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ORIGINAL ARTICLE

Stephania japonica: A Pharmacognostic Study for the *in-vivo* Wound Healing Activity using Herbal Ointment Formulated from Ethanolic Extract

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ABSTRACT

Stephania japonica plants appear to be rich in secondary metabolites, widely utilised in traditional medicine to treat and heal numerous illnesses. It is traditionally used to treat wounds, but there is no scientific data on its *in vivo* wound-healing activity. Pharmacognostical analysis evaluates a drug's identity, quality, purity, and safety through microscopic and macroscopic research. The extraction solvent dissolves significant amounts of the target material. The percentage yields of alcohol and water-soluble extractive values were calculated. After the extraction of the plant material, the herbal ointment was formulated. After checking the evaluation parameter of the ointment, the excision wound healing activity was performed on Wistar rats. The herbal extract was prepared by using a simple Soxhlet extraction process to obtain a good yield of extract, and there was no harm to the chemical constituents and their activity. This study showed that the herbal ointment heals wounds better in less time as similar with the standard ointment by applied to Wistar rats.

Keywords: Pharmacognostical, Phytochemical, Wound healing, *Stephania japonica*, Herbal ointment

INTRODUCTION

The family Menispermaceae, which has 350 species and 65 genera, includes the genus *Stephania*. This family is widely dispersed throughout the tropics. This family primarily consists of plants and shrubs, with very few trees. There are thin climbers with peltate and membrane leaves in the *Stephania* genus. Axillary inflorescences with umbelliform blooms develop from old, leafless stalks. Traditional uses of these herbs include the treatment of asthma, TB, dysentery, hyperglycemia, cancer, fever, digestive problems, sleep disorders, and inflammation [1]. *Stephania japonica* is a climbing plant that develops from a woody rhizome to generate thin stems that may eventually begin to resemble wood. The flavour of the tuberous root is astringent and unpleasant. It is used to treat hepatitis, fevers, stomach aches and dyspepsia, diarrhoea and dysentery, and urinary tract infections. It is claimed that the root might help treat itchiness because it contains picrotoxin. Discolour is bitter and extremely dangerous. Fever, diarrhoea, illnesses of the urinary system, and stomachaches can all be treated with it medicinally. Breast infections are treated with a mixture of crushed leaves and water that has a little gelatinous consistency [2]. Anti-oxidant, analgesic, antinociceptive, anti-

microbial, anti-inflammatory, anti-hyperglycemic, anti-hyperlipidemic, neuroprotective, and antidiarrheal activities of the plant of *Stephania japonica* are already documented by various scientific committees [3-9]. Although it has historically been used to treat wounds, there is no scientific evidence about its ability to cure wounds *in-vivo*. This study focuses on the identification, quality, purity, and safety of drugs for human use, specifically evaluating the physicochemical characteristics of the *Stephania japonica* plant. The herbal extract was prepared by using a simple Soxhlet extraction process with petroleum ether, benzene, acetone, ethyl acetate, methanol and ethanol according to their polarity. This study showed that the herbal ointment which is prepared from the ethanolic extract heals wounds better in less time compared with the standard ointment by applied to Wistar rats.

Figure 1: Leaves and stems of *Stephania japonica*

MATERIALS AND METHOD

Materials

Plant materials: *Stephania japonica*

Selection & collection: *Stephania japonica*'s stems were selected based on traditional uses and a literature survey [10]. The plant parts were collected from Rangpo forest, Sikkim.

Identification & authentication: The plant was identified through standard literature and authenticated by BSI, Gangtok, with accession number SHRC-5/02/2022-23/tech-200.

Plant processing

After collecting, the plant parts were washed thoroughly with tap water to remove any unwanted materials present on them. This was further dried in the shade for a few days. After complete drying, dried plant materials were coarsely powdered with the help of a mechanical grinder and passed through a sieve and stored in a tightly closed container.

Chemicals

Methanol (finar), ethyl acetate (finar), hydrochloric acid, potassium bismuth iodide, potassium mercuric iodide, alcoholic alpha naphthol, sulphuric acid, Fehling's reagent A & B (oxford lab fine chem llp), glacial acetic acid (sdfcl), ferric chloride (sdfcl), acetic anhydride, chloroform (finar), gelatin, sodium chloride, ethanol (finar), acetone (finar), petroleum ether (finar), benzene, iodine, hydrochloric acid, benedict reagent (dey's chemical works), magnesium ribbon, sodium hydroxide, metallic zinc (finar), ammonia.

