ORIGINAL RESEARCH ARTICLES

WOUND HEALING, ANTIMICROBIAL AND ANTIOXIDANT POTENTIALS OF *CEDRUS DEODARA* LOUD. AND *PINUS ROXBURGHII* SARG.

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ABSTRACT

The aim of this study was to investigate wound healing, antimicrobial and antioxidant activities of *Cedrus deodara* and *Pinus roxburghii*. The chloroform extract of *C. deodara* was found to possess significant wound healing potential, as evident from the rate of wound contraction, epithelization and hydroxyproline expression. The chloroform extract of *C. deodara* also exhibited very good antimicrobial activity and a marked dose-dependent reducing power and total antioxidant activity. Furthermore, five sesquiterpenes, namely, atlantone, himaphenolone, atlantolone, deodardione, and (*E*)-(2S, 3S, 6R)-atlantone-2, 3-diol, were also identified from this extract. A marked dose-dependent reducing power and strong total antioxidant capacity were found in favor of himaphenolone and (*E*)-(2S, 3S, 6R)-atlantone-2, 3-diol that explain the possible means of activity. The chloroform extract of *C. deodara* showed the wound healing, antimicrobial and antioxidant activities primarily due to the presence of sesquiterpenes. This study showed a scientific rationale for the traditional use of *C. deodara* and *P. roxburghii*.

Keywords: Wound, epithelization, hydroxyproline, antioxidant activity, sesquiterpenes

INTRODUCTION

Burns remain a huge public health issue, at least in terms of morbidity and long term disability, throughout the world, especially in the developing countries[1]. Burn wound healing is a complex process, causes discomfort and is prone to infection and other complications. Infection is a major complication of burn injury and is responsible for 50-75 % of hospital deaths[2]. Many of the synthetic drugs used pose problems such as allergy and drug resistance, forcing doctors to seek alternative drugs[3]. In India, medicines based on herbal sources, have been the base of treatment and cure for a variety of diseases and physiological abnormalities in practice, such as Ayurveda, Siddha and Unani. Moreover, Indian folk medicine comprises several prescriptions for therapeutic purpose such as inflammation, skin infections, wounds healing, leprosy, diarrhea, scabies, venereal disease, ulcers and snake bite. In addition, more than 80 % of the world's population still depends on traditional medicines for various skin diseases. An herbal medicine in wound management involves disinfection, debridement and providing a moist environment to encourage the establishment of the conducive environment for natural healing process[4].

The plants *Cedrus deodara* Loud. and *Pinus roxburghii* Sarg. belonging to the family Pinaceae, have long been known for their medicinal value, including wound healing activity. The plant *C. deodara* has a long history of numerous traditional and ethno botanical applications in diverse cultures[5,6]. Traditional *C. deodara* is bitter, hot, pungent, light, oleaginous, diuretic, analgesic, diaphoretic, carminative, and used in belching, inflammations, dyspepsia, insomnia, hiccough, fever, urinary discharge, epilepsy, skin disease, pulmonary disorder, urinary disorder, bronchitis, itching, tuberculous glands, leucoderma, ophthalmia, piles, disorder of mind and ulcers[7,8]. All parts of *C. deodara* are useful in Ayurveda for the treatment of insomnia, disorder of mind, disease of

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skin and blood. Recent *in vivo* and *in vitro* studies of *C. deodara* have indicated its memory-enhancing[9], anti-ulcer[10], anti-inflammatory and analgesic[11], anti-apoptotic[12], anxiolytic and anti-convulsant[13], anti-diabetic[14], immunomodulatory[15] and anti-cancer[16] activities.

The plant *P. roxburghii* is pungent, heating, oleaginous, intestinal antiseptic and several parts have been used in traditional system of medicine for disease of eye, ear, throat, skin, bronchitis, tuberculosis, diaphoresis, diuretic, rubefacient, stimulant, vermifuge, ulcer, inflammation and itching[5]. Recent *in vivo* and *in vitro* studies of *P. roxburghii* have indicated its memory enhancing[9], anti-ulcer[10], anti-dyslipidemic and anti-oxidant[17], anti-inflammatory and analgesic[18,19], hepatoprotective[20] and anti-microbial[21] activities.

These plants are such medicinal plants whose therapeutic application no doubt have a folkloric background and enjoy widespread reputation as remedy for wound[5,6]. Review of literature revealed that the wound healing property of these plants has not been subjected to scientific evaluation and there was not enough scientific data existing to support the claims made in the ancient literature. Therefore, a scientific verification of its use should be important in establishing a pharmacological basis for the claimed ethno medicinal uses of these plants. Consequently, the need for safer and effective wound healing drugs and the lack of enough scientific data to support the claims made in ancient literature prompted the present study. This study was undertaken to access the effect of the volatile oil and chloroform extract of *C. deodara* and *P. roxburghii* on biochemical, anti-microbial, and anti-oxidant expression associated to wound healing.

MATERIALS AND METHODS

Collection and authentication of plant material

The stem wood of *C. deodara* and *P. roxburghii* were collected from regions of Pauri Garhwal, Uttarakhand, India. The stem wood of *C. deodara* was authenticated by Dr. H.B. Singh, Head, Raw Materials Herbarium and Museum (RHMD), National Institute of Science Communication and Information Resources (NISCAIR), New Delhi, India. A voucher sample has been deposited at the RHMD (NISCAIR/RHMD/Consult/-2011-12/1711/11 dated April 11, 2011). The stem wood of *P. roxburghii* was authenticated by Dr. E. Roshni Nayar, Principal Scientist, National Bureau of Plant Genetic Resources (NBPGR), New Delhi, India. A voucher sample has been deposited at the NBPGR Herbarium (NHCP/NBPGR/2011-14/7288 dated April 07, 2011).

Drying and communition of plant materials

The wood samples of *C. deodara* and *P. roxburghii* were thoroughly washed and then shade dried at less than 25 ± 2 °C for 10 d. The dried plant samples were grounded well into a fine powder in a mixer grinder and sieved to get particle size 50-150 mm.

