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Evaluation of wound healing properties of bioactive fractions from the extract of *Butea monosperma* (lam) stem bark.

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Abstract

The study aims to evaluate the wound healing properties of bioactive fractions from the extract of *Butea monosperma* (Lam) stem bark. In this study the stem bark powder was extracted with ethanol, further the ethanolic extract was fractionated with different solvents (petroleum ether, benzene, chloroform and acetone) in increasing order of polarity. Thus prepared extracts were subjected to preliminary phytochemical analysis. The wound healing activity of the ethanolic extract and the fractions isolated from the stem bark of *Butea monosperma* were evaluated in excision, incision and dead space wound healing models using Albino wistar rats. The wound healing activity was assessed by the breaking strength in case of incision wounds, epithelialization and wound contraction in case of excision wound and granulation tissue dry weight, breaking strength and hydroxyproline content in case of dead space wound. The ethanolic extract and the acetone fraction showed the significant wound healing activity on all three wound models. The phytochemical investigations revealed the presence of alkaloids, tannins, flavonoids, phenolic compounds and steroids. The increased rate of wound contraction and hydroxyproline content in the ethanolic extract and the acetone fraction treated animals provides a scientific base to the ethno medicinal use of Butea monosperma, which is largely attributable to the additive or synergistic effect of their constituents.

Keywords: Butea monosperma, Dead space wound, Excision wound, Incision wound.

Introduction

Wound is a breach in the normal tissue continuum, resulting in a variety of cellular and molecular sequelae. The basic principles of optimal wound healing which include minimizing tissue damage, debriding nonviable tissue, maximizing tissue perfusion and oxygenation, proper nutrition and moist wound healing environment have been recognized for many years [1]. A number of drugs ranging from simple non-expensive analgesics to complex and expensive chemotherapeutic agents administered in the management of wound affect healing either positively or negatively [2]. Wounds are inescapable events of life which arise due to physical injury, chemical injury or microbial infections. Healing of wounds usually takes place in a direction away from its normal course and under healing, over healing or no healing of

wounds is common. Management of under healing wounds is a complicated and expensive program and research on drugs that increase wound healing is a developing area in modern biomedical sciences. Several drugs obtained from plant sources are known to increase the healing of different types of wounds. Though some of these drugs have been screened scientifically for evaluation of wound healing activity in different pharmacological models and patients, the potential of many of the traditionally used herbal agents remain unexplored. In few cases active chemical constituents were identified [3].

Butea monosperma (Lam) (Fabaceae) is a medicinal plant growing in Burma, India and Sri Lanka, The flowers are tonic, astringent, aprodiasic and diuretic. The decoction of the bark is traditionally used in cold, cough, fever, various forms of haemorrhages, in menstrual disorders and in the preparation of tonics and elixirs. The stem bark is reported to possess antitumour, antiulcer, antifungal and antidiarrhoeal activities [4-6]. It is also reported that the powder of the stem bark is used to apply on injury caused due to an axe, the juice of the stem is applied on goiter of human beings and the paste of the stem bark is applied in case of body swellings [7]. The roots are reported in the treatment of filariasis, night blindness, helmenthiasis, piles, ulcers, and tumors [8]. It is reported that the ethanolic extract of seeds of Butea monosperma, on oral administration showed antifertility activity in mice and in rats [9]. Palsonin an active principle isolated from *Butea monosperma* seeds and its piperzaine salt exhibited good anthelmintic activity in vitro on Ascaris lumbricoides and in vivo on Taxicara canis [8]. The petroleum ether extract and triterpene isolated from flowers of *Butea monosperma* exhibited anti convulsant activity [10, 11]. It has been reported that the methanolic extract of stem bark of *Butea monosperma* showed anti inflammatory and analgesic activity [12]. Recently we have reported the in vitro and in vivo anti inflammatory activity of *Butea monosperma* stem bark extract and the anti inflammatory activity of flavonoid fraction isolated from the stem bark of *Butea monosperma* [13, 14]. It is reported the efficacy of *Butea monosperma* on dermal wound healing in rats [15]. Since a little information is available about the wound healing potential of *Butea monosperma* stem bark, it was considered worthwhile to study the wound healing potential of ethanolic stem bark extract and the fractions of *Butea monosperma* (Lam) on wistar rats.

Materials and methods

Plant Material

The stem bark of *Butea monosperma* was collected during July 2009 from Manipal, Udipi district, Karnataka state, India. The samples were authenticated by Dr. Gopalakrishna Bhat, Professor of Botany, Poorna Prajna College, Udipi, India. A herbarium specimen has been deposited at the college for further reference.

