Anti-hyperbilirubinemic and wound healing activity of aqueous extract of *Calotropis procera* leaves in Wistar rats

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**ABSTRACT**

**Aims:** The aim of this study was to evaluate the bilirubin lowering and wound healing property of aqueous extract of *Calotropis procera* (AECP) leaves in Wistar rats.

**Materials and Methods:** Albino Wistar rats of either sex were used for the study. Bilirubin lowering property of *C. procera* leaves was evaluated using phenylhydrazine and paracetamol as inducing agents followed by measuring the concentration of serum total bilirubin in hyperbilirubinemic rats. Wound healing property was evaluated using incision and excision models by measuring tensile breaking strength, percentage wound contractions, and epithelization days, respectively.

**Statistical Analysis:** Statistical comparison between groups in each experiment was done with one-way analysis of variance followed by Dunnett’s test.

**Results:** AECP showed a significant (*P* < 0.05) decrease in concentrations of serum total bilirubin in hyperbilirubinemic rats as well as significant (*P* < 0.05) increase in breaking strength and percentage wound contractions with decreased epithelization period when compared to control groups.

**Conclusions:** AECP showed significant bilirubin lowering and wound healing property in Wistar rats.

**KEY WORDS:** *Calotropis procera*, excision, hyperbilirubinemia, incision, paracetamol, phenylhydrazine, wound healing

**Introduction**

The human race started using plants or plant products successfully as a mean of treatment of disease and injuries as effective therapeutic tools from early days of civilization to the modern age. *Calotropis procera* Linn (Family: Asclepiadaceae) is an ayurvedic plant with important medicinal properties. It is known by various vernacular names such as Swallow-Wort (English), Madar (Hindi), Alarka (Sanskrit), and commonly referred to as Ark, Swallow wart or milkweed. It occurs frequently in Indonesia, Malaysia, China, India as wasteland weed and also found in most parts of the world with a warm climate in dry, sandy, and alkaline soils. *C. procera* Linn is an erect, tall large, highly branched, and perennial shrub or small tree that grows to a height of 5.4 m with milky latex throughout the plant.

The phytochemistry of plant reveals presence of triterpenoids, flavonoids, cardiac glycosides, cardenolides, anthocyanins, α-amyrin, β-amyrin, lupeol, β-sitosterol, flavanols, madurane, resins, a powerful bacteriolytic enzyme calactin, a nontoxic proteolytic enzyme calotropin, and a wax. The parts of plants used in ayurvedic medicine are leaves, fresh or dried, the roots, root bark, and flowers. The powdered leaves are useful for fast healing of wounds, as purgative, to treat liver problems, to relieve stomach ache, headache, also applied in sprain to ease swelling and pain.

Traditionally, the plant has been used as anti-fungal, antipyretic, analgesic. The dried leaves are used as an expectorant, anti-inflammatory, for the treatment of paralysis.
and rheumatic pain. The dried latex and roots are used as an antidote for snake poisoning. It is also used as an abortifacient for the treatment of piles and intestinal worms. The tender leaves are also used to treat migraine. [18]

Therefore, by taking into limelight the traditional uses [19] of Calotropis procera, the present study was performed to provide a pharmacological base for use of plant in treatment of hyperbilirubinemia induced by phenylhydrazine (PHZ) and paracetamol [20, 21] and wound healing [22, 23].

**Materials and Methods**

**Animals**

Adult Wistar rats of either sex weighing about 180–250 g were purchased from Bharat serum and vaccines, Thane. The animals were housed in standard laboratory conditions in groups of three at 25°C ± 2°C, humidity 60% ± 2%, and 12 h light: Dark cycle. Animals had a free access to standard laboratory food purchased from Amrut rat and mice feed, Nashik, India and water. The animals were acclimatized to the laboratory conditions 1 week prior to the experimentation. All experiments were performed during a light portion of 12–12 h. The protocol was approved from Institutional Animal Ethics Committee of MGV’S Pharmacy College, Nashik (MGV/PC/XXVIII/01/2013-14).