Isolation of wood oil

The powder plants material (stem wood of *C. deodara* and *P. roxburghii*), were subjected to steam distillation by using Clevenger apparatus. On cooling, the wood oil was separated from the aqueous layer, dried over anhydrous sodium sulphate and stored in an amber colored glass bottle in a cool place[22,15].

Preparation of plant extract

The powderd plants material (stem wood of *C. deodara* and *P. roxburghii*) were extracted with chloroform using Soxhlet extraction apparatus. The solvents were completely removed under reduced pressure till a semi solid mass was obtained. The extracts were stored in amber colored bottle and kept in refrigerator until further use.

Phytochemical testing

An attempt was made to observe the presence and absence of different phytochemical constituents in the wood oil and chloroform extract of stem wood of *C. deodara* and *P. roxburghii*, viz. alkaloids (Dragendorff's, and Hager's test), glycosides (Borntrager's and modified Borntrager's test), flavonoids (Shinoda, alkaline reagent and zinc hydrochloride test), terpenoids (vanillin sulphuric acid test, 5 % phosphomolybdic acid in ethanol test and 10 % ammonium molybdate sulphuric acid test), steroids (Salkowski, Libermann-Burchard reaction and Liebermann's reaction), carbohydrates (Molish and Fehling's solution test), proteins (biuret and ninhydrin test), fats and fixed oil (spot test and solubility test), and phenolic compounds (ferric chloride and dilute iodine solution test) according to the standard methods[23].

Thin-layer chromatography (TLC) profiling of wood oil and extract

The wood oil and extract were applied to silica gel G plates for TLC profiling. For TLC profiling, plates were developed using various ratios of solvent system in *n*-hexane: ethyl acetate as mobile phase. The spots were visualized using anisaldehyde-sulphuric acid at 120 °C[24,9].

Animals

Swiss albino mice (30-35 g) were obtained from central animal house facility of Swami Vivekanand Subharti University, Utter Pradesh, India. The animals were housed in a polypropylene cage under standard conditions (25 ± 2 °C, 12 h light and dark cycle) and animals were fed on standard chow diet and water *ad libitum*. All the experimental procedures and protocols involving animals were reviewed and approved by the Institutional Animal Ethical Committee (registration number: 1204/PO/ac/2009/CPCSEA) and were in accordance with the guidelines of CPCSEA.

Ointment formulation

The ointments (1 % and 2.5 % w/w) were formulated using soft white paraffin obtained from S.D. Fine Chemical, India. White soft paraffin was melted on a hot plate at 60 ˚C and mixed with oil and chloroform extracts of *C. deodara* and *P. roxburghii* with continuous stirring until samples and soft paraffin completely miscible[25].

Acute dermal toxicity

The acute dermal toxicity testing was done by applying the ointment containing oil and extract of the highest concentration 2.5 % (w/w) on the shaved back of the mice. The OECD guideline no. 402 was followed in the study[26].

In vivo wound healing evaluation

Burn wound model

Partial thickness burn wounds were inflicted on overnight-starved animals under thiopental sodium anesthesia, by placing hot cylindrical metal rod (10 mm diameter) on the shaven back of the animals[27,28]. Immediately after the injury and on subsequent day, Ringers' lactate (1 mL kg^{-1}) was administered *i.p.* daily for resuscitation. Other than drugs under investigation, no local or systemic chemotherapeutic cover was provided to animals. Animals showing signs of infection were excluded from the study and replaced with fresh animals.

Experimental protocol

Animals bearing the partial thickness burn wounds were randomly divided into ten groups. Five animals were taken for each group. Group-I (control) was treated with ointment base. Group-II (SSD) received the standard drug (silver sulphadiazine and chlorhexidine gluconate cream; 1.0 % w/w). Groups-III (Cd O 1 %) and IV (Cd O 2.5 %) were treated with ointment containing 1 % and 2.5 % oil of *C. deodara*, respectively. Groups-V (Pr O 1%) and VI (Pr O 2.5%) were treated with ointment containing 1 % and 2.5 % oil of *P. roxburghii*, respectively. Groups-VII (Cd C 1%) and VIII (Cd C 2.5%) were treated with ointment containing 1 % and 2.5 % chloroform extract of *C. deodara*, respectively. Groups-IX (Pr C 1%) and X (Pr C 2.5%) were treated with ointment containing 1 % and 2.5 % chloroform extract of *P. roxburghii*, respectively. The treatments (100 mg $mouse^{-1}$) were applied topically once a day, starting from the wound induction until complete healing.

Assessment of burn healing

Animals were inspected daily and the healing was assessed on the basis of the parameters; rate of wound contraction, epithelization time and hydroxyproline content[29].

Rate of wound contraction

The wound contraction rate was measured as percentage reduction in wound size at every 4 d interval. Progressive shrink in the wound size was monitored from time to time by tracing the boundary and the wound area was assessed graphically. Wound contraction was expressed as reduction in percentage of the original wound size.

Area of wound in mm^2 (wound closure rate) was calculated by the following formula:

Area of wound = square of radius of wound in mm × 22/7

On the day zero, circular 10 mm wound was made on the animals. Therefore, on day zero the wound area was 78.5 mm^2.

$$\text{Percentage of wound healing} = \frac{\text{Initial wound size-Specific day wound size}}{\text{Initial wound size}} \times 100$$

Epithelialization time

Falling of eschar leaving no raw wound area was considered as end point of complete reepithelization and the time required for this was taken as period of epithelialization.

