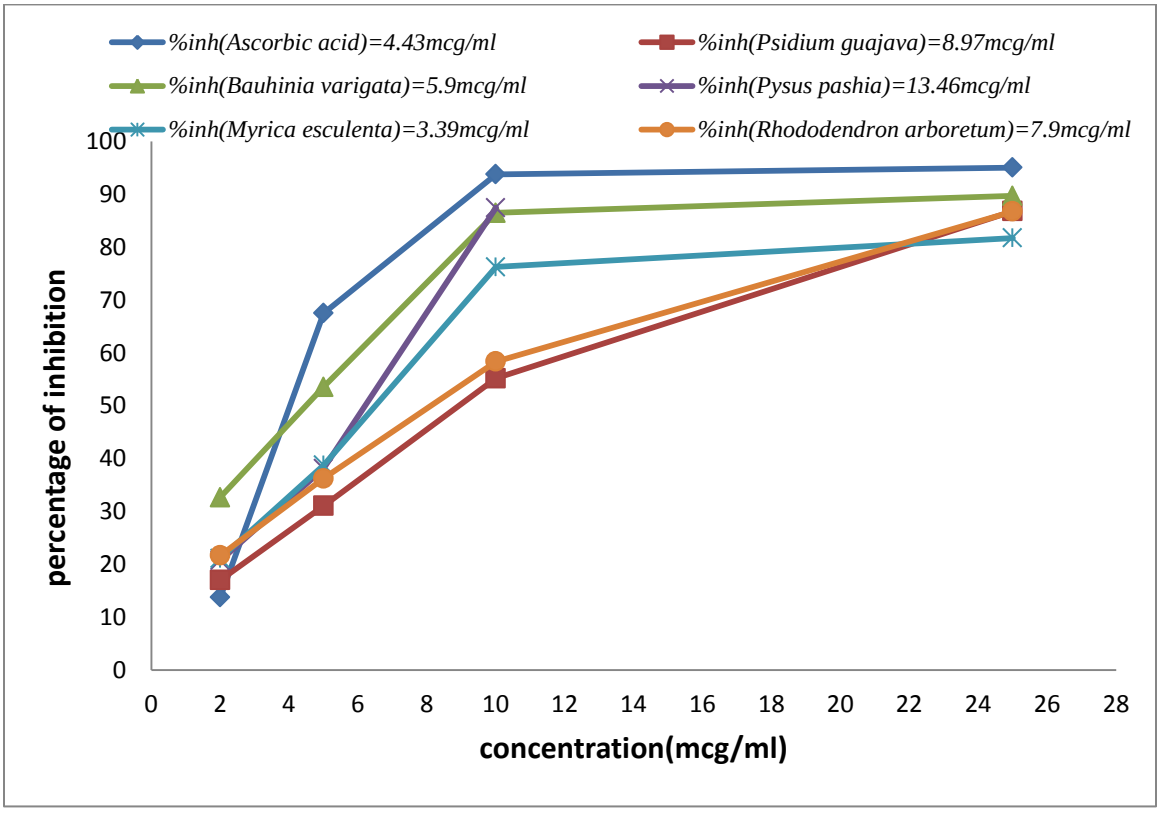
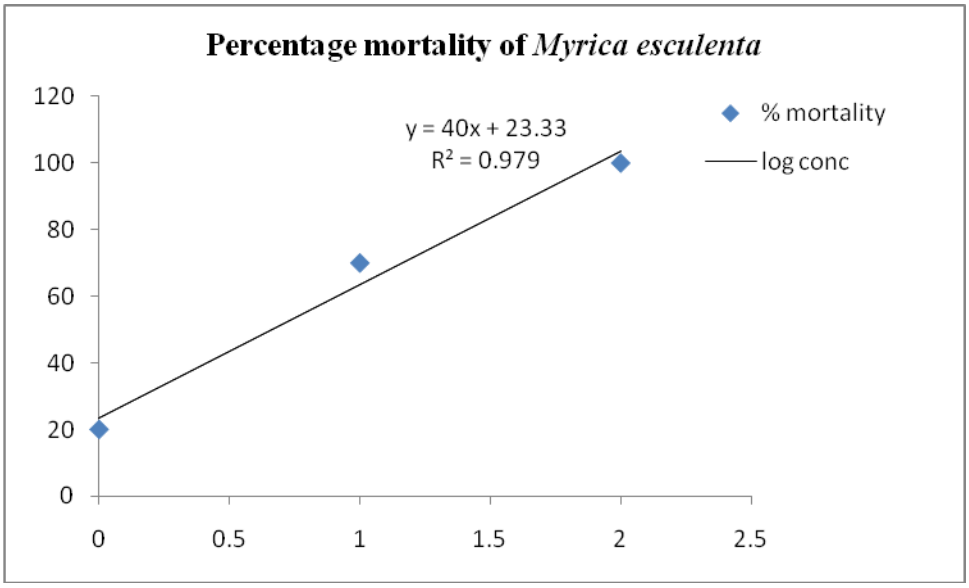