Equipment

Digital balance, heating mantle (sunsim), hot air oven, rotary evaporator (hahnvapor), water bath, Soxhlet apparatus.

Glass apparatus

Beaker, measuring cylinder, funnel, glass rod, petri-dish, china-dish, conical flask, round-bottom flask, glass slide, test-tube, test-tube holder, test tube stand, spatula, etc.

Methods

Pharmacognostical studies

Macroscopic examination:

The macroscopical observation was carried out according to the standard methods to determine the shape, size, colour, taste and odour.

Powder microscopy:

After dissolving powdered plant material in distilled water, the drug's minute particles were dipped in colouring agents such as rheuthenium red, quick green, Safranine, Sudan IV, and iodine, cleaned, mounted, and examined under an electron microscope. The microscopic characteristics were recorded.

Total ash:

A tar-coated platinum or silica dish containing precisely two grams of air-dried medicament was ignited at a temperature of not more than 800 °C until the substance was carbon-free [11]. After cooling, the weight was recorded. If a carbon-free ash could not be obtained, the burned mass was cleansed with hot water, and the residue was gathered. On ashless filter paper, the residue was burned together with the filter paper until the ash was white or almost white. Once the filtrate had totally evaporated, it was put in the dish. It was calculated as a proportion of the overall ash content of the medication.

Acid-insoluble ash:

Using 25 millilitres of 2M HCL acid to boil the ash (from total ash) for five minutes, the insoluble debris was collected in an Ashless Filter Paper or Gooch Crucible. Following a hot water cleaning, it was ignited, allowed to cool in a desiccator, and then weighed. The percentage of acid-

insoluble ash was calculated using the drug base that was dried.

Water-soluble ash:

The ash (total ash technique) was boiled for five minutes with 25 cc of water, and the insoluble materials were collected in a Gooch crucible or on ashless filter paper. Following a fifteen-minute burn at a temperature not to exceed 800 °C, the mixture was washed with hot water. Water-soluble ash displays the weight differential following the deduction of the insoluble material's weight from the ash's weight. Next, using dry medications as a base, the percentage of water-soluble ash was computed [12].

Moisture content:

The loss-on-drying method, which involves heating a substance at 105 ℃ for 4 hours and weighing. Again, heat for 30 mins and weigh again. Repeat this process until the weight of plant material is constant, and calculate the percentage of weight loss after complete drying using the following formula:

$$\% \text{ moisture content} = \frac{(Wi - Wf)X\ 100}{Wi}$$

Where Wi = sample initial weight.

Wf = sample weight after drying.

Physiochemical evaluation

Extractive value:

The extracts obtained by exhausting crude drugs are indicative of approximate measures of their chemical constituents. Taking into consideration the diversity in chemical nature and properties of drug contents, alcohol and water were used for the determination of extractive values.

Preparation of plant extract:

Stephania japonica was extracted using a Soxhlet apparatus. 35 g of the powdered plant material was packed in a thimble and extracted using 300 mL of solvent. The plant material was extracted using a successive solvent extraction process with petroleum ether, benzene, acetone, ethyl acetate, methanol and ethanol. After completion of the extraction, concentrated the extract was concentrated using a rotary evaporator and a water bath.

Phytochemical screening of *Stephania japonica*:

Different extracts of *S. japonica* were evaluated phytochemically as per standard protocol to find out the presence of different phyto-constituents [13].

Chromatographic analysis

Thin-layer chromatography:

TLC is a crucial separation technique in plant chemistry, aiding in the identification of known and unknown compounds. Pre-coated silica plate was used as the stationary phase, and ethyl acetate: acetic acid: formic acid: water (100:11:11:27) was used as the mobile phase for the Flavonoid identification using Rutin as a standard compound.