Hydroxyproline content

On the 11th d, the animals from each group were used to determine hydroxyproline content using the techniques described by Neuman and Logan, 1950[30]. The wound tissue was excised and its weight was recorded. The tissue was dried in an oven at 60 °C

for 12 h and the dry weight was again noted. It was hydrolyzed in 6N hydrochloric acid for 24 h at 110 °C in sealed glass tube. The hydrolysate was neutralized to pH 7. The samples (200 µL) were mixed with 1mL of 0.01M copper sulphate followed by the addition of 1 mL of 2.5N sodium hydroxide and then 1 mL of 6 % hydrogen peroxide. The solution was mixed and shaken occasionally for 5 min. All the tubes were incubated at 80 °C for 5 min with shaking. On cooling, 4 mL of 3N sulfuric acid was further added with agitation. Finally, 2 mL of 5 % p-dimethylaminobenzaldehyde was added. The samples were incubated at 70 °C for 16 min, cooled by placing the tubes in water at 20 °C, and the absorbance was measured at 500 nm using colorimeter. The amount of hydroxyproline in the samples was calculated using a standard curve prepared with pure L-hydroxyproline[29].

Antimicrobial screening

Agar dilution method was used to determine the minimum inhibitory concentration (MIC) of oil and chloroform extracts of *C. deodara* and *P. roxburghii*. In the agar dilution tests, microorganisms were tested for their abilities to produce visible growth on a series of agar plates containing dilutions of the antimicrobial agents. The lowest concentration of an antimicrobial agent that inhibits the visible growth of a microorganism was noted as MIC[31]. The antimicrobial study of oils and extract was conducted against various microorganisms. The pure cultures of the gram negative bacteria (*Escherichia coli* and *Pseudomonas aeruginosa*), gram positive bacteria (*Staphylococcus aureus, Streptococcus pyogenes* and *Bacillus subtilis*) and fungi (*Candida albicans, Aspergillus clavatus* and *Aspergillus niger*) were used for the study. Ampicillin, chloramphenicol and griseofulvin were used as positive control. Bacteria were cultured at 37 °C for 48 h in nutrient agar medium and fungi at 28 °C for 72 h in potato dextrose agar and used as inoculums.

Determination of *in vitro* antioxidant activity

Total antioxidant capacity

The total antioxidant capacity (TAC) was measured using phosphomolybdenum method[32]. The wood oil and extract were dissolved in methanol (2 mg mL^{-1}) and 0.3 mL of each sample was added to 3 mL of reagent solution (0.6 M sulphuric acid, 28 mM sodium phosphate and 4 mM ammonium molybdate mixture). All the tubes were incubated for 90 min at 95 °C. After that, the mixture was cooled to room temperature and the absorbance was examined at 695 nm against blank. Ascorbic acid equivalents were calculated using the standard graph of ascorbic acid. The experiment was conducted in triplicates and values were expressed as equivalents of ascorbic acid in mg per g of sample.

Reducing power assay

This method was based on the reduction of the Fe(III)/ferricyanide complex to the ferrous form by one-electron-donating antioxidant. The ferrous ion (Fe^{2+}) is monitored by measuring the formation of Perls Prussian blue at a wavelength of 700 nm. The ferric ion (Fe^{3+}) reducing power of the wood oil and chloroform extract of *C. deodara* and *P. roxburghii* was determined by the previously described method[33]. Aliquots (50 µL) of different concentrations of samples (5-25 µg mL^{-1}) were mixed with 50 µL phosphate buffer (0.2 M, pH = 6.6) and 50 µL potassium ferricyanide (1%), followed by incubation at 50 °C for 20 min in dark. After incubation, 50 µL of TCA (10 %) was added to terminate the reaction and the mixtures were subjected to centrifugation at 3000 rpm for 10 min. For final reaction mixture, the supernatant (50 µL) was mixed with 50 µL distilled water and 10 µL $FeCl_3$ solution (0.1 %). The reaction mixture was incubated for 10 min at room temperature and the absorbance was examined at 700 nm against a proper blank solution. A higher absorbance of the reaction mixture indicated greater reducing power ability, confirming quantitative increase in the reduction of Fe^{3+} to Fe^{2+} in the reaction mixture and *vice versa*. All tests were run in triplicate in this assay. Ascorbic acid as positive control was also tested for the reducing power assay.

Isolation and characterization of phytoconstituents

Column chromatography

The dry extract (10 g) was combined with a minimum amount of solvent and mixed thoroughly with silica gel (100 to 200 mesh size) in ratio of 1:1.5 (drug: silica) to make a slurry. The slurry was dried under reduced pressure using rotary evaporator so that the extract gets adsorbed on silica. Column was packed using silica gel 100 to 200 mesh size in *n*-hexane by wet packing method. Adsorbed silica was loaded to the prepared column. The column was eluted with suitable solvent gradient system[34]. Each fraction of 50 mL volume was collected and fractions were pooled on the basis of TLC profile to make different fractions.

Processing of the isolated fractions

Pooled fractions were collected on the basis of TLC profile. The fractions collected were dried in a rotary evaporator and weighed. The compounds obtained from the different fraction were given a specific code.

Table I: Phytoconstituents present in wood oil and chloroform extract of *C. deodara* and *P. roxburghii*

Phytoconstituents	*Cedrus deodara*		*Pinus roxburghii*	
	Wood oil	Chloroform extract	Wood oil	Chloroform extract
Alkaloids	-	+	-	+
Glycosides	-	+	-	+
Flavonoids	-	+	-	+
Terpenoids	+	+	+	+
Steroids	+	+	+	+
Carbohydrates	-	+	-	+
Proteins	-	-	-	-
Fats and Oils	+	-	+	-
Phenolic compound	+	+	-	-

+: present; -: not detected

Characterization of isolated compounds

The isolated compounds were subjected to spectroscopic analysis. The analyses were done at Sophisticated Analytical Instrument Facility (SAIF), Central Drug Research Institute, Lucknow, UP. FT-IR spectra were recorded on the PE Service (Excalibur HE 3600, PERKIN ELMER Spectrum Version 10.03.06A). 1H NMR spectra were recorded on Bruker DRX-300; SWITZERLAND spectrometer. Samples were dissolved in $CDCl_3$ based on the solubility of the sample. Mass spectra were recorded on DART-MS (JMS-T100LC, Accu TOF).