**Drugs and Chemicals**

Phenylhydrazine (Sigma-Aldrich, USA), paracetamol (Merck), Silymarin (Silibyn, Micro labs), Dexamethasone (DXM) injection (Life care pharmaceuticals), ketamine HCl (Anket, Neon labs), Total Bilirubin kit, alanine transaminase (ALT), and aspartate transaminase (AST) (Coral Clinical Systems, Mumbai) were used in present study. Drug solutions were prepared fresh in distilled water and stored in a refrigerator at 4°C.

The doses prepared were based upon the previous studies and shown to be pharmacologically active [10-12].

**Preparation of Plant Extract**

Leaves of *C. procera* were collected from Tapovan garden, Panchvati, Nashik. The leaves were authenticated by Dr. (Mrs.) A. G. Bhaskarwar, Ayurvedic Seva Sangh, Panchvati, Nashik. The collected leaves were shade dried and powdered using a grinder. The aqueous extract of *C. procera* (AECP) was prepared by boiling the powdered leaf matter with 16 times of its weight in distilled water and reducing its volume up to 1/32 times. The extract obtained was stored in refrigerator till used.

**Phenylhydrazine Induced Hyperbilirubinemia**

The animals were treated with PHZ (5 mg/kg i.p.) for 5 days to develop jaundice following standard procedure [24] with slight modification. LD₅₀ (993 mg/kg) was found to be reported in rats [25]. Hence, the doses selected for study were 25 mg/kg and 50 mg/kg.

The animals were randomly distributed into five groups (n = 5). Group I received vehicle (distilled water, 5 ml/kg, p.o.), Group II received PHZ (5 mg/kg, i.p.), Group III received PHZ (5 mg/kg, i.p.) and Silymarin suspension (100 mg/kg, p.o.), Group IV received PHZ (5 mg/kg, i.p.) and AECP (25 mg/kg, p.o.), and Group V received PHZ (5 mg/kg, i.p.) and AECP (50 mg/kg, p.o.). The concentration of serum total bilirubin was determined by Mod. Jendrassik and Grof’s method [26].

**Paracetamol Induced Hyperbilirubinemia**

The animals were treated with paracetamol (2 g/kg p.o.) for 5 days to develop jaundice following standard procedure [27] with slight modification. The animals were randomly distributed into five groups (n = 5). Group I received vehicle (distilled water, 5 ml/kg, p.o.), Group II received paracetamol (2 g/kg p.o.), Group III received paracetamol (2 g/kg p.o) and Silymarin suspension (100 mg/kg p.o.), Group IV received paracetamol (2 g/kg p.o) and AECP (25 mg/kg, p.o.), and Group V received paracetamol (2 g/kg p.o) and AECP (50 mg/kg, p.o.). The concentration of serum total bilirubin was determined by Mod. Jendrassik and Grof’s method, ALT and AST by Reitman and Frankel’s method [28] on day 1 and day 5 after 6 h of administration of PHZ to confirm jaundiced condition of animals. The treatment of jaundiced groups with Silymarin (100 mg/kg, p.o.) and AECP (25 and 50 mg/kg, p.o.) was started on day 6 and continued up to day 10. Blood was collected from tail vein of rats on day 1 (normal), day 5 (after 6 h of PHZ administration), and on day 10 to determine serum concentration of total bilirubin and Hb level.

**Excision Wound Model**

Animals were randomly distributed into four groups (n = 5) as follows: Group I received vehicle (distilled water, 5 ml/kg, p.o.), Group II received DXM (0.34 mg/kg i.m. on 1st day and 0.17 mg/kg i.m. on alternative days for 7 days), Group III received AECP (25 mg/kg, p.o.), Group IV received AECP (50 mg/kg, p.o.). All procedures were carried out under ketamine anesthesia (60 mg/kg i.p.) [29]. On the debrided backs of Wistar rats, two paravertebral incisions of 2.5 cm were made cutting through the full thickness of the skin. Interrupted sutures, 1 cm apart were placed to approximate the cut edges of skin by ethilon 4-0. The sutures were removed after 7 days and skin breaking tensile strength was measured on day 10 by continuous water flow technique of Lee [30].

