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# Stability studies and evaluation of the semi solid dosage form of the rutin, quercitin, ellagic acid, gallic acid and sitosterol isolated from the leaves of *Tectona grandis* for wound healing activity.

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## ABSTRACT

*Gallic acid (GA), rutin(R), quercitin (Q), ellagic acid (EA) and sitosterol(S) isolated from the methanolic extract of the leaves of Tectona grandis were formulated as 0.2 % ointment in emulsifying base and were evaluated for their wound healing activity. The animals were divided into eight groups of six animals each. Groups 1 used as control, group 2 used as a standard i.e. nitrofurazone ointment (0.2 % w/w), group 3 for emulsifying ointment base, groups 4,5,6, 7 and 8 were used for gallic acid,rutin ,quercitin, ellagic acid and sitosterol . The formulation containing the most active phytoconstituent was packed in air tight bottle and evaluated for itsstability. The ointment was stored at $0^0$ ,$30^0$, $45^0$ at different predetermined intervals i.e. 30, 60 and 90 days and was evaluated for its appearance, spreadability, texture, pH and phase separation. The animals treated with rutin showed complete healing on the $10^{th}$ day, followed by gallic acid, Ellagic acid, Sitosterol, Quercitin. The formulation containing rutin was subjected to stability testing .There was no change in color, pH and no phase separation at $0^0$ and $30^0$ for 90 days. The pH of the ointment was found to be in the range of 5.95-6.36 .The spreadability of the formulation was found to lie between 33.92- 52.77 and the spreading time ranges between 18 sec to 28 sec. The texture of the ointment remained smooth through out the stability studies. The drug content of the ointment was found with in the range of 100%-97.07% at $30^0$.*

**Keywords:** Rutin, ointment, stability, wound healing, *Tectona grandis*

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## INTRODUCTION

The plant under investigation is *Tectona grandis*, Verbinaceae and is commonly called teak. The literature survey has revealed that the plant posses various pharmacological properties 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, , wound healing, analgesic and anti inflammatory [1-5]. Juglone, Betulin aldehyde, Lapchol, apocarotenoids, Gallic acid, rutin, quercitin, ellagic acid [5-10] 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, analgesic and anti inflammatory activity and this was attributed to the presence of the phyto constituents like phenolic acids, flavonoids, tannins etc. The present study was aimed at identifying the isolated compounds possessing the best wound healing activity using excision and incision wound healing models and to determine the stability of the formulation containing the constituent which has shown the best activity.

## MATERIALS AND METHODS

**Plant material:**
The frontal leaves of Tectona grandis were collected from the rural areas of Bangalore. Identified and authenticated by the Regional Research Institute, Bangalore where the specimen voucher (RRCBI Acc no 12474) has been deposited. The material was shade dried, pulverized and preserved in air tight containers until further use.

**Chemicals:** Chemicals were obtained from Merck and SD fine chemicals

**Preparation of the extracts:**
The methanolic extract of dried powder (1 kg) of the leaves was prepared by using Soxhlet apparatus which was then concentrated and dried to give dark brown mass.

**Phytochemical screening;**
The extract was then subjected to preliminary phytochemical analysis using standard procedures and majority of the constituents were found to be polar in nature.

**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.Sprague-Dawley (SD) rats of either sex weighing 250-275 gm were used. The animals were maintained under standard conditions and were fed with commercial diet and water adlibitum during the experiment.

**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 eight groups of six animals each. Groups 1 was used as control, group 2 was used as a standard i.e. nitrofurazone ointment (0.2 % w/w), group 3 was for emulsifying ointment base and groups 4,5,6, 7 and 8 were used for gallic acid ,rutin ,quercitin, ellagic acid and sitosterol respectively. 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[11,12,13].

**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.

**Incision wound model:** A paravertebral straight incision of 6 cm length was made on both sides of the vertebral column in the incision wound model. The wound was closed by suturing at equidistance of about 1 cm apart. Animals were treated daily from 0 to 9 $^{th}$ post-wounding day. The breaking strength of wound was measured on 10 $^{th}$ day by continuous, constant water flow technique [5].