Determination of *in vitro* antioxidant activity of isolated compounds

Total antioxidant capacity

The total antioxidant capacity (TAC) was measured using phosphomolybdenum method[32]. The most active extract and isolated compounds were dissolved in suitable solvent individually and 0.3 mL of each sample was added to 3 mL of reagent solution (0.6 M sulphuric acid, 28 mM sodium phosphate and 4 mM ammonium molybdate mixture). All the tubes were incubated for 90 min at 95 °C. The mixture was cooled to room temperature and the absorbance was read at 695 nm against blank. Ascorbic acid equivalents were calculated by using the standard graph of ascorbic acid. The experiment was conducted in triplicate and values were expressed as equivalents of ascorbic acid in mg per g of sample.

Reducing power assay

Aliquots (50 µL) of different concentrations of samples (5-25 µg mL^{-1}) were mixed with 50 µL phosphate buffer (0.2 M, pH = 6.6) and 50 µL potassium ferricyanide (1 %), followed by incubation at 50 °C for 20 min in dark. After incubation, 50 µL of TCA (10 %) was added to terminate the reaction and the mixture was subjected to centrifugation at 3000 rpm for 10 min. For final reaction mixture, the supernatant (50 µL) was mixed with 50 µL distilled water and 10 µL $FeCl_3$ solution (0.1 %). The reaction mixture was incubated for 10 min at room temperature and the absorbance was measured at 700 nm against an appropriate blank solution. A higher absorbance of the reaction mixture indicated greater reducing power ability, confirming quantitative increase in the reduction of Fe^{3+} to Fe^{2+} in the reaction mixture and *vice versa*. All tests were run in triplicate in this assay. Ascorbic acid as positive control was also tested for the reducing power assay[33].

Statistical analysis

All values are expressed as mean ± SD. The data obtained from the various groups were statistically analyzed using One-way ANOVA followed by Tukey multiple comparisons test. $*p < 0.05$, $***p < 0.001$ *vs* control group.

RESULTS

Phytoconstituents of wood oil

Phytochemical testing showed that the wood oil of *C. deodara* contains terpenoids, steroids, phenolic compound, fixed oil and fats, whereas, wood oil of *P. roxburghii* contains terpenoids, steroids, fixed oil and fats (Table I).

Phytoconstituents of chloroform extract

Phytochemical testing showed that the chloroform extract of *C. deodara* contains alkaloids, glycoside,

flavonoids, terpenoids, steroid, phenolic compound and carbohydrates, whereas, the chloroform extract of *P. roxburghii* contains alkaloids, glycosides, flavonoids, terpenoids, steroids and carbohydrate (Table I).

TLC profiling of wood oil and extract

The TLC analysis showed that a large number of compounds present in the chloroform extract of *C. deodara* as compared to others. These unidentified constituents are likely to be terpenoids and flavonoids since our method of extract preparation was similar to that used in the previously reported isolation and characterization of constituents from *C. deodara*[21].

Wound healing study

Effect of wood oil and chloroform extract of *C. deodara* and *P. roxburghii* on wound contraction

The healing patterns of SSD, Cd O 1 %, Cd O 2.5 %, Cd C 1 %, Cd C 2.5 %, Pr O 1 %, Pr O 2.5 %, Pr C 1 %, and Pr C 2.5 % groups revealed that SSD and Cd C 2.5 % treated groups showed better healing patterns with

Table II: Effect of wood oil and chloroform extract of *C. deodara* and *P. roxburghii* on epithelialization time and hydroxyproline content

Treatment	Epithelialization time (days)	Hydroxyproline content (µg 100 mg^{-1} tissue)
Group I (Burn + Empty base)	37.8 ± 1.304	3031.6 ± 97.032
Group II (Burn + Standard drug)	25.8 ± 1.304***	4248.6 ± 85.702***
Group III (Burn + Cd O 1 %)	35.2 ± 0.837*	3113.8 ± 48.054
Group IV (Burn + Cd O 2.5 %)	34.8 ± 0.837*	3249.2 ±50.92***
Group V (Burn + Pr O 1 %)	35.6 ± 1.140	3120.2 ± 38.17
Group VI (Burn + Pr O 2.5 %)	35.2 ± 1.304*	3141.4 ± 67.32
Group VII (Burn + Cd C 1 %)	30.0 ± 1.225***	3982.6 ± 102.97***
Group VIII (Burn + Cd C 2.5 %)	27.6 ± 1.140***	4137.4 ± 59.28***
Group IX (Burn + Pr C 1 %)	35.2 ± 0.837*	3121.8 ± 37.81
Group X (Burn + Pr C 2.5 %)	34.8 ± 1.643*	3361.4 ± 82.82***

*Values are represented as mean ± SD. Statistical differences between the treated groups and control group were evaluated by One-way Analysis of Variance followed by Tukey Multiple Comparisons Test. *p < 0.05, ***p < 0.001 vs control group.*

Table III: Antimicrobial activity of wood oil and chloroform extract of *C. deodara* and *P. roxburghii* on different microbes

Micro organism	Minimum Inhibitory Concentration (MIC) in µg mL^{-1}						
	Cedrus deodara		*Pinus roxburghii*		Standard Drugs		
	Cd O	Cd C	Pr O	Pr C	Ampicillin	Chloramphenicol	Greseofulvin
E. coli	250	200	250	250	100	50	NA
P. aeruginosa	200	200	250	200	100	50	NA
S. aureus	250	200	250	250	250	50	NA
B. subtilis	500	250	500	250	100	50	NA
S. pyogenes	500	250	200	250	200	50	NA
C. albicans	>1000	1000	1000	1000	NA	NA	500
A. niger	>1000	-	1000	-	NA	NA	100
A. clavatus	1000	1000	500	500	NA	NA	100