Figure 3. Antioxidant property of selected plants

For brine shrimp, following range has been prescribed for toxicity. LC50 <1.0mcg/ml: Highly toxic; LC50= 1-10mcg/ml: Toxic; LC50= 10-30 mcg/ml: Moderately toxic; LC50= 30-100mcg/ml: Mildly Toxic; LC50>100mg/ml: Non toxic. Moderate toxicity of plant extracts were observed (**Fig 4**) and probably have no obvious danger of outright toxicity. Thus topical application of the extract from these plants is unlikely to have any acute ill effect on patients as they are not in highly toxic category.



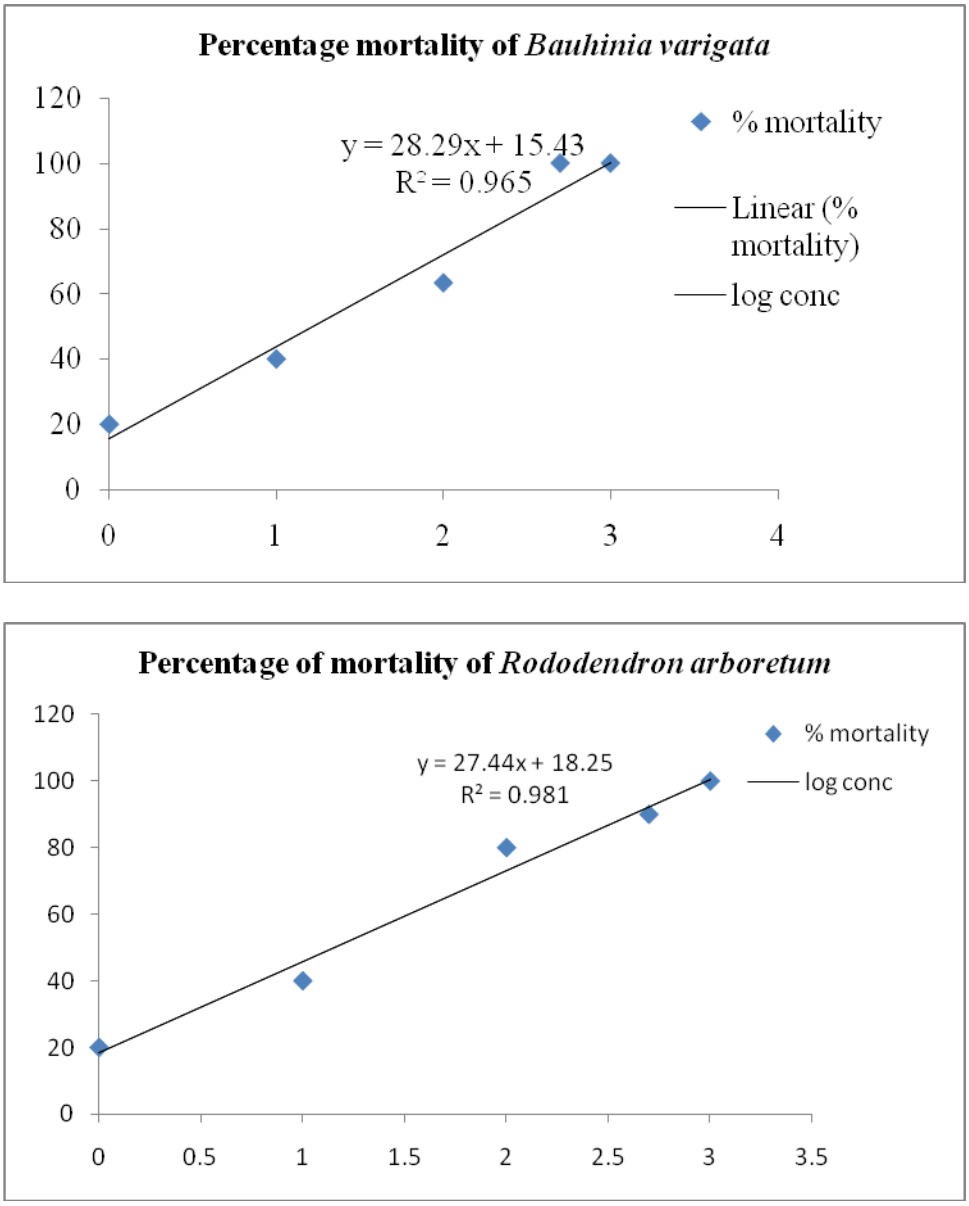


Figure 4. Result of brine shrimp assay of medicinal plants

The measurements of the wounds treated with Framycetin and herbal ointments was carried out on daily basis. It is observed that the wound contracting ability of the herbal ointment with the methanolic extract is comparable with that of the standard drug. The ointment made from the methanolic extract of *this triherbal formulation* displayed significant wound healing activity. These results were compared to the group treated with 1% Framycetin (Standard), blank (mixture of PEG400 and PEG4000) and control (liquid paraffin). Complete wound healing activity of the formulation was found on 9th day of observation.