Preparation of Plant Extracts

The bark was dried in the shed and coarsely powdered. The powder was extracted with ethanol in a soxhlet apparatus for 72h. The ethanolic extract was evaporated in vacuo giving the residue (24%). The ethanolic extract obtained was suspended in distilled water in small amounts and was extracted successively and exhaustively with petroleum ether (60-80°C), benzene, chloroform and acetone in the order of increasing polarity. The extract and fractions were concentrated in a rotary evaporator at reduced pressure.

Preliminary Phytochemical Analysis

The ethanolic extract and the fractions isolated from it were screened for the presence of various phytoconstituents

according to the phytochemical methods described by Harborne [16].

Experimental Animals

Adult Wistar strain rats (150 to 200 gm) were used for all the experiments in the present study. The animals were maintained under standard husbandry conditions in the animal house of the institute (temperature 25 ± 2°C) in a natural light-dark cycle and fed with standard rodent diet and water ad libitum. Ethical committee clearance was obtained from IAE (Institutional Animal Ethics Committee) of CPCSEA (Ref. No./IAEC/XII/08/CLBMCP/2009-2010).

Acute toxicity studies

The acute toxicity of ethanolic extract and the various fractions of *Butea monosperma* stem bark extract was determined as per the OECD guideline no. 423 (Acute toxic class method) [17]. It was observed that the ethanolic extract and the fractions were not mortal even at 2000 mg kg^{-1} dose. Hence, $1/10^{th}$ (200 mg kg^{-1}) of this dose was selected for this study.

Excision wound model

The rats were inflicted with excision wounds as described by Morton and Malon [18]. An excision wound was inflicted by cutting away 500 mm^2 full thickness of a pre-determined area on the depilated back of the rat. The rats were divided into seven groups of six animals each. Group 1(control) animals were topically applied with simple ointment base, Group 2 animals were topically applied with soframycin ointment and the remaining groups were topically treated with 200 mg kg^{-1} b.w. of test substances mixed with ointment base. Treatments were given once daily till the wound was completely healed. Epithelialization period was noted as the number of days after wounding required for the dead tissue remnants to fall off leaving no raw wound behind. Wound contraction rate was monitored by planimetric measurement of the wound area on alternate days. This was achieved by tracing the wound on a graph paper. Reduction in the wound area was expressed as percentage of the original wound size [19].

Incision wound model

The method of Ehrlich and Hunt was adopted for incision wound study [20]. The animals were anaesthetized under light ether, on the depilated backs of the animals, two paravertebral incisions of 6 cm length were made cutting through the full thickness of the skin. Interrupted sutures, 1 cm apart, were placed to approximate the cut edges of the skin. The rats were divided into seven groups of six animals each. Group 1(control) animals were topically applied with simple ointment base, Group 2 animals were topically applied with soframycin ointment and the remaining groups were topically treated with 200 mg kg^{-1} b.w. of test substances mixed with ointment base. The sutures were removed on the 8th post wound day and skin breaking strength was measured on the 10th day by continuous water flow technique [21].

Dead Space Wound model

Dead space wounds were created through a small transverse incision made in the lumbar region. A polypropylene tube (2.5 × 0.5 cm) was implanted subcutaneously beneath the dorsal paravertebral lumbar skin [22]. The day of the wound creation was considered as day zero. The animals were divided in to six groups of six animals each. Group 1 was the control group that received 2 mL of 1% carboxymethyl cellulose. The remaining groups were administered each with 200 mg kg^{-1} b.w. of test substances orally, once daily for 10 days. Granulation tissue formed on the polypropylene tube was harvested by careful dissection on day 10 and the breaking strength of the granulation tissue was measured. The granulation tissue was dried in an oven at 60°C overnight and the

dry weight was noted. Acid hydrosylate of the dry tissue was used for the determination of the hydroxyproline content [23].

Statistical Analysis

The experimental results were expressed as mean ± S.E.M. Results were analyzed by the one- way ANOVA followed by Tukey-kramer post hoc multiple comparison test using Graph pad InStat version 3.00. P value of <0.05 was considered as statistically significant.

Results

Preliminary Phytochemical Analysis

The preliminary phytochemical analysis of the ethanolic extract and the various fractions showed the presence of alkaloids, tannins, flavonoids, phenolic compounds and steroids (Table 1).

Table 1: Phytochemical screening of ethanolic extract and its fractions of stem bark of Butea monosperma.