-: not detected; *NA: not applicable*

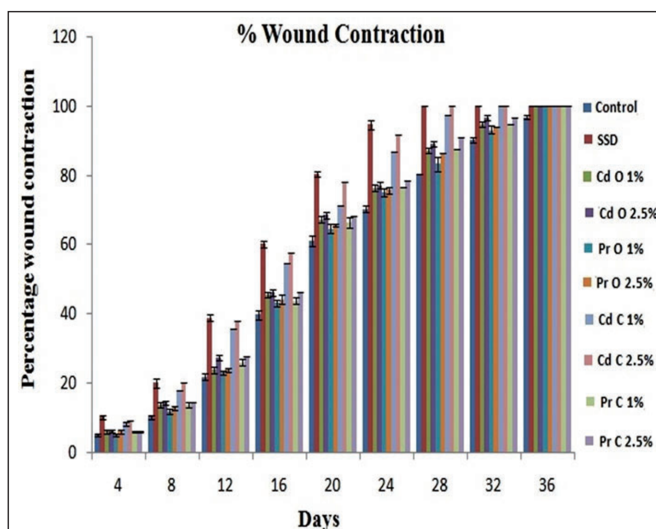

Fig. 1: Effect of wood oil and chloroform extract of *C. deodara* and *P. roxburghii* on percentage wound contraction. Values are represented as mean ± SD.

complete wound closure within 28 d, while it was about 40 d in control group. In addition, there was significant reduction in wound size from day four onwards in SSD, Cd C 1% and Cd C 2.5% treated groups and also on later d the closure rate was much faster as compared with control group. Interestingly, wound closure was also faster in Pr C 2.5 %, Cd O 2.5 % and Cd O 1 % treated groups as compared with control group. Collectively, results showed that chloroform extract of *C. deodara* incorporated in soft white paraffin was superior to other group (Fig. 1).

Effect of wood oil and chloroform extract of *C. deodara* and *P. roxburghii* on epithelialization time

The epithelialization time of SSD, Cd O 1%, Cd O 2.5%, Cd C 1%, Cd C 2.5%, Pr O 1%, Pr O 2.5%, Pr C 1%, and Pr C 2.5% groups revealed that SSD, Cd C 1% and Cd C 2.5% treated groups showed significant ($p < 0.001$) reduction in epithelialization time as compared to control group. Interestingly, treatments with Cd O 1%, Cd O 2.5%, Pr O 2.5%, Pr C 1% and Pr C 2.5% also reduced the epithelialization time with a significant of $p < 0.05$. In contrast, the Pr O 1% treated group did not show any significant reduction (Table II). Collectively, results showed that chloroform extract of *C. deodara* was superior to other group.

Effect of wood oil and chloroform extract of *C. deodara* and *P. roxburghii* on hydroxyproline content

The hydroxyproline content of SSD, Cd O 1%, Cd O 2.5%, Cd C 1%, Cd C 2.5%, Pr O 1%, Pr O 2.5%, Pr C 1%, and Pr C 2.5% treated groups revealed that SSD, Cd O 2.5%, Cd C 1%, Cd C 2.5%, and Pr C 2.5% significantly ($p < 0.001$) increased the hydroxyproline content (Table II). However, Cd O 1%, Pr O 1%, Pr O 2.5% and Pr C 1% slightly increases the hydroxyproline content. Among all the treated groups, SSD and Cd C 2.5%, treated groups showed to be comparable and most effective for the increase in hydroxyproline content.

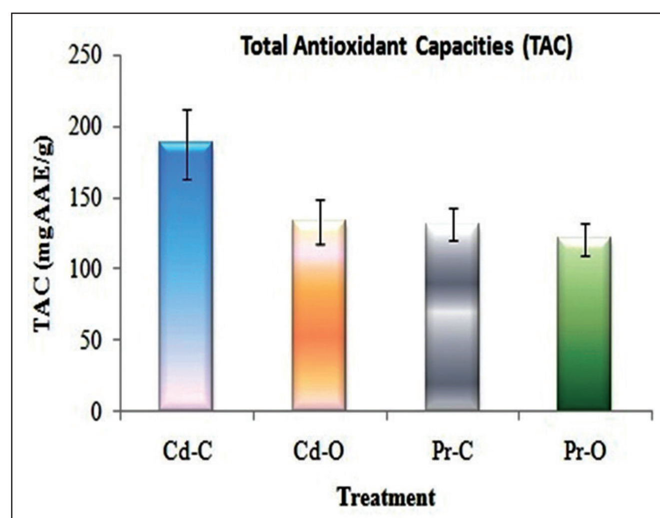

Fig. 2: Total antioxidant capacity of wood oil and chloroform extract of *C. deodara* and *P. roxburghii.* Values are reported as means ± S.D. of three parallel measurements.

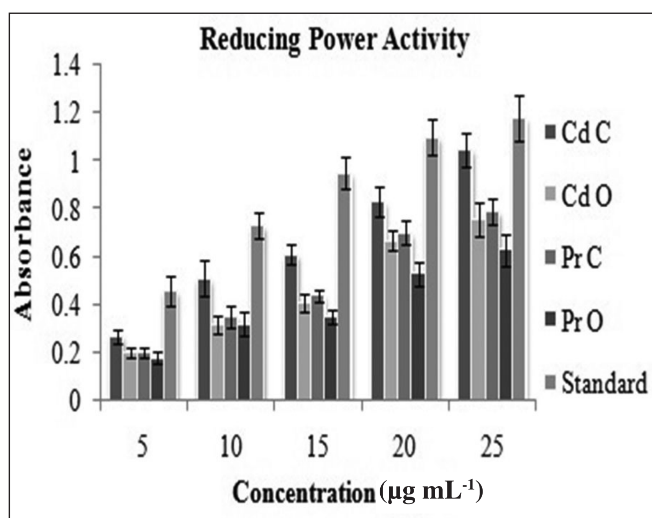

Fig. 3: Reducing power activity of the wood oil and chloroform extract of *C. deodara* and *P. roxburghii.* Values are reported as means ± S.D. of three parallel measurements.