Ointment formulation:

Based on higher extractive value and results of phytochemical analysis, the ethanolic extract was used for ointment formulation. The ointment was formulated according to the British Pharmacopoeia using the following ingredients [14]:

Table 1: List of ingredients

Ingredients	Quantity (gm)
White soft paraffin	85
Cetostearyl alcohol	4.5
Wool fat	2.5
Hard paraffin	2.5
Plant extract	5
Methyl paraben	0.5
Lavender oil	1-2 drops
Total	100

Evaluation of the herbal ointment

Physical evaluation: Physical parameters like colour and odour were examined by visual examination.

Viscosity: The viscosity of the formulated ointment was measured using a Brookfield Viscometer.

pH: The pH of the prepared herbal ointment was determined in triplicate using a digital pH meter, and the average value was calculated.

Spreadability: Spreadability was assessed by placing an extra sample within two glass slides, then compressing it to a consistent thickness with a specific weight for a predetermined amount of time. The measure of spreadability was the amount of time needed to separate the two slides. Improved spreadability is the outcome of separating two slides in less time. The formula below was used to calculate spreadability.

$$S = \frac{ML}{T}$$

Where, S= Spreadability, M= Weight tide to the upper slide, L= Length of glass slide, T= Time taken to separate the

slides.

Centrifugation: For assessing accelerated ointment deterioration, it is thought to be a special tool. By centrifuging at 10,000 rpm for 10 minutes, it was ascertained.

Washability: Formulation was applied on the skin, and then the ease of removal of water was checked.

Loss on drying: The sample's weight loss is primarily due to the presence of water, which was determined.

Acute Skin Irritation Test: The formulated ointment was applied topically to Human skin, and the results were noted down.

In-vivo wound healing activity

For this investigation, Wistar albino rats weighing between 180 and 200 g were employed. Animals were purchased from the Himalayan Pharmacy Institute's Majhitar authorised animal house after approval from the Institutional Animal Ethical Committee with the approval number HPI/2023/60/IAEC/PP-0208. The rats were placed into 3 groups, and each group had six rats (n=6). Group 1 referred to the disease control group, where no treatment was done; group 2 was referred to as the standard group, which was treated with povidone-iodine ointment [15] and group 3 was treated with ointment prepared from *Stephania japonica* extract [16]. In a cross-ventilated animal housing with a temperature of 25°C, a relative humidity of 44-56%, and a light-to-dark cycle of 12:12 hours, the animals were acclimated to the typical laboratory settings. They were then fed regular food and given access to water whenever needed throughout the study. According to the technique outlined below, excision wounds were made. Six rats (n = 6) from each of three groups of animals will have their dorsums shaved before being given 50 mg/kg (i.p.) of ketamine hydrochloride to put them to sleep. An impression was created on the shaved dorsal region, and the location of the wound was indicated. A 2.5 cm long full-thickness excision wound was made along the marking with toothed forceps, a surgical blade, along sharp scissors. Rats were released into their natural environment unclothed. From the day of the procedure until complete healing, the simple ointment bases, designed extract ointment, and standard drug were used once daily [17, 18].

Percentage wound healing = (healed area/total wound area) x 100.

RESULT

Pharmacognostical Evaluation

Table 2: Macroscopic Examination

Shape	Elongated
Taste	Spicy flavor
Odour	Indistinct
Colour	Brownish green

Table 3: Ash value detection

S. No	Parameters	Values (in percentages)
1.	Total ash % w/w	7.75
2.	Acid insoluble ash %w/w	2.35
3.	Water soluble ash %w/w	5.15

Physicochemical Evaluation

Extractive Value of *Stephania japonica*:

Table 4: Extractive values of *Stephania japonica*

Sl. No	Types of solvent	Colour	Extractive value in percentage (w/w)
1	Alcohol soluble extractive value	Reddish brown	7.94%
2	Water-soluble extractive value	Brown	4.12%

Table 5: Different phytochemical tests

Phyto-constituent	Pet. ether extract	Benzene extract	Acetone extract	Ethyl acetate extract	Methanol extract	Ethanol extract
Alkaloid	-	+	+	+	+	+
Carbohydrates	+	+	+	+	+	+
Saponins	-	-	-	-	-	-
Glycosides	-	-	-	-	-	-
Tannins	-	-	-	+	-	-
Triterpenoid & Steroid	+	+	+	+	+	+

Phyto-constituent	Pet. ether extract	Benzene extract	Acetone extract	Ethyl acetate extract	Methanol extract	Ethanol extract
Flavonoids	-	-	-	-	+	+
Phenols	-	+	+	+	+	+

