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Wound Healing and *In Vitro* Antioxidant Activities of *Croton bonplandianum* Leaf Extract in Rats

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Abstract: *Croton bonplandianum* has been credited with potential to cure liver diseases, cure against ring worms and skin diseases. Leaves of *Croton bonplnadianum* are highly medicinal and used for controlling B.P and for the treatment of skin diseases, cuts and wounds. Phytochemically the plant has been reported to contain Rutin ($C_{18}H_{36}O_{19}$) as main constituent together with crotsparinine, crotosparine and its methyl derivatives aphorbol which play a key role in wound healing. The present study was carried out to investigate the effect of *Croton bonplandianum* leaves on experimental wounds and *invitro* antioxidant activities like effect on DPPH and Nitric oxide. Ethanolic and aqueous extract of shade dried leaves of *Croton bonplandianum* extract is formulated as 10% ointment and topically applied to experimental wounds in rats. The plant showed a definite, positive effect on wound healing, with significant increase in wound contraction. The efficacy of this plant in wound healing may be due to its chemical constituent rutin and antioxidant enzymes, thereby justifying the traditional claim.

Key words: *Croton bonplandianum* · Rutin · Wound healing · DPPH and Nitric oxide

INTRODUCTION

Herbal and natural products of folk medicine have been used for centuries in every culture through out the world [1]. Data from WHO show that 70-80% of the world's population use herbal medicines as alternative medicine [2]. Medicinal plants owe curative value to hoard of chemical substances present in various plant tissues. Plants at the same time also synthesize toxic chemicals apparently as primary defense against pathogens and predators. There is considerable evidence indicating that many of plant products are toxic to human. Hence every herbal product needs through testing [3]. Euphorbiaceae family in the plant kingdom is a complex heterogeneous family consisting of about 322 genera & 8900 species in the world. In India, this family is represented by 73 genera &410 species. Here is a plant of Euphorbiaceae family, sub family crotonoideae. *Croton bonplandianum* was selected to investigate its antioxidant and wound healing property [4]. *Croton bonplandianum*, commonly known as three –leaved caper (English), ban tulasi, jungle tulasi (Bengali), kalabhangre (Hindi), eliamanakkau (Tamil), kukka mirapa (Telugu),alpabedhi soppu (Kannada). This plant is about 60 cm high perineal herbs and can be found in waste lands and road side areas. Following and fruiting time of this plant is September to December [5].The plant has been credited with potential to cure liver diseases and swelling of the body, cure against ring worms and skin diseases [6]. Bark and roots are alternative and chologogue [7, 8]. Leaves are simple ovate –lanceolate serrate with two glands at the base. Leaves of this plant are highly medicinal and used for controlling B.P & for the treatment of skin diseases and cut & wounds and it is antiseptic and antidote. The pharmacological properties of leaf extracts have been evaluated for antioxidant and wound healing properties. The effect of leaf residue of *Croton bonplandianum* was studied on growth and metabolism of parthenium [9-11]. The part which has medicinal value is seed and seed oil. The seeds are used for the treatment of jaundice, acute constipation abdominal dropsy and internal abscesses [5]. The seed of *Croton bonplandianum* contains diterpines, phorbol ester, including 12-orthotrideconeoly-phorbol-13-acetate (TPA) and myristoyl phorbol acetate (MPA). TPA is a

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carcinogen, affecting prostaglandin metabolism [12, 13]. The fresh juice of the plant is used against head ache by ethnic groups. Latex of plants has healing effect on wounds and cuts [7, 8]. The latex of the plant (1:5 v/v in 50% acetone) showed anti fungal activities by causing absolute inhibition against two ring worms fungi, viz- microsporium gypsum and trichophyron mentagrophyt [14]. Croton is rich in secondary metabolites including alkaloids and terpinoids, the latter include irritant co-carsinogenic phorbol esters [15, 16]. The degree of presence or absence of various phytochemicals depends on the type of organic solvent employed for the phytochemical screening of *Croton bonplandianum*. Organic solvents usually employed are petroleum ether, chloroform, acetone and methyl alcohol. Compared to all other extracts, methanol extracts has higher number of 20 metabolites with higher contents. Phytochemically the plant has been reported to contain rutin ($C_{18}H_{36}O_{19}$) as main constituent, crotsearinine, crotasparine and its methyl derivatives aphorbol [17-19].

MATERIALS AND METHODS

Plant Material: The leaves of *Croton bonplandianum* were collected from the Regional Forest Research Centre (RFRC), Rajahmundry and it was authenticated by RFRC and Pharmacognosy Department of GIET.