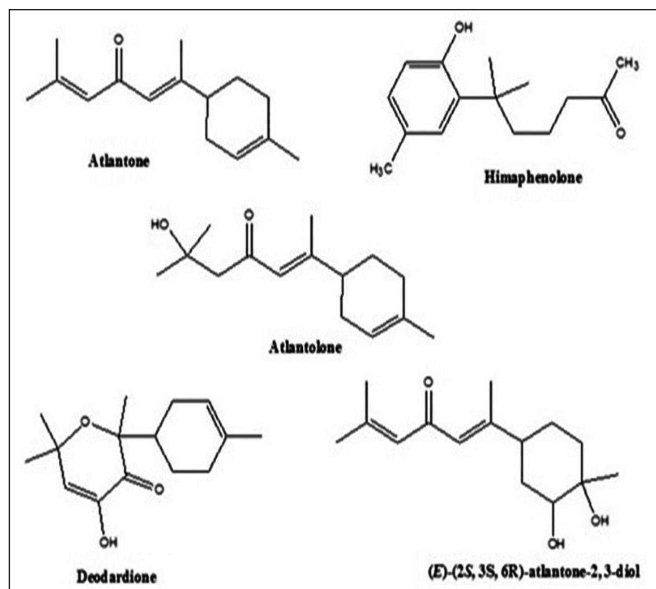

Fig. 4: Structure of compounds isolated from chloroform extract of *C. deodara*

Antimicrobial activity

The antimicrobial assay of wood oil and chloroform extract of *C. deodara* and *P. roxburghii* reveals that Cd C exhibit better effectiveness as compared to Cd O, Pr O and Pr C, against all tested organisms[21]. The antibacterial effect of Cd C was nearly equal to ampicillin against *S. aureus* and *C. albicans*. However, the antibacterial effect of chloramphenicol was better among all the therapeutic interventions. Interestingly, Cd C was found to be most effective against *S. aureus* as compared to Cd O, Pr C, Pr O and ampicillin (Table III).

Antiantioxidant activity

Total antioxidant capacity

Total antioxidant capacity of wood oil and chloroform extract of *C. deodara* and *P. roxburghii* reveals that Cd C exhibits utmost total antioxidant capacity. In contrast, Cd O, Pr O exhibits least total antioxidant capacity among all four tested groups (Fig. 2).

Reducing power ability

The reducing power ability of wood oil and chloroform extract of *C. deodara* and *P. roxburghii* were significant and in concentration-dependent manner (Fig. 3). As the concentration increased from 5 to 25 μg mL^{-1}, there was an increase in absorbance values for all the tested samples. Increasing absorbance of the reaction mixture indicates an increase in the reducing power ability. Reducing power ability of Cd C was noted to be the best among the Cd O, Pr O and Pr C. However, Pr O exhibit least reducing power activity at all concentrations. The reducing power activity of Cd C was nearly equal to the ascorbic acid, a standard antioxidant agent.

Spectral identification of isolated compounds

Compound CdC-B

Compound CdC-B was isolated as a light yellowish gum. It's positive ESI-MS showed molecular ion peak at m/z 219.12 [M^+], 220 $[M+1]^+$, 221 $[M+2]^+$, 217 $[M-2H]^+$, 215 $[M-4H]^+$ and 201$[M-H_2O]^+$ corresponding to the molecular formula $C_{15}H_{22}O$, exact mass 218.17 and molecular weight 218.33. FT-IR spectrum indicated absorption maxima for C-H (2925, 1401, 799 cm^{-1}), C = O (1671 cm^{-1}), and C = C (1620 cm^{-1}). 1H NMR spectrum showed 6.11-6.00 (2H, m, C-H at 10 and 2 position), 5.40 (1H, s, C-H at 8 position), 2.09-2.0 (4H, m, C-H at 6 position and CH_3 at 7 position), 1.95-1.88 (6H, m, two CH_3 at 11 position), 1.81-1.78 (4H, m, CH_2 at position 1 and 4) and 1.71-1.66 (5H, m, CH_3 at position 3 and CH_2 at position 5). Thus, on the basis of above spectral data and comparison with earlier reported spectral values[35] the structure of compound CdC-B was characterized as atlantone (Fig. 4).

Compound CdC-C

Compound CdC-C was also isolated as a light yellowish gum. It's positive ESI-MS showed molecular ion peak at m/z 234.13 [M^+], 233.12 $[M-1]^+$ and 231.11 $[M-3H]^+$ corresponding to the molecular formula $C_{15}H_{22}O_2$ exact mass 234.16 and molecular weight 234.33. FT-IR spectrum indicated absorption maxima for C-H of CH_3 and CH_2 (2928, 1405 cm-1), C = O (1666 cm^{-1}), Ar-H (3020, 758 cm^{-1}), O-H (3428 cm^{-1}) and C-O (1112, 1216 cm^{-1}). 1H

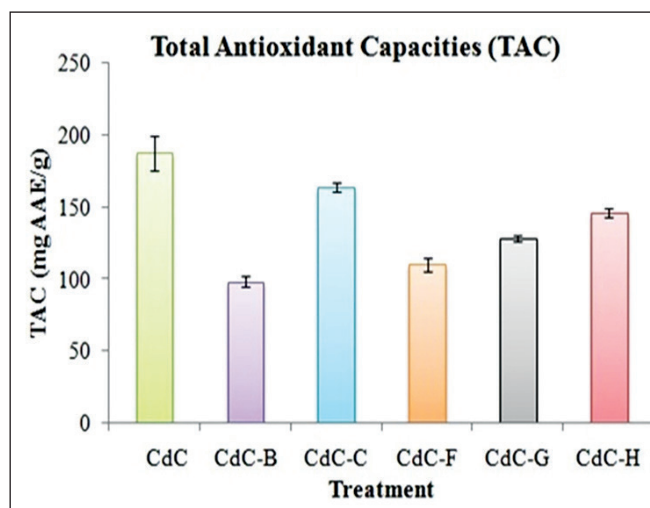

Fig. 5: Total antioxidant capacity of chloroform extract of *C. deodara* and isolated compounds. Values are reported as means ± S.D. of three parallel measurements

NMR spectrum showed 6.62 (1H, d, Ar-H at 9 position), 6.28 (1H, d, Ar-H at 8 position), 6.44 (1H, s, Ar-H at 11 position), 2.43 (3H, s, CH_3 at 10 position), 2.27-2.23 (2H, m, CH_2 at position 2), 2.09-2.06 (3H, m, CH_3 at position 1), 1.78-1.64 (4H, m, CH_2 at 3 and 4 positions) and 1.46-1.37 (6H, m, CH_3 at position 5). Thus, on the basis of above spectral data and comparison with earlier reported spectral values[36] the structure of compound CdC-C was characterized as himaphenolone (Fig. 4).

