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COMPARATIVE PHYTOCHEMICAL AND PHARMACOLOGICAL SCREENING OF THE METHANOLIC EXTRACTS OF THE FRONTAL AND MATURE LEAVES OF *TECTONA GRANDIS.*

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ABSTRACT

The ethnopharmacological survey has revealed that only the frontal leaves of *Tectona grandis* have been used for the treatment of wounds. Comparative study of the activity and phytoconstituents of the mature and the frontal leaves of *Tectona grandis w*as carried out. Wound healing activity of both the extracts was evaluated by the excision wound model. The wound healing was 100 % on the 12th day and 16th day in the frontal leaves ($P<0.001$) and the mature leaves ($P<0.05$) respectively which was quite significant. A comparative phytochemical analysis was carried out to prove that the amount of phytoconstituents varied with the stages of development of the plants which in turn may be contributing to the activity of the extract. Total phenolic acid and flavonoid content of both the extracts was estimated and was found to be more in the frontal leaf extract, giving scientific proof to the use of frontal leaves.

KEY WORDS

Flavonoid, phenolic acid, *Tectona grandis*, wound healing.

INTRODUCTION

The plant under investigation is *Tectona grandis*, which belongs to the family Verbinaceae. The literature survey has revealed that the plant posses various activities i.e. in the treatment of urinary discharge, in the treatment of the common cold and headache, as a laxative and sedative, in bronchitis, as diuretic, anti diabetic, in scabies, diabetes, analgesic and anti inflammatory [1-4]. Juglone, Betulin aldehyde, Lapchol, apocarotenoids [5-8] are some of the important phytoconstituents that have been reported from this plant. We had earlier demonstrated that the hydro alcoholic extract of *Tectona grandis* leaves possesses significant wound healing activity in different wound models [9] .To give scientific proof for the difference in activities a comparative screening of the wound healing was carried out for both the frontal and the mature leaves. It has also been reported that the various stages of development of the plant brings about the changes in the nature, amount and the constituents in plants and this variation in turn

could bring about a change in the activity [10].Literature survey has revealed that the amount of phenolic acids is high in young fruits of red raspberries, black currents and strawberries [11]. Hence the study was designed and aimed to carry out a comparative study of the activity and the phytoconstituents of the mature and the frontal leaves of *Tectona grandis*. The results obtained in this work prove and give scientific justification for the traditional use of frontal leaves for treatment of wounds.

MATERIALS AND METHODS

***Collection and extraction of the frontal and mature leaves of Tectona grandis*:** The frontal and the mature leaves of the plant were collect from the rural areas of Bangalore. The plant was identified and authenticated by the Regional Research Institute, Bangalore where the specimen voucher (RRCBI Acc no 12474) has been deposited for future reference. The leaves were dried and pulverized and stored until further use.1 kg each of the frontal and mature leaves were extracted in a Soxhlet' s extractor using methanol. The extracts were then concentrated and dried. The percentage yield of the frontal leaves was found to be 6.6% and that of the mature leaves was 5.6%. The extracts were then subjected to preliminary phytochemical analysis [12].

***Selection of animals*:** The Institutional Animal Ethical Committee (No Krp/IAEC-27/2006) approved the experimental protocol and the guidelines for the animal care were strictly adhered to during the experimentation as recommended by committee for the purpose of control and supervision of experiments on animals (CPCSEA), Govt of India. The animals were maintained under standard conditions and were fed with commercial diet and water *ad libitum* during the experiment.

Preparation of the formulation: The methanolic extracts of frontal and the mature leaves were formulated as 5% ointment in emulsifying base. The emulsifying base was prepared using emulsifying wax, white soft paraffin and liquid paraffin. The fusion method was used for the preparation of the medicated ointments. The required quantity of the emulsifying base was melted at 70 ^{0} using a thermostatic water bath and a weighed quantity of the extracts was incorporated into the melted base and stirred to get a homogeneous ointment [13].

***Total phenolic content in the frontal and mature leaves of Tectona* grandis:** The total phenolic content was determined by Folin ciocalteu method [14].The extract (1mg/ml) and different dilutions of standard gallic acid were mixed separately with 1ml of Folin ciocalteu reagent. To these 10ml of 7% sodium carbonate was added. The mixtures were incubated at room temperature for 90 mts and the total phenolic content was determined by colorimetry at 750 nm.A standard curve was prepared using 100 µg/ml, 200 µg/ml, 300 µg/ml, 400 µg/ml, 500 µg/ml, 600 µg/ml and 700 µg/ml solutions of gallic acid in methanol. The total phenolic content was expressed in terms of gallic acid equivalents (Table 1).