Animals: Healthy young Wistar Albino rats of either sex weighing about (150-200g) obtained from animal house of GIET School of Pharmacy. They were maintained under standard laboratory conditions of food and water and kept for quarantine for 24 hours.

Preparation of Extract: After the collection of leaves they were placed in a clean tray and allowed for shade drying under sunlight. The dried leaves are powered coarsely and stored in airtight containers in order to avoid contact with moisture &air. The powdered leaves were taken & weighed; they were packed in to a muslin bag, in soxhlet extractor, using alcohol as solvent. The aqueous extract was obtained by cold maceration method. The extracts were carried out for 4 days. The extract was concentrated, collected & stored in the refrigerator & it was used for pharmacological investigation.

Experimental Protocol: The herbal extracts (10% ointment) were administered topically in the form of ointments. Albino rats of either sex were distributed into 4 groups consisting of 6 rats per group, Group-1: Control rats, treated by plain ointment base for 4 weeks daily, Group-2: Standard rats were treated by tocopheral ointment for 4 weeks daily, Group-3: Rats were treated with 10% aqueous extract of *Croton bonplandianum* as ointment.(0.5g) for 4 weeks daily, Group-4: Rats were treated with 10% alcoholic extract (0.5g) of *Croton bonplandianum* for 4 weeks daily.

Excision Wound Healing Model: In excision wound healing model, the rats inflicted with excision wounds under light ether anesthesia. A circular wound of about 250 mm^2 was made on depilated ethanol sterilized dorsal thoracic region of rats. The group 1 was considered as control, group 2 as reference standard (10% tocopherol ointment) and groups 3&4 animals treated with 10% w/w ointment prepared from aqueous and alcoholic extracts of *Croton bonplandianum* respectively. The ointment was applied once a day, till the complete epithelization starting from the day of operation. The parameter studied was wound closure. The wounds were traced on mm^2 graph paper on (7^{th}, 14^{th}, 21^{st}, &28^{th} days), there after daily until healing was complete. The% wound closure was calculated.

In vitro Antioxidant Studies

Assay for Nitric Oxide Scavenging Activity: NO was generated as a result of decomposition of sodium nitroprusside in aqueous medium, interacts with oxygen at physiological pH to produce nitrite ions, which are measured by using Griess reaction [20, 21]. The absorbance of the chromophore formed during the diazotization of nitrite with sulphanilamide and subsequent coupling with Naphthyl ethylenediamine was read at 546 nm and referred to the absorbance of standard solution of potassium nitrite, treated same way with Griess reagent.

The reaction mixture (3ml) containing sodium nitroprusside (10μM) in phosphate buffer and various concentrations of drug i.e., 10, 20, 40, 80, 100 μg/ml of ethanolic extract of *Croton bonplandianum* or ascorbic acid were incubated at 25° C for 120 minutes. Control without test compound kept in an identical manner. After incubation 0.5 ml of the incubation solution was removed and diluted with 0.5 ml of the incubation solution was removed and diluted with 0.5 ml of Griess reagent (1% w/v sulphanilamide, 2% w/v H_3Po_4 and 0.1% w/v Naphthyl ethylene diamine dihydrochloride). The absorbance of the chromophore was read at 546 nm. The percentage inhibition of nitric oxide generation was measured by comparing the absorbance values of control and those of test compounds.

$$\text{NO scavenged (\%)} = \frac{\text{A control- A test}}{\text{A control}} \times 100$$

Diphenly-2-picrylhydrazyl(DPPH) scavenging effect: Diphenyl-2-picrylhydrazyl (DPPH) forms a stable molecule on accepting an electron or a hydrogen atom and thus has applications in the determination of radical scavenging activity of natural products [22]. Antioxidant reacts with DPPH and converts it into 1, 1-diphenyl-2-picrylhydrazine free radical. The degree of decolourisation indicates the scavenging potential of the antioxidant drug [23].

0.1 mM solution of DPPH in ethanol was prepared and 1ml of this solution was added to 3 ml of extract solution in ethanol at different concentrations i.e. 10, 20, 40, 80, 100 µg/ml. After 30 min absorbance was measured at 517 nm. Lower absorbance of the reaction mixture indicates higher free radical scavenging activity. The capability to scavenge the DPPH radical was calculated using following formula.

$$\text{DPPH scavenged (\%)} = \frac{\text{Acontrol- Atest}}{\text{Acontrol}} \times 100$$

***In vitro* Antioxidant Activity:** The antioxidant activity of the extract was expressed as IC_{50}. The IC_{50} value was defined as the concentration (µg/ml) of extracts that inhibits the formation of free radicals by 50%.