**Statistical Analysis**

All data expressed as mean ± standard error of mean (SEM) of value for corresponding parameters. Statistical comparison
Results

Phenylhydrazine Induced Hyperbilirubinemia

Serum total bilirubin level

Animals treated with PHZ (5 mg/kg i.p.) showed significant ($P < 0.05$) increase in serum bilirubin level on day 5 compared to day 1 and significant ($P < 0.05$) decrease in serum bilirubin on day 10 compared to day 5. Animals showed significant ($P < 0.05$) increase in the level of serum total bilirubin compared to vehicle treated group. Hyperbilirubinemic rats treated with Silymarin (100 mg/kg p.o) and AECP (25 and 50 mg/kg p.o.) showed significant ($P < 0.05$) decrease in levels of serum total bilirubin as compared to PHZ treated group on day 10 of study [Table 1].

Hemoglobin

Animals treated with PHZ (5 mg/kg i.p.) showed significant ($P < 0.05$) decrease in Hb level on day 5 compared to day 1 and significant ($P < 0.05$) increase in Hb level on day 10 compared to day 5. Animals showed significant ($P < 0.05$) decrease in levels of Hb compared to vehicle treated group. Hyperbilirubinemic rats treated with Silymarin (100 mg/kg p.o) and AECP (25 and 50 mg/kg p.o.) showed significant ($P < 0.05$) increase in levels of Hb as compared to the PHZ treated group on day 10 of study [Table 1].

Paracetamol Induced Hyperbilirubinemia

Serum total bilirubin level

Animals treated with paracetamol (2 g/kg p.o.) showed significant ($P < 0.05$) increase in serum bilirubin level on day 5 compared to day 1 and significant ($P < 0.05$) decrease in serum bilirubin on day 10 compared to day 5. Animals showed significant ($P < 0.05$) increase in the level of total bilirubin compared to vehicle treated group. Hyperbilirubinemic rats treated with Silymarin (100 mg/kg p.o) and AECP (25 and 50 mg/kg p.o.) showed significant ($P < 0.05$) decrease in levels of serum total bilirubin as compared to paracetamol treated group on day 10 of study [Figure 1].

Serum alanine transaminase and aspartate transaminase level

Animals treated with paracetamol (2 g/kg p.o.) showed significant ($P < 0.05$) increase in serum level of ALT and AST on day 5 compared to day 1 and significant ($P < 0.05$) decrease in serum level of ALT and AST on day 10 compared to day 5. Animals showed significant ($P < 0.05$) increase in serum levels of ALT and AST compared to vehicle treated group. Hyperbilirubinemic rats treated with Silymarin (100 mg/kg p.o) and AECP (25 and 50 mg/kg p.o.) showed significant ($P < 0.05$) decrease in serum levels of ALT and AST compared to the paracetamol treated group on day 10 of study [Figures 2 and 3].

Wound Healing Property

Incision model

Animals treated with DXM (0.34 mg/kg i.m. on first day and 0.17 mg/kg i.m. on alternative days for 7 days) and AECP (25 and 50 mg/kg p.o.) showed significant ($P < 0.05$) increase in tensile breaking strength of sutured skin compared to control group [Table 2].

Excision model

Percentage wound healing and epithelization days: Animals treated with DXM (0.34 mg/kg i.m. on 1st day and 0.17 mg/kg i.m. on alternative days till epithelization) and AECP (25 and 50 mg/kg p.o.) showed significant ($P < 0.05$) increase in wound contractions [Figure 4] and decrease in epithelization period as compared to control group [Table 2].

Discussion

In order to establish a scientific basis for utilization of *C. procera* in the treatment of hyperbilirubinemia, it was decided to evaluate the bilirubin lowering activity in PHZ and paracetamol-induced hyperbilirubinemic rats. Earlier reports revealed that PHZ induced hyperbilirubinemic rats showed marked increase in serum total bilirubin levels,$^{[21]}$ the reason for which would be excess hemolysis of the RBC’s leading to over production of the bilirubin causing hyperbilirubinemia. Paracetamol treated rats showed marked increase in serum total bilirubin, ALT and AST levels,$^{[22]}$ the mechanism of which is acute hepatocyte necrosis due to formation of N-acetyl-p-benzoquinoneimine (NAPQI) and saturation of sulfate and glucuronide pathways of paracetamol metabolism.$^{[23]}$

Silymarin a unique flavonoid complex is a substance with documented hepatoprotective property by its cell membrane stabilizing property.$^{[24]}$ Similarly, the number of flavonoid components, flavonolignans were found to be present in *C. procera* leaves.$^{[25]}$ Hence, the bilirubin lowering activity of