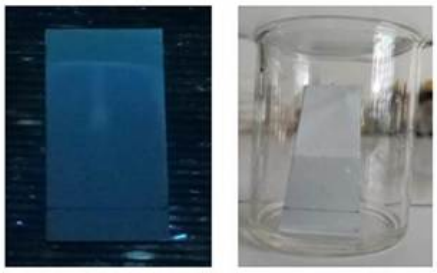
'+' indicates present and '-' indicates absent

Chromatographic analysis

Thin Layer Chromatography: TLC of the ethanolic plant extract was performed using Rutin as a standard compound.

Table 6: Data showing R_f values of TLC study

Sl. No.	TLC study	Solvent system	R_f
1	Ethanolic plant extract	Ethyl acetate: acetic acid: formic acid: water (100:11:11:27)	0.42
2	Rutin	Ethyl acetate: acetic acid: formic acid: water (100:11:11:27)	0.44



(a) (b)

Figure 2: Thin layer chromatography: (a) UV (254 nm) detection of TLC plate for flavonoids, and (b) TLC chamber

Formulation of Simple Ointment

Table 7: Formula for simple ointment as per BP

Ingredients	Master Formula (g)	Reduced Formula (g)
White soft paraffin	850	85
Cetostearyl alcohol	45	4.5
Hard paraffin	25	2.5
Wool fat	25	2.5
Plant extract	50	5
Methyl paraben	5	0.5
Lavender oil	2-3 drops	1-2drops
Total	1000	100

(a) (b)

Figure 3: Preparation of herbal ointment: (a) Herbal ointment, and (b) Pulverisation of ointment bases

Evaluation parameter of herbal ointment

Table 8: Evaluation parameter

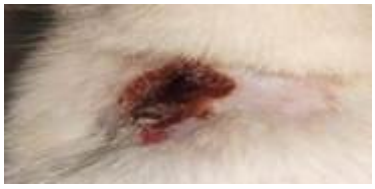
Physiochemical Parameter	Observation
Colour	Pearl white
Odour	Characteristic
Viscosity	Cp 5303
pH	5.5
Spreadability	0.334 g.cm/sec
Washability	Good
Centrifugation	No phase separation
Loss on drying	8.23
Acute skin irritation	No irritation

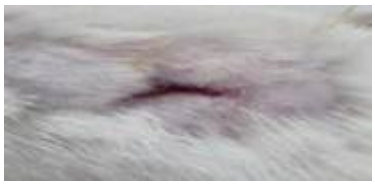
Wound Healing Activity

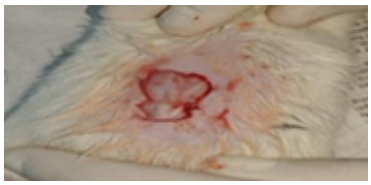
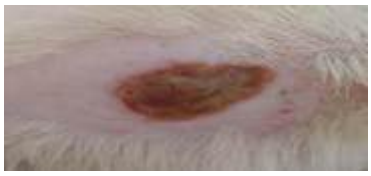
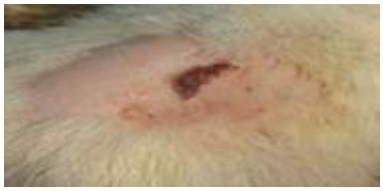
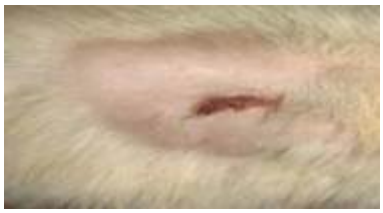
The current research will utilise healthy Wistar albino rats weighing between 180 and 200 g. Animals must be obtained from the Himalayan Pharmacy Institute's licensed animal shelter in Majhitar, Sikkim, India. The rats were divided into three groups, with six rats in each group.

- Group-1 referred to the control group,
- Group-2 represented the standard group that received povidone iodine ointment treatment, and
- Group-3 treated with ointment prepared from *Stephania japonica* extract.

