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ORIGINAL ARTICLE

Antihypertensive activity of flowering twigs of *Calotropis procera* (Ait.) is predominantly mediated through vasorelaxant pathway

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ABSTRACT

Objective: To evaluate the crude methanolic (Cp.Cr) and aqueous (Cp.Aq) extracts of *Calotropis procera* Aiton for acute toxicity and antihypertensive effects, validating its ethnomedicinal use in cardiovascular disorders.

Methods: Phytochemical screening was conducted to determine the chemical constituents of C. *procera*. Acute toxicity was assessed in BALB/C mice using oral doses of 10, 100, 1000, 1500, and 2000 mg/kg. Vasorelaxant effects were evaluated using rabbit aortic strips pre-contracted with 1 μM norepinephrine and 80 mM potassium chloride (high K^+) to explore the mechanism of action. Antihypertensive activity was assessed in hypertensive Sprague-Dawley rats at doses of 1 and 10 mg/kg orally, using the tail-cuff method to measure systolic blood pressure.

Results: Phytochemical analysis revealed the presence of flavonoids, saponins, cardiac glycosides, and terpenoids. The extract was non-toxic up to 1500 mg/kg, while a 75% mortality rate was observed at 2000 mg/kg. Both Cp.Cr and Cp.Aq induced dose-dependent relaxation in K^+ and norepinephrine-induced contractions. The EC_{50} for Cp.Aq was 0.15 mg/ml for KCl-induced and 0.13 mg/ml for NE-induced contractions. A significant reduction in systolic blood pressure ($p<0.05$) was observed, particularly with the aqueous fraction of C. *procera*.

Conclusion: The flowering twigs of C. *procera* exhibit significant antihypertensive effects, likely mediated through vasorelaxation via inhibition of voltage-gated calcium channels. These findings support its potential use in hypertension management and provide evidence of its safety and pharmacological efficacy.

Keywords: Calotropis (MeSH); Antihypertensive Agent (MeSH); Hypertension (MeSH); Toxicity Tests, Acute (MeSH); Vasodilation (MeSH).

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INTRODUCTION

Calotropis procera is an evergreen perennial shrub belonging to the family Apocynaceae. It typically thrives in dry and xerophytic conditions. The plant exudes a white gluey latex from its stem or leaves upon incision or cuts to the aerial parts. Its flowers are fleshy and range in color from white to pinkish, clustered at the ends of twigs (Figure 1).[1] C. *procera* is native to Africa but is also widely distributed in Asia, where it is commonly known as "Calotropis," "dead sea fruit," and "giant milkweed." In Pakistan, it is referred to as "Aak".[2] Traditional uses of C. *procera* include remedies for rheumatism, eczema, jaundice, fever, cold, and diarrhoea, with various parts of the plant being used medicinally. The roots are known to treat ailments such as leprosy, cough, asthma, diarrhoea, eczema, and dysentery, while the latex alleviates joint pain and swelling. Additionally, the flowers are employed in managing asthma, loss of appetite, stomach problems, and cough, and they serve as a general tonic. The root bark is used to address intestinal worms, skin disorders, and ascites.[1] Phytochemical studies reveal that C. *procera* contains numerous bioactive compounds, including alkaloids, steroids, terpenoids, tannins, phenols, flavonoids, and sugars.[4] The flowers of C. *procera* have demonstrated notable anthelmintic properties in sheep,[5] and their pharmacological activities include analgesic, antipyretic, anti-inflammatory, and antimicrobial effects.[6] Hepatoprotective properties of the flowers have also been reported.[7] Furthermore, the latex exhibits antifungal, antibacterial, and antioxidant activities.[8] The roots possess antifertility activity in female rats.[9] Despite extensive documentation of these pharmacological properties, the potential antihypertensive effects of C. *procera* remain underexplored.