Compound CdC-F

Compound CdC-F was isolated as a brownish oil. It's positive ESI-MS showed molecular ion peak at m/z 235.10 [M^+], 236 $[M+1]^+$, 217 $[M-H_2O]^+$ and 207 $[M-C_2H_4]^+$ corresponding to the molecular formula $C_{15}H_{24}O_2$ exact mass 236.18 and molecular weight 236.35. FT-IR spectrum indicated absorption maxima for C-H (3019, 2933, 1384, 757 cm^{-1}), C = O (1619 cm^{-1}), O-H (3424 cm^{-1}) and C-O (1215, 1046 cm^{-1}). 1H NMR spectrum showed 6.07 (1H, s, C-H at 8 position), 5.59 (1H, br s, C-H at 2 position), 2.62-2.40 (2H, m, CH_2 at 10 position), 2.24-2.14 (3H, m, CH_3 at 7 position), 2.06-1.89 (5H, m, CH_2 at position 1 and 4, C-H at position 6), 1.81-1.79 (2H, m, CH_2 at position 5), 1.72-1.57 (3H, m, CH_3 at 3 position) and 1.37-1.18 (6H, m, two CH_3 at 11 position). Thus, on the basis of above spectral data and comparison with previously known spectral values[35], the structure of compound CdC-F was characterized as atlantolone (Fig. 4).

Compound CdC-G

Compound CdC-G was isolated as brownish gum. It's positive ESI-MS showed molecular ion peak at m/z 251.13 $[M+H]^+$, 249.12 $[M-1]^+$, 235.14 $[M-CH3]^+$ and 233.12 $[M-OH]^+$ corresponding to the molecular formula $C_{15}H_{22}O_3$ exact mass 250.16 and molecular weight 250.33. FT-IR spectrum indicated absorption maxima for O-H (3434 cm^{-1}), C-H (3018 cm^{-1}), C-H of CH_3 and CH_2 (2934 and 1382 cm^{-1}), C = O (1678 cm^{-1}), C = C (1622 cm^{-1}) and C-O (1217, 1117 cm^{-1}). 1H NMR spectrum showed 7.25 (1H, s, C-H at 5 position), 2.52 (1H, m, C-H at 3' position), 2.17-2.15 (1H, m, C-H at 1' position), 2.10-1.85 (4H, m, CH_2 at 2' and 5' position), 1.65-1.55 (5H, m, CH_3 at position 4' and CH_2 at position 6'), 1.42 (3H, s, CH_3 at position 2) and 1.25-1.21 (6H, m, CH_3 at 4 position). Thus, on the basis of above spectral data and comparison with previously known spectral values[37], the structure of compound CdC-G was characterized as deodardione (Fig. 4).

Compound CdC-H

Compound CdC-H was obtained as a light brownish gum. It's positive ESI-MS showed molecular ion peak at m/z 253.14 $[M+H]^+$ and 235.14 $[M-OH]^+$ (calculated 253.3572) corresponding to the molecular formula $C_{15}H_{25}O_3$. FT-IR spectrum indicated absorption maxima for O-H (3423 cm^{-1}), C-H (3018 cm^{-1}), C-H of CH_3 and CH_2 (2929 cm^{-1}), C = O (1665 cm^{-1}) and C = O (1217, 1117 cm^{-1}). ^1H NMR spectrum showed 7.26 (2H, s, C-H at 8 and 10 position), 2.76-2.74 (1H, m, C-H at 2 position), 2.65-2.59 (1H, m, C-H at 6 position), 2.24-2.07 (6H, m, CH_3 at 11 and 7 position), 1.91-1.79 (4H, m, one proton of CH_2 at position 1 and CH_3 at position 11), 1.71-1.56 (3H, m, one proton of CH_2 at position 1, 4 and 5) and 1.31-1.18 (5H, m, one proton of CH_2 at position 4 and 5 and CH_3 at 3 position). Thus, on the basis of above spectral data and comparison with previously known spectral values[38], the structure of compound CdC-G was characterized as (*E*)-(2*S*, 3S, 6R)-atlantone-2, 3-diol (Fig. 4).

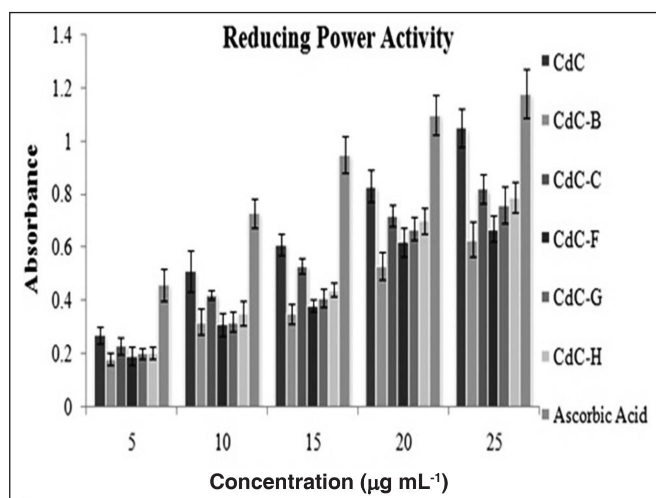

Fig. 6: Reducing power activity of chloroform extract of *C. deodara* and isolated compounds. Values are reported as means ± S.D. of three parallel measurements

Antimicrobial activity

Total antioxidant capacity

All the five isolated compounds exhibit total antioxidant capacity, but himaphenolone and (*E*)-(2S, 3S, 6R)-atlantone-2, 3-diol exhibit maximum antioxidant capacity among the five isolated compounds. It's worth noting that the total antioxidant capacity of both himaphenolone and (*E*)-(2S, 3S, 6R)-atlantone-2, 3-diol were found to be nearly equal to the total antioxidant capacity of chloroform extract of *C. deodara*. However, atlantone showed least total antioxidant capacity (Fig. 5).