Extracts	Steroids	Alkaloids	Flavonoids	Phenolic compounds	Tannins
ETEXT	+	+	+	+	+
PETFR	+	-	-	-	-
BENFR	-	-	-	-	-
CHLFR	-	+	-	-	+
ACEFR	+	-	+	+	-

ETEXT: ethanolic extract, PETFR: petroleum ether fraction BENFR: benzene fraction, CHLFR: chloroform fraction, ACEFR: acetone fraction.
+ denotes the presence of the respective class of compounds.
- denotes the absence of the respective class of compounds.

Table 2: Effect of ethanolic extract of *Butea monosperma* stem bark and its fractions on percentage of wound contraction and period of epithelization in excision wound model.

Groups	% Wound contraction				Period of epithelization (days)
	4th day	8th day	12th day	16th day	
Control	5.12±0.09	56.67±0.67	83.0±0.58	85.17±0.79	22.0±0.37
Standard	61.83±1.52**	58.67±0.72	85.17±0.95	98.33±0.33**	15.83±0.31**
ETEXT	54.33±1.50**	58.0±0.86	85.0±0.52	95.50±0.76**	17.0±0.37**
PETFR	32.67±1.23**	54.67±1.38	84.17±0.60	86.83±0.87	21.17±0.48
BENFR	38.67±1.15**	55.0±0.52	84.50±0.43	86.67±0.67	21.67±0.42
CHLFR	41.67±0.88**	55.67±0.84	83.67±0.49	88.0±0.57	21.83±0.48
ACEFR	58.50±0.76**	58.5±0.85	85.50±0.56	96.0±0.37**	16.67±0.42**

Standard: Soframycin ointment, ETEXT: ethanolic extract, PETFR: petroleum ether fraction BENFR: benzene fraction, CHLFR: chloroform fraction, ACEFR: acetone fraction. Each value is the Mean ± S.E.M for 6 rats. **P<0.001 compared with control.

Excision wound model

Excision wounds heal by contraction (wound closure) and epithelization, the percentage of wound closure or closure rate includes by recording the changes in wound area at fixed intervals of time, viz. 4^{th}, 8^{th}, 12^{th} and 16^{th} day after treatment with ethanolic extract and its different fractions. The percentage of wound contraction in all the test groups was significant statistically when compared to control on the 4^{th} day, there was no statistical significance in the percentage of wound contraction during the 8^{th} and 12^{th} days. But on 16^{th} day there was a statistically significant difference in percentage of wound contraction. The maximum percentage of wound closure on the 16^{th} day was observed with standard drug, soframycin (98.33%) and ethanolic extract of *Butea monosperma* stem bark (95.5%). Amongst different fractions, the acetone fraction (96.0%) showed maximum percentage of wound closure than the other fractions and it was comparable to that of ethanolic extract. The ethanolic extract and the acetone fraction were also significantly ($P < 0.05$) reduced the epithelialization period of excision wounds (Table 2).

Incision wound model

Incision wounds heal by granulation and collagenation. The mean wound breaking strength or tensile strength of wound in control group was 147.33 g, while in the case of ethanolic extract (218.33 g) and the acetone fraction treated group (212.83 g), it was found that the mean time for epithelialization and mean scar area were reduced significantly, there by increasing the mean tensile strength compared to control group. The mean wound breaking strength in case of soframycin treated group was 333.83 g (Table 3).

Table 3: Effect of ethanolic extract of *Butea monosperma* stem bark and its fractions on wound breaking strength in incision wound model.

Groups	Mean wound breaking strength (g)
Control	147.33±1.23
Standard	333.83±5.12**
ETEXT	218.33±1.71**
PETFR	155.83±2.26
BENFR	151.0±2.59
CHLFR	163.33±1.33*
ACEFR	212.83±2.02**

Standard: Soframycin ointment, ETEXT: ethanolic extract, PETFR: petroleum ether fraction BENFR: benzene fraction, CHLFR: chloroform fraction, ACEFR: acetone fraction. Each value is the Mean ± S.E.M for 6 rats. *P < 0.01; **P<0.001 compared with control.

Table 4: Effect of ethanolic extract of *Butea monosperma* stem bark and its fractions on dry weight, tensile strength and hydroxyproline content in dead space wound model .