Hypertension is a prevalent global health challenge, contributing significantly to cardiovascular morbidity and mortality. Although traditional knowledge associates C. *procera* with antihypertensive benefits,[10] comprehensive scientific validation of this effect, particularly for flowering twigs, is lacking. Existing studies predominantly focus on the plant's antimicrobial, anti-inflammatory, and hepatoprotective properties, leaving a critical gap in understanding its cardiovascular effects. Addressing this gap is essential to harnessing its potential for therapeutic applications in managing hypertension.

This study aims to investigate the antihypertensive activity of the flowering twigs of C. *procera* (Ait.) through in vivo screening. Additionally, acute toxicity assessments will be conducted to establish a safe dosage range. All experiments were conducted in the Pharmacology Laboratory of Institute of Basic Medical Sciences,

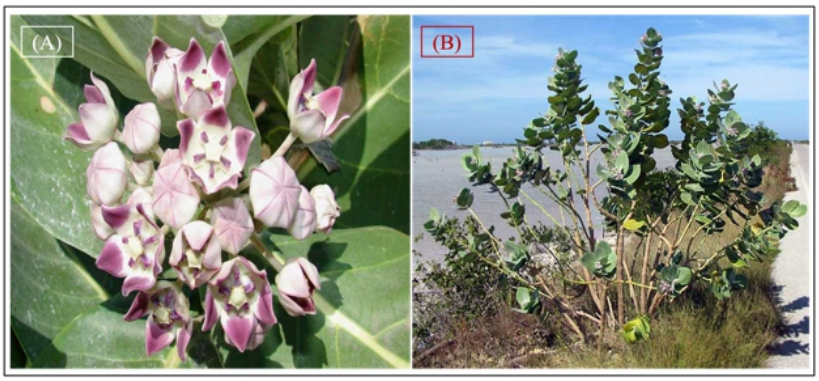

Figure 1: Flowering twigs **(A)** and aerial parts of C. procera **(B)**.

Khyber Medical University. Animals were housed in the animal facility of Khyber Medical University, and in vivo experiments were performed within the laboratory to minimize disturbances.

METHODS

Plant materials and preparation of extract: Fresh flowering twigs were collected from Bannu region of Khyber Pakhtunkhwa, Pakistan. Professor Hashim Khan, Department of Botany, Government Post Graduate College Bannu identified the plant specimen. The flowering twigs were air-dried in the shade at room temperature for 25 days. Once dried, 2.0 kg of the plant material was finely ground using a standard grinder, yielding 1.8 kg of powdered material. This powdered sample was then macerated with 80% hydro-methanol at room temperature for seven days. Following the maceration process, the mixture was filtered using standard-grade filter paper. The resulting filtrates were concentrated under reduced pressure using a rotary evaporator, producing 150 g of a semi-solid crude methanolic extract (designated as Cp.Cr). The solubility of the extract was subsequently tested in normal saline and distilled water.

Phytochemical analysis and isolation of abundant phytochemicals: Standardized protocols were employed to identify various classes of phytochemicals including alkaloids, terpenes, sterols, flavonoids, saponins, cardiac glycosides, and tannins. For fractionation of abundant phytochemicals, a series of organic solvents were employed. First, 150g of Cp.Cr was suspended in distilled water. Then the dissolved materials along with water were successively fractionated in a higher order of polar solvents, yielding *n*-hexane, ethyl acetate, chloroform, *n*-butanol, and residual aqueous portions respectively.

Drugs and standards: Analytical-grade chemicals were used in experiments. Norepinephrine was bought from Merck, Germany. Acetylcholine was purchased from BDH, England. Acetylcholine and norepinephrine were purchased from England. Test solutions and suspensions were prepared on the same day of experiments in the pharmacology laboratory.

Experimental animals: BALB/C mice weighing between 25 and 40 g were procured from RAS Traders, Lahore. Male Sprague Dawley rats, weighing between 150 and 250 g, were also obtained from the same supplier. Additionally, rabbits were purchased from the local market in Peshawar, Khyber Pakhtunkhwa, Pakistan. All the animals were maintained in the animal house in their respective cages. Advanced Study & Research Board and Ethical Board of the Khyber Medical University approved the study protocols (Approval No. Dir/KMU-EB/AA/000419). The research was carried out according to the globally accepted guidelines for the use of laboratory animals and care as found in European Community guidelines (EEC Directive of 1986).