**Skin irritation study**:
Healthy rabbits were selected and were shaved in two different areas of the dorsal side, each about 500mm$^2$.The rabbit was kept in rabbit holder and the 1$^{st}$ area was kept as a control, to which emulsion ointment base was applied, the 2 $^{nd}$ area was treated with gallic acid in emulsion ointment base. After 4 hrs the skin was observed and compared with the control .The same was repeated using the other isolated constituents in emulsifying ointment base [5]

**Stability studies of the most potent ointment [14, 15]:**
The formulation containing the most active phytoconstituent was packed in air tight bottles and evaluated for its stability.
It were stored at $0^0$ ,$30^0$, $45^0$ at different predetermined intervals i.e. 30, 60 and 90 days and was evaluated for its appearance, spreadability, texture, pH and phase separation.

**pH measurement**:
The pH of the formulation was measured using digital pH meter during the stability studies at 0, 30, 60, and 90 days.

**Physical changes:**
The formulation was packed in an air tight container and stored at different temperature i.e. $0^0$,$30^0$ and $45^0$ for 90 days.
Physical changes like color, odor, smoothness and phase separation were observed visually during the study.

**Estimation of drug content:**
The formulation containing the active constituent was accurately weighed (1gm) and transferred to a 100ml volumetric flask and the volume was made up to 100ml with methanol. This was filtered and 1ml of the filtrate was taken and diluted and the content was estimated using UV/Visible spectrophotometer at 271 nm, using the initial reading (0day) as 100% content.

**Spreadability:** An excess of sample was placed between two glass slides and 1000 g weight was placed on glass slide for 5 min to compress the sample to uniform thickness. Weight (250 g) was

added to the pan. The time in sec. required to separate two slides was taken as a measure of spreadability

Spreadability was calculated by using the following formula.
S= M.L/T.
Where S = spreadability, M = Weight tied to upper slide L = Length of glass slides
T =Time taken to separate the slides from each other.
In this experiment, M = 250 gm, L = 3.82 cm and time T was recorded

## RESULTS AND DISCUSSION

**Comparative effect of isolated compounds from the leaves of *Tectona grandis* on period of epithelization and wound contraction 50% in excision wound model**:
The animals treated with rutin showed complete healing on the 10$^{th}$ day, followed by gallic acid, Ellagic acid, Sitosterol, Quercitin.

**Table no 1**

| Treatment | 50% wound contraction (days) | period of epithelization (days) |
|---|---|---|
| Control | 9.6±0.04 | 18.80 ± 0.48 |
| EO base | 8.8±0.48 | 18.40 ± 0.40 |
| Gallic acid | 6.0±0.00** | 12.40 ± 0.40** |
| Ellagic acid | 6.0±0.20** | 14.00 ± 0.00** |
| Rutin | 4.2±0.20** | 10.20 ± 0.48** |
| Quercitin | 7.2±0.48** | 16.00 ± 0.00** |
| Sitosterol | 7.2±0.48** | 14.80 ± 0.48** |
| nitrofurazone | 7.2±0.48** | 15.20 ± 0.48** |

*All values are mean ±SEM* ***P<0.001 indicates extremely significant when compared with respective control.*

**Fig 1: Comparative effect of isolated compounds from the leaves of *Tectona grandis* on period of epithelization in excision wound model**:

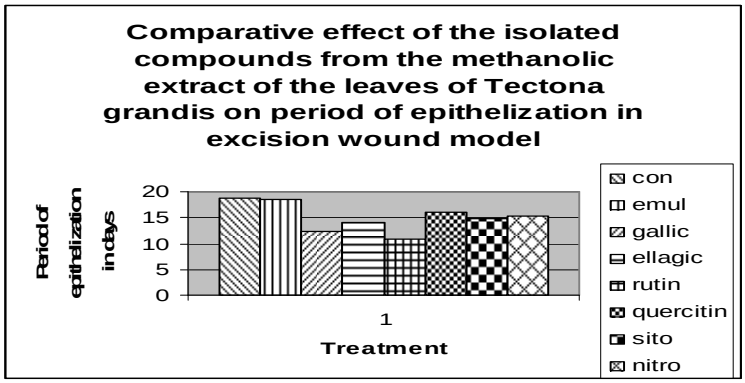
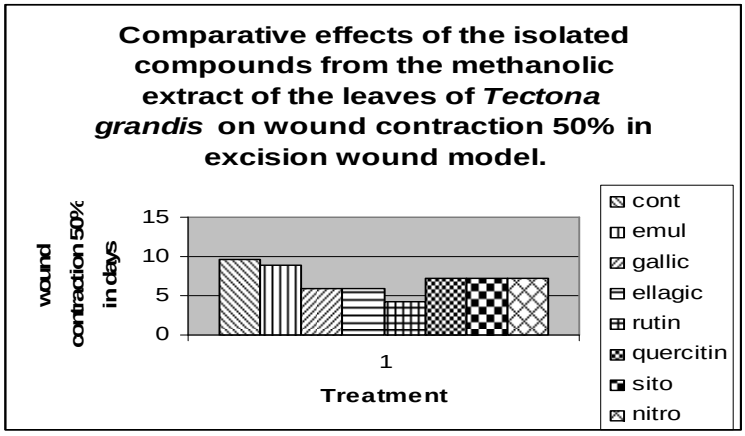



**Fig 2: Comparative effect of isolated compounds from the leaves of *Tectona grandis* on wound contraction 50% in excision wound model:**



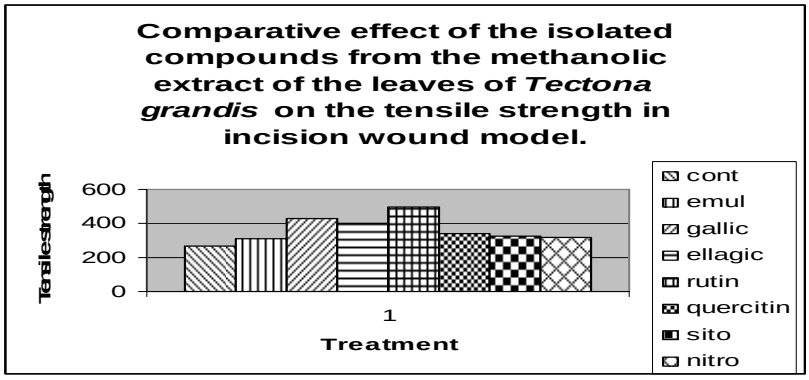

The animals treated with rutin showed maximum tensile strength of 499.2±12.9, followed by gallic acid, ellagic acid, quercitin and sitosterol.

**Table 2: Comparative effect of isolated compounds from the leaves of *Tectona grandis* on tensile strength in Incision wound model.**

| Treatment | Tensile strength |
|---|---|
| Control | 270 ±9.4 |
| EO base | 310±10.4 |
| Gallic acid | 430±12.25** |
| Ellagic acid | 402±13.84** |
| Rutin | 499.2±12.9 ** |
| Quercitin | 344.6±6.7** |
| Sitosterol | 326.2±16.2* |
| nitrofurazone | 316.8±9.6* |

*All values are mean ± SEM, n=5-6, *P<0.05 indicates significant and **P<0.001 indicates extremely significant when compared with respective control*

**Fig 3: Comparative effect of the isolated compounds from the methanolic extract of *Tectona grandis* on the tensile strength in the incision wound model.**




## Skin irritation study:

The ointments containing the gallic acid, ellagic acid, rutin and sitosterol did not show any severe type of irritation and there was no evidence of any noticeable inflammation. However a

slight redness was observed in the case of the ointments prepared from quercitin. This could be one of the reasons for the delay in wound healing activity of the quercitin.

**Table 3: Effect of the isolated compounds from the methanolic extract of the leaves of *Tectona grandis* on the skin irritation study**

| Group | Sign | Score |
|---|---|---|
| EO base | No change | 0.0 |
| Gallic acid | No change | 0.0 |
| Ellagic acid | No change | 0.0 |
| Rutin | No change | 0.0 |
| Quercitin | No noticeable inflammation, but slight redness | 0.5 |
| Sitosterol | No change | 0.0 |
| Nitrofurazone | No change | 0.0 |

## Stability testing of the formulation:

The formulation containing rutin was subjected to stability testing .There was no change in color and no phase separation at $0^0$ and $30^0$ for 90 days. However there was a slight change in color in the formulation at $45^0$ and phase separation occurred after 3 months at the same temperature. There was no significant change in the pH during the entire period of stability studies. The pH of the ointment was found to be in the range of 5.95-6.36 .The spreadability of the formulation was found to lie between 33.92- 52.77 and the spreading time ranges between 18 sec to 28 sec.The texture of the ointment remained smooth through out the stability studies with no grittiness. The drug content of the ointment was found with in the range of 100%-97.07% at $30^0$which is in normal (95%-105%) permitted range of variation.