Groups	Dry weight (mg)	Tensile strength (g)	Hydroxyproline (µg/mg)
Control	42.83±0.60	326.5±4.75	21.48±0.17
ETEXT	68.0±0.58**	393.0±5.16**	23.06±0.28**
PETFR	43.5±0.92	321.5±3.55	21.57±0.21
BENFR	45.17±0.70	328.0±3.35	20.96±0.08
CHLFR	45.5±0.85	329.83±5.16	21.84±0.08
ACEFR	69.33±0.42**	405.83±4.73**	23.50±0.17**

ETEXT: ethanolic extract, PETFR: petroleum ether fraction BENFR: benzene fraction, CHLFR: chloroform fraction, ACEFR: acetone fraction. Each value is the Mean ± S.E.M for 6 rats. **P<0.001 compared with control.

Dead Space Wound model

The mean dry weight of granulation tissue in the control group was 42.83 mg, which was significantly ($P < 0.05$) increased to 68.0 mg in ethanolic extract treated group followed by acetone fraction (69.33 mg) when compared to the control group. The breaking strength in control group was 326.5 g, where as the ethanolic extract (393.0 g) and acetone fraction (405.83 g) showed significant increase in breaking strength when compared to the control group. The hydroxyproline content was significantly increased ($P < 0.05$) in ethanolic extract and the acetone fraction treated groups when compared to the control group (Table 4).

Discussion

The results of the present investigations revealed that the ethanolic extract of the stem bark of *Butea monosperma* and its acetone fraction possess significant wound healing activity in excision, incision and dead space wound models. In spite of tremendous development in the field of synthetic drugs during recent era, they are found to have some or other side effects, whereas plants still hold their own unique place, by the way of having no side effects. Therefore, a systematic approach should be made to find out the efficacy of plants against wounds so as to exploit them as herbal wound healing agents.

Experimental assessment of the wound healing activity of ethanolic extract and its fractions showed increased rate of wound contraction and epithelialization and increased granuloma tissue formation in ethanolic extract and acetone fraction treated animals. Topical application of the ethanolic extract and the acetone fraction on excision wounds accelerated wound contraction and reduced epithelialization period in rats. Wound healing involves regeneration of specialized cells by proliferation of surviving cells and connective tissue response characterized by the formation of granulation tissue [24]. It is also characterized by haemostasis, reepithelialization and remodeling of the extracellular matrix. Epithelialization, which is the process of epithelial renewal after injury, involves the proliferation and migration of epithelial cells towards the centre of the wound while wound contraction is largely due to the action of myofibroblasts [25, 26]. Thus, the effect of ethanolic extract and the acetone fraction on wound contraction and epithelialization suggest it may enhance epithelial cells migration and proliferation, as well as the formation, migration and action of myofibroblasts. On chronic oral administration, ethanolic extract and the acetone fraction enhanced the granuloma tissue formation in dead space wounds. Granuloma tissue formed on an inert foreign body in a dead space comprises an accumulation of modified macrophages [24], histological giant cells and undifferentiated connective tissue consisting largely of collagen [24, 26, 27]. Increase in granuloma tissue in dead space wound is associated with enhanced collagen maturation and increased protein content as well as angiogenesis [28-30]. in the wound. These processes are indicators of new tissues generation and suggest that ethanolic extract and the acetone fraction may stimulate mechanisms associated with tissue regeneration. Closely related to this is the effect of growth factors secreted by macrophages on wounds. Macrophages secrete peptide growth factors that exert pro-healing effect by stimulating regeneration, fibroblast proliferation and activation and angiogenesis [24]. It is, therefore, likely that in addition to enhancing wound contraction and epithelialization, the ethanolic extract and the acetone fraction may also stimulate processes associated with tissue regeneration.

Ethanolic extract and the acetone fraction significantly increased the skin breaking strength and hydroxyproline content which was a reflection of increased collagen levels by increased cross linking of collagen fibres. In addition, increase in dry granulation tissue weight indicated the presence of higher protein content [31]. The breakdown of collagen liberates free hydroxyproline and its peptides and elevated level of hydroxyproline is the index of increased collagen turnover.

From the above studies it is quite apparent that the ethanolic extract and its acetone fraction of *Butea monosperma* stem bark possesses significant wound healing activity, which was evident by the increased rate of wound contraction, reduction in the period of epithelialization, increase in collagen deposition, breaking strength and hydroxyproline in granulation tissue.

Conclusion

The ethanolic extract of *Butea monosperma* stem bark and its acetone fraction showed wound healing property. We propose that the additive and synergistic activity of phytochemicals such as flavonoids, steroids, tannins, phenolic compounds and alkaloids present in the ethanolic extract and the acetone fraction of *Butea monosperma* were responsible for its potent wound healing property. The present investigation offers scientific evidence to the folkloric accounts of the use of stem bark in treating cuts and wounds.

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