Reducing power ability

The reducing power ability of chloroform extract

of *C. deodara* and all the isolated compounds was observed in concentration-dependent manner (Fig. 6). Interestingly, all the five isolated compounds also exhibit reducing power activity, but himaphenolone and (*E*)-(2S, 3S, 6R)-atlantone-2, 3-diol showed the utmost reducing power activity among the five isolated compounds. It's worth noting that the reducing power activity of both himaphenolone and (*E*)-(2S, 3S, 6R)-atlantone-2, 3-diol were found to be nearly equal to the reducing power activity of chloroform extract of *C. deodara* and ascorbic acid as well. However, atlantone showed least reducing power activity.

DISCUSSION

In extension of our earlier investigations on *C. deodara* and *P. roxburghii*, the present study explored the wound healing, antimicrobial and antioxidant potentials of wood oil and chloroform extract of *C. deodara* and *P. roxburghii.* Chloroform extract of *C. deodara* was found to more efficient amongst the tested groups. Numerous studies revealed that terpenoids and flavonoids have the most prominent wound healing potentials due to their astringent and antimicrobial property, which seems to be responsible for wound contraction and increased rate of epithelialization[39,14]. The preliminary phytochemical test and TLC profile revealed a number of compounds including terpenoids and flavonoids in the chloroform extract of *C. deodara* as compared to oil and chloroform extract of *P. roxburghii*. Moreover, we have found that chloroform extract of *C. deodara* contains several sesquiterpenes and use isolated five sesquiterpenes, namely atlantone, himaphenolone, atlantolone, deodardione, and atlantone-2, 3-diol, from that extract. Interestingly, all the isolated compounds showed prominent *in vitro* antioxidant activity in total antioxidant capacity and reducing power assay model[40]. Therefore, the chloroform extract of *C. deodara* exhibited significant antioxidant potential, mainly due to the presence of sesquiterpenes, which may be responsible for various pharmacological activities of this plant. The use of *C. deodara* in India for various skin infections is justified by this work.

Wound healing is characterized by three phases namely inflammation, proliferation and remodeling. Proliferative phases are characterized by epithelialization, angiogenesis, formation of granulation tissue and collagen deposition[28]. Cellular proliferation and increased collagen synthesis are the basic hallmarks involved in wound healing and are depending on the hydroxyproline content[41]. Moreover, increase in hydroxyproline content indicates increased collagen synthesis which in turn leads to enhanced wound healing[42]. In the present study, the hydroxyproline content was noted to be significantly increased with the topical application of chloroform extracts of *C. deodara,* which is a reflection of increased cellular proliferation and there by increased collagen synthesis, the possible mechanism of action involved in wound healing potential of *C. deodara.* Collagen not only confers strength and integrity to the tissue matrix, but also plays an important role in homeostasis and in epithelialization at the later phase of healing[43]. Hence, enhanced synthesis of collagen in *C. deodara* treated animals could provide strength to repaired tissue and also healing pattern. Based on the results of the study, it could be understood that chloroform extract of *C. deodara* might have enhanced the strength of the scar by increasing the collagen levels, which could stick the wound edges together at the repaired site. Several data indicated that number of phases of healing especially coagulation, inflammation, macrophagia, fibroplasias, collagenation, wound contraction and epithelization are intimately interlinked[44-46]. Therefore, a treatment could influence the healing of a wound by intervening in any one or many phases of healing.

Wound contraction, a process of shrinkage of area of the wound, depends on the reparative abilities of the tissue, type and extent of the damage and general state of the health of the tissue[47,48]. In the present study, enhanced rate of wound contraction and drastic reduction in healing time was observed in chloroform extract of *C. deodara* treated group as compared to other treated groups. The epithelialization time was also found to be shorter in animals treated with ointments containing chloroform extract of *C. deodara,* which promoted epithelialization either by facilitating the proliferation of epithelial cells or by increasing the viability of epithelial cells.

Though healing process takes place by itself and does not require much help, various risk factors such as infection and delay in healing have brought attention to promote this process[42,49,50]. When a wound occurs and is exposed to external environment, it is more prone to attack by microbes, which invade through the skin and delay the natural wound-healing process. The significant antimicrobial effect of the chloroform extract of *C. deodara* against all the pathogens potentiate the wound healing activity and justified the highest wound healing effect of this extract observed in the present study.

Antioxidant capacity and reducing power are the index of oxidative defense cascade. In addition, antioxidant capacity and reducing power of any herbal intervention have gained recognition for their ability to play a crucial role in the process of wound healing as these serve as

cellular messengers, which drive numerous aspects of molecular and cell biology[51]. In the present study, the chloroform extract of *C. deodara* was noted to possess utmost antioxidant capacity and reducing power, which clearly signifies the prominent wound healing potential of *C. deodara*. It has been observed that numerous essential oils containing terpene hydrocarbons and oxygenated terpenes exhibit antioxidant capacity through their reductive capacity in a Fe^{3+}–Fe^{2+} system[33]. This contention supports the antioxidant potential of chloroform extract of *C. deodara* as in the present study TLC profiling reveals that chloroform extract of *C. deodara* is supplemented with the terpenoids. Thereby, it may be suggested that the antioxidant activity of this plant may contribute to their claimed wound healing property.

The present study explored the wound healing, antimicrobial and antioxidant potentials of wood oil and chloroform extract of *C. deodara* and *P. roxburghii.* The high content of terpenoids in the chloroform extract of *C. deodara* may be responsible for utmost wound healing activity, probably due to their antioxidant, antimicrobial, proliferative and anti-inflammatory properties. Thus, the folklore claim for the rationale use of the *C. deodara* and *P. roxburghii* in skin diseases can be justified by the present study.

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