Determination of flavonoids in the frontal and mature leaves of Tectona grandis:The flavonoid content of the extracts was determined by aluminum chloride colorimetric method [14]. The plant extracts(1mg/ml) in methanol were separately mixed with 1.5ml of methanol, 0.1ml of 1M potassium acetate, 0.1ml of 10% aluminum chloride and 2.8ml of distilled water and was maintained at room temperature for about half an hour. The absorbance of this mixture was measured at 415 nm. A calibration curve for the standard quercitin was obtained by taking 12.5ml, 25ml, 50ml, 75ml and 100ml in methanol. The total flavonoid contents were calculated as quercitin equivalent from the calibration curve by plotting the absorbance versus concentration (Table 1).

HPLC analysis of the cinnamic acid, tannic acid and gallic acid in the frontal and mature leaves of Tectona grandis:Quantative analysis

of the phenolic acids was performed using the Shimadzu High Performance Liquid Chromatographic system equipped with LC-10ATVP pump, a rheodyne injector, SPD M10AVP Photo Diode Array Detector in combination with CLASS-VP 6.12 SP5 integration software. Reverse-phase chromatographic analysis was carried out in isocratic conditions using a C-18 reverse phase column C18 – ODS (Octadecylsilane), Lichrospher RP 18e particle size 5µm (Merck) at 25℃.Running conditions included: injection volume 20 µl, mobile phase Methanol: 0.5% Orthophosphoric acid (60:40) flow rate, 1 ml/min, detection at 270-280nm.The samples were filtered through ultra membrane filters (pore size 0.45 micro; Merck, Germany) before injection. Tannic, gallic and cinnamic acids were used as standards. The phenolic acids present in each of the samples were identified by comparing the chromatographic peaks with the retention time of the standards. The amount of the phenolic acid is expressed as micrograms per gram of fresh weight. The HPLC chromatograms are as shown (Fig 2 and 3).

Pharmacological screening: Comparative evaluation of wound healing activity of the methanolic extracts of frontal leaves and mature leaves of *Tectona grandis*:
The excision wound model was used for the comparative study of wound healing activity of the mature and frontal leaves [9].

Excision wound model: The animals were anesthetized using ether and an impression was made on the dorsal thoracic region 1 cm away from the vertebral column and 5 cm away from the ear. The skin was excised to the full thickness to obtain a wound area of about 500 mm^2. Haemostasis was achieved by blotting the wound with a cotton swab soaked in normal saline. The animals were divided into four groups of six animals each. Groups 1 used as control for emulsifying ointment base, group 2 was used as a standard i.e. nitrofurazone ointment (0.2 % w/w),group 3 were used for methanolic extract in emulsifying ointment base of mature leaves, and group 4 was frontal leaves extract in emulsifying ointment. The wound area was measured by tracing the wound on a millimeter scale graph paper on every alternate day till complete falling of scar. The wound contraction-50% was calculated of the original wound size (500mm^2) for final analysis of the results. Complete healing i.e. no leaving of the wound was considered as the end point of complete epithelization and the days required for this was taken as the period of epithelization.

Statistical analysis: Results are expressed as mean ± SEM. The difference between experimental groups were determined using one way analysis of variance (ANOVA) followed by Dunnet test. $P<0.05$ was considered significant.

RESULTS AND DISCUSSION

Wound healing is a complicated process, which involves a series of stages like inflammation, granulation, wound contraction and epithelization followed by tissue remodeling. Any extract that is used for its wound healing activity may exhibit one or more of the biological activities which could be the basis for it to be used traditionally as a medicine; some of these activities may be analgesic, anti inflammatory, anti microbial, antioxidant etc.
The methanolic extract of the mature leaves was formulated as 5 % emulsifying ointment and was evaluated comparatively with the ointment prepared using the methanolic extract of the frontal leaves for its wound healing activity using excision model. The results revealed that the wound healing activity of the frontal leaves methanolic extract in emulsifying base was significantly increased when compared to the mature leaves methanolic extract in emulsifying base. The wound healing was 100 % on the 12^{th} day in case of the frontal leaves ($P<0.001$), while complete epithelization was achieved on the 16^{th} day in case of the mature leaves which was quite significant ($P<0.05$) (Table 1, Fig 1).