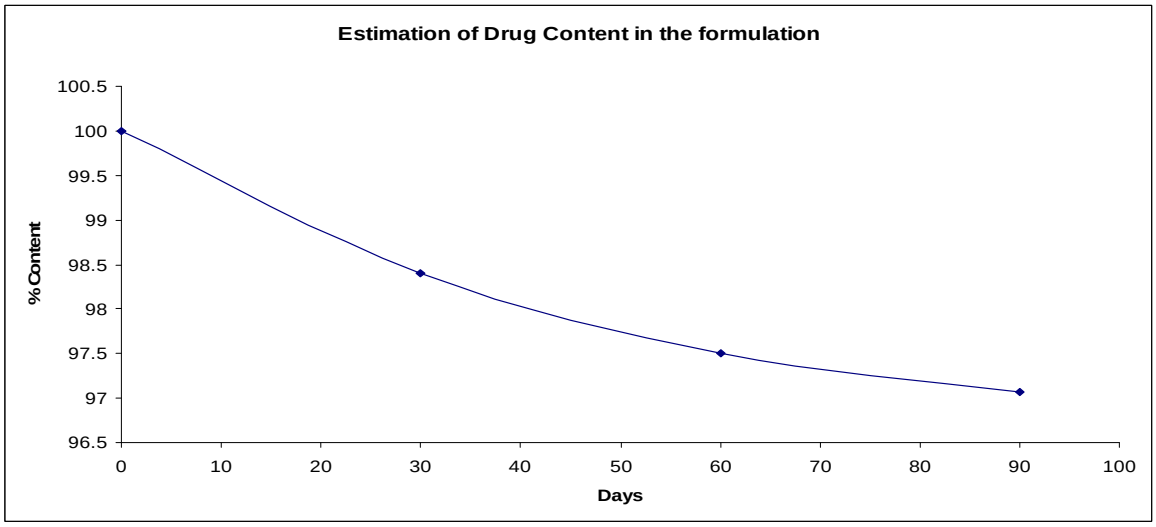
**Table 4: Comparison of the different parameters of the formulation after storage at various temperatures 30, 60 and 90 days**

| Characteristics of the formulation | Temp ($C^0$) | Time( in days) | | | |
|---|---|---|---|---|---|
| | | Initial | 30 | 60 | 90 |
| Color | $0^0$<br>$30^0$<br>$45^0$ | Pale yellow<br>Pale yellow<br>Pale yellow | Pale yellow<br>Pale yellow<br>Pale yellow | Pale yellow<br>Pale yellow<br>Pale yellow | Pale yellow<br>Pale yellow<br>Darker yellow |
| Spread ability (spreading time in sec) | $0^0$<br>$30^0$<br>$45^0$ | 52.77 (18)<br>52.77 (18)<br>52.77 (18) | 39.58 (24)<br>50.00 (19)<br>41.38 (23) | 39.58 (24)<br>50.00 (19)<br>39.58 (24) | 33.90 (28)<br>50.00 (19)<br>38.00 (25) |
| Texture | $0^0$<br>$30^0$<br>$45^0$ | No grittiness<br>No grittiness<br>No grittiness | No grittiness<br>No grittiness<br>No grittiness | No grittiness<br>No grittiness<br>No grittiness | No grittiness<br>No grittiness<br>No grittiness |
| pH | $0^0$<br>$30^0$<br>$45^0$ | 6.36<br>6.36<br>6.36 | 6.29<br>6.28<br>6.10 | 6.23<br>6.08<br>5.95 | 6.13<br>5.98<br>5.91 |
| Phase separation | $0^0$<br>$30^0$<br>$45^0$ | NPS<br>NPS<br>NPS | NPS<br>NPS<br>NPS | NPS<br>NPS<br>NPS | NPS<br>NPS<br>PS |

*NPS: No phase separation; PS: Phase separation*

**Estimation of drug content in the formulation:**
The drug content of the ointment was found with in the range of 100%-97.07% at $30^0$ which is in normal (95%-105%) permitted range of variation.