Table 9: Mean wound length measurement and percentage wound healing in the excision wound model

Day	Date	Mean Wound length(cm)	Percentage Wound Healing	Picture
Control group:				
1st	20.05.2023	2.5±0.058	-	
3rd	23.05.2023	2.2±0.060	12	
6th	26.05.2023	1.8±0.052	28	
9th	29.05.2023	1.6±0.037	36	
12th	01.06.2023	1.3±0.037	48	
15th	04.06.2023	1.1±0.037	56	
18th	07.06.2023	1±0.075	60	
21st	10.06.2023	0.8±0.089	68	

Day	Date	Mean Wound length(cm)	Percentage Wound Healing	Picture
Standard group:				
1st	20.05.2023	2.5±0.036	-	
3rd	23.05.2023	2.0±0.048	20	
6th	26.05.2023	1.5±0.027	40	
9th	29.05.2023	1.3±0.025	48	
12th	01.06.2023	1.2±0.038	52	
15th	04.06.2023	1.0±0.012	60	
18th	07.06.2023	0.8±0.02	68	
21st	10.06.2023	0.6±0.009	76	

Day	Date	Mean Wound length(cm)	Percentage Wound Healing	Picture
Herbal ointment:				
1st	20.05.2023	2.5±0.039	-	
3rd	23.05.2023	1.6±0.025	36	
6th	26.05.2023	1.4±0.030	44	
9th	29.05.2023	1.3±0.029	48	
12th	01.06.2023	1.1±0.018	56	
15th	04.06.2023	0.8±0.017	68	
18th	07.06.2023	0.4±0.007	84	
21st	10.06.2023	0.1±0.015	96	

The excision wound is set to be formed using the procedure described below. Three groups of six rats each will be trimmed on the dorsum and sedated with ketamine hydrochloride (50 mg per kg, i.p., weight of body weight). After shaving the dorsal region, an impression must be created, and the place where the wound will be made must be indicated. The wound area was measured on 3th, 6th, 9th, 12th, 15th, 18th, and 21st day compared with the wound area on the first day. The produced extract ointment and normal medication should be used once a day from the beginning of the procedure. Using the following formula, the degree of wound recovery was determined as the percentage closure in the wound area relative to the initial wound area:

Percentage wound contraction = 100-[Final diameter (cm) x 100/ initial diameter (cm)]

Wound area was measured in individual animals, and mean wound length was calculated for each group.

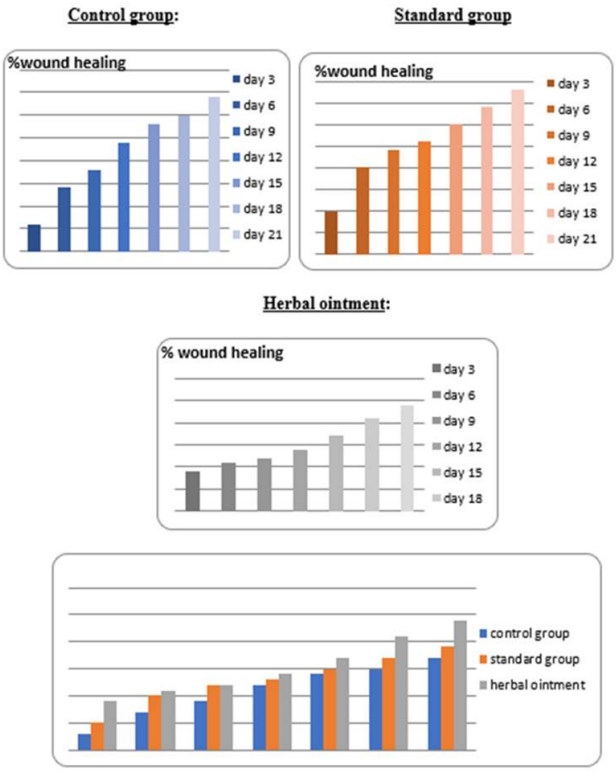


Figure 4: Graphical representation of the wound healing activity of different groups: Comparison of control, standard and test group (ointment formulation)