Table1.
Comparative effect of the frontal and the mature leaves of Tectona grandis on period of epithelization and wound contraction in excision wound model.

Treatment	50% falling of scar (days)	Wound Contraction (days)
EO Control	9.60±0.74	19.2 ± 0.48
Standard	8.00 ± 0.00	15.6 ± 0.24**
Frontal leaves (oint 5%)	6.88 ± 0.48*	11.6 ± 0.40**
Mature leaves (oint 5%)	8.8 ± 0.48	16.2 ± 0.48*

All values are mean ± SEM, *P<0.05 indicates significant and **P<0.001 is extremely significant when compared with control

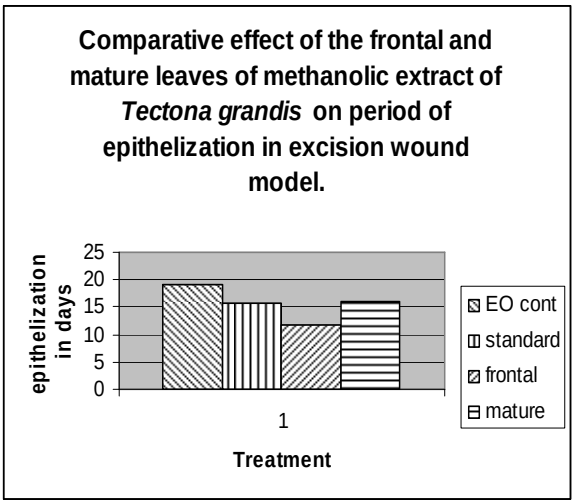

Figure 1
Comparative effect of the frontal and the mature leaves of Tectona grandis on period of epithelization and wound contraction in excision wound model

The quantity of the different phytoconstituents may vary with the different stages of development in plants, which can affect the pharmacological activity. The phytochemical analysis of both the extracts has revealed the presence of sterols, glycosides, tannins, flavonoids, phenolic acids, carbohydrates and proteins. Literature survey has revealed that tannins promote wound healing activity through several mechanisms that include chelation of free radicals; antioxidant, antimicrobial and astringent property [15].Phenolic acids have been reported to posses anti inflammatory, analgesic, antioxidant and wound healing properties [16]. Lipid peroxidation, results in cellular membrane damage which leads to swelling and cell death. The free radicals attract the different inflammatory mediators that are responsible for the general inflammatory response and tissue damage. During injury there is an increase in the consumption of the endogenous anti oxidants that bring about a decrease the amount of anti oxidants. Flavonoids may contribute an additive effect to the endogenous anti oxidants and to inhibit the eicosanoid biosynthesis therefore decreasing the formation of the inflammatory metabolites which is responsible for its anti inflammatory property .Hence by virtue of their free radical scavenging, antioxidant and

anti-inflammatory properties, flavonoids may help in healing of wounds [17].Considering the importance of these classes of drugs the total phenolic acids and the flavonoid contents were estimated for both the extracts .The total phenolic acid content of both the extracts was estimated using the Folin ciocalteu method . The phenolic acid content of the frontal leaves extract was found to be 26 µg/g while that in the mature leaf extract was found to be 17 µg/g. The amount of flavonoids was evaluated using aluminum chloride and was found to be more in the frontal leaves i.e. 15.07 µg/g when compared to the mature leaves i.e. 9.2µg/g(table 2).

Table 2.
Phenolic acid content and total flavonoid content of the mature and frontal leaf extracts

Extract	Phenolic content µg/g.	Total flavonoids µg/g
frontal	26	15.07
mature	17	9.2

HPLC analysis is one of the important ways for chemical characterization therefore this study establishes the fingerprint for the phenolic acids that are important phytochemicals. HPLC analysis of the frontal and the mature leaf extract has shown peaks at retention times (mts) of 17.73, 33.51 and 27.26 respectively for Gallic acid, Cinnamic acid, Tannic acid. The results revealed a difference in the amount of phenolic acids i.e. the amount of tannic acid, cinnamic acid and gallic acid is 2.96 µg/g, 0.69 µg/g, and 2.12 µg/g respectively in the frontal leaf extract while it is 1.55 µg/g, 0.34 µg/g, 1.25 µg/g in case of mature leaves. This difference in the phytoconstituents could be one of the contributing factors for the difference in activities of the two extracts. The amount, type and the quantity of the plant metabolites vary and depend on the different stages of development. This was evidenced by the difference in the amount of phenolic acids and the flavonoids in the mature and the frontal leaves of the methanolic extracts.