**Fig 4: Estimation of drug content in the formulation**




## DISCUSSION

The various phytochemicals present in plants have been reported to posses great potential in treatment of various diseases, many of them have been shown to be very useful in wound care i.e. promoting the rate of wound healing, bringing about decrease in pain, discomfort, and scarring. These constituents owe their activity to direct effect on the wound healing processes and sometimes to their other activities like anti oxidant, anti-inflammatory and anti-microbial properties or it could be a synergistic effect due to the combination of these properties.

Of the five isolated compounds rutin has exhibited the best wound healing activity followed by gallic acid, ellagic acid, sitosterol and quercitin. Significant decrease in the period of epithelization in the excision wound model and a significant increase in the tensile strength in the incision wound model was noticed in case of the isolated compounds, the increase in tensile strength of treated wounds may be due to the increase in collagen concentration and stabilization of the fibers.

Flavonoid rutin differs from quercitin in the presence of a sugar rutinose at position 3, so it can be predicted that this sugar could be contributing to the pharmacokinetic factor there by showing better activity then quercitin. It has been reported that although glycosides are usually weaker antioxidants than aglycones their bioavailability is increased due to the increase in solubility by the presence of a sugar moiety. Both quercitin and rutin are highly effective chelators of transition metals implying little difference between aglycones and glycosides in the ability to complex metal. It has also been reported that the topical application of quercitin could result in toxic response to the wound [16, 17] which was proved by the skin irritation studies where slight redness was seen when the formulation was tested for its skin irritation, the delay in wound

healing could be due to its irritant activity on the wound. It has been reported that rutin also possesses significant anti inflammatory activity when compared to quercitin and hesperidin

There fore it can be predicted that the formulation containing rutin has shown the best wound healing activity by virtue of its synergistic effects i.e. anti inflammatory, analgesic, anti microbial and anti oxidant properties. Hence this formulation containing rutin was considered for stability testing studies

The various physicochemical properties used to evaluate the prepared ointment formulation are shown in Table no 7. From the results, it is evident that the ointment showed good homogeneity. The pH of the ointment was found to be in the range 5.95-6.36 which lies in the normal range of the pH (5.5-6.50) of the skin therefore making the formulation less sensitive and ensures better acceptability to the skin. From the stability studies it is clear that the formulated ointment showed no significant changes in pH, spread ability, consistency and phase separation after keeping at different temperatures for 90days at $0^0$ and $30^0$ however there was a slight change in color in the formulation at $45^0$C and phase separation occurred after 90 days. The phase separation was attributed to the loss of emulsifying property due to storage at high temperature.

The slight color change that might indicate a small degree of degradation which may not be significant practically since the storage directions for ointments is to keep at a cool place at temperatures < $45^0$, no such degradation was apparent during the accelerated stability studies at temperatures below $45^0$C. It has also been reported that the color of rutin changes at higher temperature due to degradation [18, 19]. Ointment base should spread easily without much drag and should not produce friction in the rubbing process. Spread ability is a term to denote the extent of area to which the ointment and gel readily spreads on application to skin or affected part. It is expressed in terms of seconds taken by two slides to slip off from ointment placed in between the slides under the direction of certain load. Lesser the time taken for separation of two slides, the better spread ability. The formulation showed good spreadability. Therefore the formulation is stable at topical conditions and must be stored in air tight container at temperature below $45^0$C .

## CONCLUSION

The isolated compounds were formulated in emulsifying ointment base as 2% ointments and were evaluated for their wound healing activity. The formulations were evaluated for its skin irritation and the results revealed no skin irritation except for quercitin as mentioned earlier. The formulation containing rutin was found to be most potent as wound healing agent. The formulation containing rutin was subjected to stability studies and the results of the stability studies revealed that the formulation was stable and there were no significant changes in any of the parameters studied at temperatures $30^0$ up to three months. The formulation containing rutin was found to be stable at topical conditions and must be stored at temperature below $45^0$C .

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