Table 1:

| Treatment (mg/kg) | Day 1 (mg/dL) | Day 5 (mg/dL) | Day 10 (mg/dL) | Serum total bilirubin (mg/dL) |
|------------------|--------------|--------------|---------------|----------------------------|
| D.W. (5 mL/kg)   | 10.8±0.52    | 10.83±0.57   | 10.4±0.43     | 1.08±0.08                  |
| PHZ (5)          | 11.1±0.65    | 6.3±0.45     | 6.56±0.26     | 0.95±0.02                  |
| Silymarin (100)  | 11.97±0.21   | 7.03±0.06    | 7.33±0.23     | 1.10±0.08                  |
| AECP (25)        | 12.53±0.35   | 8.03±0.33    | 8.2±0.05      | 1.0±0.05                   |
| AECP (50)        | 11.53±0.5    | 7.86±0.75    | 8.46±0.23     | 1.02±0.07                  |

*NS. All data subjected to one-way ANOVA followed by Dunnett’s test. $^{[21]}$P<0.05 considered significant as compared to control group. $^{[22]}$P<0.05 considered significant as compared to PHZ group. $^{[23]}$P<0.05 considered significant as compared to day 1, $^{[24]}$P<0.05 considered significant as compared to day 5. D.W.=Distilled water, PHZ=Phenyhydrazine, AECP=Aqueous extract of *Calotropis procera*, Hb=Hemoglobin, ANOVA=Analysis of variance.
AECP was studied along with Silymarin and comparing both with jaundiced groups.

In the present study, results of both hyperbilirubinemic model viz., PHZ and paracetamol, revealed a significant ($P < 0.05$) decrease in the serum total bilirubin levels in PHZ and paracetamol treated animals with increase in Hb in PHZ treated animals and decrease in Serum ALT and AST levels when compared with vehicle-treated group and also results were observed to be similarly effective as that of Silymarin treated groups.

Dexamethasone a glucocorticoid possesses, a marked anti-inflammatory property along with its capacity to stimulate connective tissue growth factor (CTGF) expressions in normal tissues and organs causing fibroblast proliferation and extracellular matrix deposition\cite{26} which may serve as a basis for wound healing.

| Treatment    | Incision wound model | Excision wound model |
|--------------|----------------------|----------------------|
| (mg/kg)      | (g)                  | (days)               |
| D.W. (5 mL/kg) | 395.7±13.33          | 16±1.23              |
| DXM (5)      | 512.3±8.82*          | 11±1.58*             |
| AEC (25)     | 619±5.77*            | 11±0.92*             |
| AEC (50)     | 709±52.92*           | 11±1.12*             |

$n=5$. All data subjected to one-way ANOVA followed by Dunnett’s test. \(P<0.05\) considered significant as compared to control group. D.W.=Distilled water, DXM=Dexamethasone, AECP=Aqueous extract of Calotropis procera, ANOVA=Analysis of variance.
for its use as wound healing agent for a preclinical study. Wound healing property of \textit{C. procera} was studied using two different models viz., incision and excision wound model. The results of incision wound showed a significant increase in breaking strength of sutured skin. In excision study, the animals treated with AECP showed a significant increase in wound contraction, increased percentage wound healing with a decrease in epithelization period. This result was in agreement with that of a previous study by Shilpa et al. who reported that treatment with \textit{Crinum deflexum} possesses potent wound healing activity in excision and incision wound model.\textsuperscript{272}

Results of the present study revealed that AECP possess a marked bilirubin lowering property which resulted in decrease in serum concentration of total bilirubin in both models of hyperbilirubinemia and also possess a wound healing property which resulted in an increased tensile breaking strength of sutured skin, increased percentage of wound healing with decreased epithelization period in incision and excision wound model, respectively.

The above obtained results may be possibly due to presence of various phytochemicals which play role as antioxidants thereby stabilizing the cellular membrane of hepatocytes exhibiting bilirubin lowering property, as well as chemical moieties which cause increase in fibrinogenesis leading to wound healing property.

Hence, the further detailed scientific study of \textit{C. procera} such as screening, isolation, and characterization along with anti-oxidant and histopathological study can discover the particular chemical moiety with its possible mode of action for above-obtained results.

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Conflicts of Interest

There are no conflicts of interest.

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