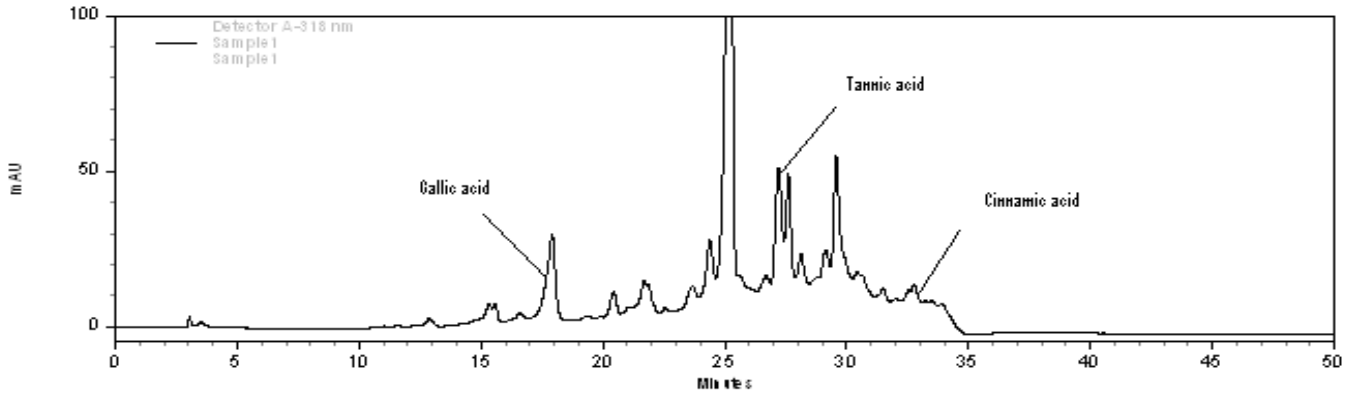

Figure 2

HPLC chromatogram for methanolic extract of mature leaf

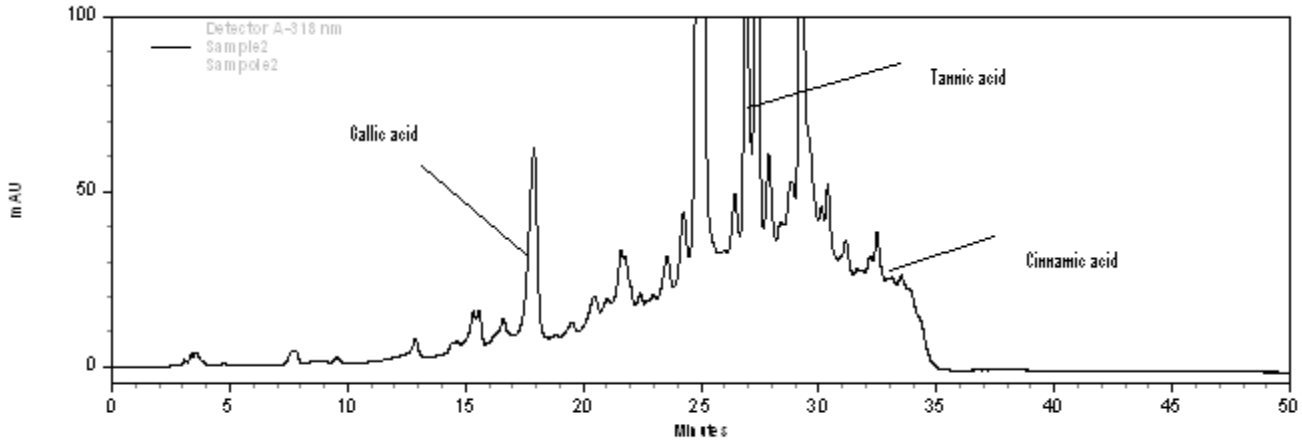

Figure 3

HPLC chromatogram for methanolic extract of frontal leaf

CONCLUSION

The present study was carried out to investigate the pharmacological activity and the phytochemical profile for the both the mature and frontal leaves of *Tectona grandis*. The outcome of this work give scientific support to the folkloric accounts to the use of the frontal leaves of *Tectona grandis* in the treatment of wounds. The frontal leaves contain more amount of the phytoconstituents when compared to the mature leaves, which helps us to authenticate the traditional use of frontal leaves for treatment of wounds.

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