It was rapid and complete healing of wound was observed but the total healing was not observed in other groups of animals [13]. Single or multiple factors may play a role in any one or more individual phases, contributing to the overall outcome of the healing process. Tannin and total phenolic compounds that are typically act as astringent agent, were found to variety of herbal medicine and shown healing activity along with antioxidant activity [14, 15]. Accordingly, in the present work, application of phenolic rich formulated product showed the significant increase on healing of the incision wound.

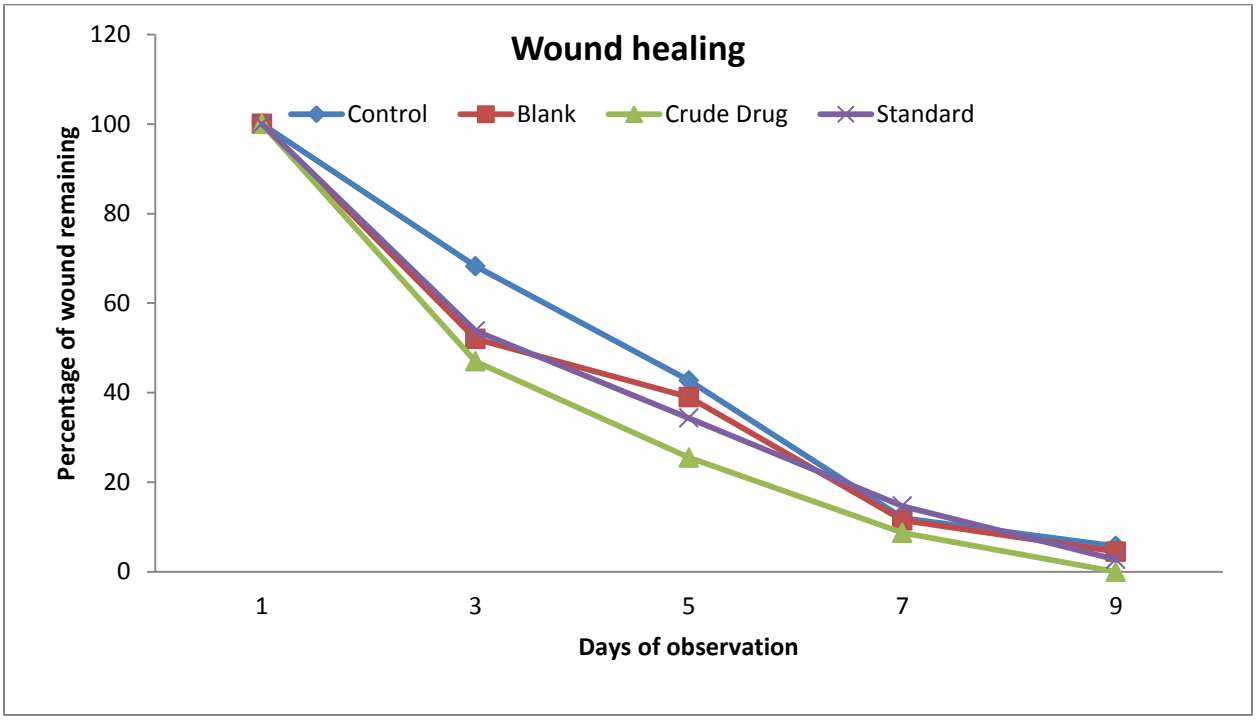


Figure 5. Wound area in different test groups of Albino rats

CONCLUSIONS

After the preliminary phenolic estimations, plants *Bauhinia variegata*, *Rhododendron arboreum*, and *Myrica esculenta* were formulated into 10% w/w ointment in the ratio of 1:1:2 respectively. In excision wound model, wound was totally healed after treatment of herbal ointment while 2.72%, 4.5%, and 5.73% wound area was found remaining in framycetin treated, blank and control group of rats, respectively. The prepared ointment exhibited good wound healing effect comparable with that of 1% Framycetin cream.

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