Statistical Analysis: Results were expressed as ± SEM: differences in mean values are estimated by the use of ANNOVA followed by Dunnett's post hoc test. The minimum level of significance was set up at $P<0.05$.

RESULTS

Using Aqueous Extract of *Croton bonplandianum*: The results of studies conducted with standard antioxidant. Tocopherol and aqueous extract of croton bonplandianum applied as 10% ointment (0.5g of the formulated ointment was applied on wound). It was observed for 28 days. The percentage of wound contraction was observed 29.2% in 14 days and 63.1 in 21 days and 89.2 in 28 days respectively (Table 1).

Using Alcoholic Extract of *Croton bonplandianum*: The results of studies were conducted with standard Tocopherol ointment and 10%w/w ointment (0.5g of the formulated ointment was applied on wound).It was observed for 28 days. The percentage of wound contraction was observed 36.6% in 14 days and 66.2 in 21 days and 91.6% in 28 days respectively (Table 1). Finally it was referred that alcoholic extract of *Croton bonplandianum* has more% of wound contraction, compared to aqueous extract of *Croton bonplandianum*.

The free radical scavenging activity of ethanolic extract of *Croton bonplandianum* was measured *in vitro* by:

- 1,1-di phenyl-2-picryl hydrazyl (DPPH) [23].
- Nictic oxide scavenging (NO)

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The NO generated from sodium nitroprusside in aqueous solution at physiological pH interacts with oxygen to produce nitrite ions, which was measured by Griess reagent. Scavenges of NO compete with oxygen leading to a reduced product of NO (20). The results of the present study postulate that ethanolic extract of *Croton bonplandianum* has ability to scavenge NO by the above mentioned mechanism.

Table I: Effect of *Croton bonplandianum* Leaf extract On the Excision wound healing mode

		% Wound Contraction			
Groups	Drug	7th Day	14th Day	21st Day	28th Day
1.	Control	10.6±0.26	24.9±0.13	36±0.05	42.3±0.03
2.	Standard	19.5±0.28	36.5±0.14	65.4±0.08***	87.1±0.06***
3.	10% Aqueous Extract as Ointment (0.5 g)	15.7±0.36	29.2± 0.18*	63.1±0.14***	89.2±0.05***
4.	10% Alcoholic Extract (0.5 g)	20.8±0.32	36.6 ±0.16**	66.2±0.1***	91.6±0.02***

Values are expressed as±SEM of six animals in each group. P* > 0.05- significant, P** < 0.01-moderately significant and P*** < 0.001- highly significant

DISCUSSION

Wounds are common clinical entities in day to day life, which may be major or minor.The process of wound healing can be classified into five phases, cellular phase (collagenation), narrowing of wound area (wound contraction), collagen deposition (collogenation), epithelial covering (epithelialisation), scar remodling (cicatrixisation).

These are concurrent but in dependent on each other. Any reagent which accelerates the process is a promoter of wound healing. Wound healing is a process by which damaged tissue is restored as closely as possible to its normal state and wound contraction is the process of shrinkage of area of the wound. It depends upon the reparative abilities of the tissue, type & extent of the damage and general state of the health of the tissue. The granulation tissue of the wound is primarily composed of fibroblast, collagen, edema &small new blood vessels. The undifferentiated mesenchymal cells of the wound margin modulate themselves into the wound gap along with the fibrin stands. The collagen is the major component of extra cellular tissue, which gives support &strength &is composed of amino acid (hydroxyproline). In our study the alcohol leaf extract of *C.bonplandianum* significantly increased the rate of wound contraction, according to Ramachandran *et al.,* it was concluded that herbal extract ointments using Croton bonplandianum and Vitex negundo leaf extracts increased the significant rate of wound contraction [24]. The constituent of the *C.bonplandianum* include several phenolic compounds, primarily the flavonoids conformed by the qualitative chemical tests. Recent studies shown that phytochemical constituents like flavonoids & other phenolics have been reported to have multiple biological effects such as antioxidant activity, anti-inflammatory action, inhibition of platelet aggregation and antimicrobial activities [25]. Antioxidant property of the extract is confirmed by DPPH and Nitic oxide scavenging activity. A significant reduction in the period of epithelization was observed when compared to control. From this study it was concluded that ethanolic extract of *Croton bonplandianum* has wound healing properties and the presence of flavonoids indicates that it has antioxidant activity, by correlating these two properties that became responsible for the healing of the wound in 28 days of the study when compared to control and standard group of animals.

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