DISCUSSION

As a result of this search, these were what emerged as the most interesting parts of previous works focused on research: evaluating antioxidant, anti-nociceptive [4, 19, 20] and anti-inflammatory" properties [21] or cytotoxicity potential derived from leaves extract allocation type [22] of *Stephania japonica* instead of concentrating on wound application effects [23]. Researchers have concluded that enough steroids, alkaloids, carbohydrate phenols and flavonoids exist in *Stephania japonica* extracts after phytochemical screening. An herbal balm was formulated using the *Stephania japonica* extracts, and its wound-healing activity was assessed in an animal (Wistar rat) excision wound model. The results of the study revealed that the herbal ointment belonging to the *Stephania Japonica* group possessed wound healing potential. From day 0 to the end of the study, wound healing was observed and recorded, in which the herbal ointment group showed superior progress than that of the control group (Fig. 1) [24]. The findings of the present study, taken together, suggest that the wound healing potential of *Stephania japonica* is promising when formulated as an ointment. The plant owes its wound healing properties to its immense number of phytochemical constituents [25]. For proper application in managing injuries, including pressure ulcers, more research is needed to understand the body's wound healing mechanisms and thoroughly optimise the ointment formulation [26].

The following are some of the most important findings from the most recent research on the use of *Stephania japonica* extract in wound healing ointments:

1. **The scope of the inquiry:** Past examinations zeroed in on assessing the cancer prevention agent, pain-relieving, and cytotoxic properties of *Stephania japonica* leaf extricates, without directly exploring wound recuperating applications. Current research made an herbal ointment from Stephania japonica and tested how well it healed wounds in an animal model.

2. **Phytochemical investigation:** Steroids, alkaloids, carbohydrates, phenols, and flavonoids were found in Stephania japonica extracts in previous research. Flow research based upon the phytochemical profile to foster an injury recuperating balm definition [27].

3. **Evaluation of wound healing:** Previous studies did not evaluate the ability of *Stephania japonica* extracts to heal wounds. Current research developed an in vivo excision wound model using Wistar rats to demonstrate the effectiveness of the Stephania japonica-based ointment for wound healing [28].

4. **Outcomes:** *Stephania japonica* extracts were found to have antioxidant, analgesic, and cytotoxic properties in previous research [29]. Momentum research showed that the *Stephania japonica*-based treatment fundamentally worked on injury healing in

the animal model, with the injury healing notice and recorded for more than 21 days [30, 31].

CONCLUSION

These studies help to ensure the identification, quality, purity, and safety of the drug for human use. The physicochemical characteristics of the *Stephania japonica* plant have been attempted to be evaluated. It is critical to identify plant material both taxonomically and pharmacognostically in order to establish pharmacognostical standards and prevent the use of fake and adulterated medications. From West Bengal, the plant is collected. The plants used in this study were chosen based on how well they work in traditional medical systems. The extractive value was afterwards calculated using a Soxhlet extraction. The goal of this study was to create and assess an herbal ointment. To achieve a good yield of extract and avoid harming the chemical contents and their activity, the herbal extracts were made using a straightforward Soxhlet extraction procedure. Then the simple ointment base was formulated and kept in a beaker for further formulation of the herbal ointment. The phytochemical analysis of extracts of *Stephania japonica* stem revealed the presence of Steroids, alkaloids, Carbohydrates, phenols, flavonoids. After the extraction of the plant material the herbal ointment was formulated. The herbal ointment evaluation parameter was performed. After checking the evaluation parameter, the wound healing activity was performed on wistar rats. The animals are divided into three groups. Group-I control group, group-II standard group and group-III herbal ointment. From the zero day it started to be observed, then at 3^{rd}, 6^{th}, 9^{th}, 12^{th}, 15^{th}, 18^{th} and 21^{st} day it was observed and results found. From this experiment of wound healing activity of excision model, it was observed that the herbal ointment have the good healing properties and heals the wound similar to the standard drug, so this can be useful for the further use in wound healing activity.

Abbreviations

FDA; Food and Drug Administration, TM; Traditional Medicine, BSI; Botanical Survey of India, Cp; Centipoise, IL; Interleukin, VEGE; Vascular Endothelial Growth Factor, FE; Iron, BHA; Beta Hydroxy Acid, DPPH; 2,2-diphenylpicrylhydrazyl, ABTC; 2,2'- azino-bis(3-ethylbenzothiazoline-6-sulphonic acid), H_2SO_4; Sulfuric Acid, ml; Milliliter, nm; Nanometer, NaOH; Sodium hydroxide, mg; Milligram

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