Data recording: Lab chart 7 was used for recording changes in isometric tension using a Force Transducer (Model No: MLT 0225 Pan Lab S.l) coupled with a bridge amplifier connected with 4 channels Power lab (Model No: 4/25T). Antihypertensive activity was recorded using a non-invasive blood pressure (NIBP) controller system supplied with 4 channels Power Lab (Model No: 4/25T).

Physiological solutions used in the experiments: Three types of Krebs solutions were used in the experiments. Normal Krebs solution contains (mM) NaCl 118.2, KCl 4.7, KH2PO4 1.3, MgSO4 1.2, NaHCO3 25.0, Glucose 11.7, and CaCl2 2.5. While the composition of K-Normal Calcium free Krebs solution was: K-Normal (Ca++ free) Krebs solution with ingredients in (mM): NaCl 118.2, KCl 4.7, KH2PO4 1.3, MgSO4 1.2, NaHCO3 25.0, Glucose 11.7, and ethylenediamine tetraacetic acid (EDTA) 0.1. The concentration of Krich (Calcium-free) Krebs solution was: (NaCl 50.58, KCl 50, KH2PO4 1.26, MgSO4 3.10, NaHCO3 23.8, Glucose 11.1, and EDTA) 0.1. Fresh solutions were used in the experiments.

Stock solutions used in the experiments: Stock solutions of C. *procera* were prepared at concentrations of 10, 100, 150, and 200 mg/ml for acute toxicity evaluation. For the assessment of vasorelaxation effects, stock solutions with concentrations of 3, 30, and 300 mg/ml were prepared and used in the experiments. Isolated aortae were maintained in Krebs normal solution for tissue studies. The observed vasorelaxant effects were further translated into in vivo experiments using rats. Additionally, stock solutions of 1 mg/ml and 10 mg/ml were prepared for antihypertensive activity and subsequent dosing.

Acute toxicity studies: Acute toxicity studies of Cp.Cr were conducted using BALB/C mice. The animals were divided into four groups, each consisting of four mice. One group was denoted as negative control group which was administered with normal saline (10 mL/kg), while three groups were denoted as treated groups which were given the extract at doses 10, 100 and 1000 mg/kg orally. Signs and symptoms of toxicity and mortality were observed over a 24-hour follow-up period,

following our previously reported methodology.[12] Further, the extract was checked in test doses 1500 and 2000 mg/kg for mortality and morbidity. The LD50 value was calculated according to our reported procedure.[12,13]

Determination of vasorelaxation effects: The test samples were evaluated for their potential vasorelaxation effects using rabbit aortic strip preparations. Briefly describing, rabbits were slaughtered. Their thoracic aorta was isolated and maintained in Krebs solution continuously aerated with carbogen gas. The aortae were carefully cleared of connective tissues where rings measuring 2-3 mm in thickness were prepared. These segments were maintained in Krebs solution with the following concentrations (in mM): NaCl 118.2, KCl 4.7, $MgSO_4$ 1.2, KH_2PO_4 1.3, Glucose 11.7, $NaHCO_3$ 25.0, $CaCl_2$ 2.5 with pH 7.4. The preparations were mounted in a 10 mL organ bath maintained at 37°C and continuously aerated with carbogen gas (95% oxygen and 5% carbon dioxide). One end of each aortic strip was attached to a stainless-steel triangle connected to a force-displacement transducer, while the other end was secured to the organ bath to stabilize the isolated tissue. An initial tension of 1 g was applied to the aortic strips and the preparations were allowed to stabilize for 1 hour. Force Transducer (Model No: MLT 0225 Pan Lab S.I) coupled with bridge amplifier connected with 4 channels Power lab (Model No: 4/25T) was used for measuring changes in isometric tension.[14]

The aorta was considered intact with functional endothelium if it exhibited relaxation greater than 80% of its control response upon the application of acetylcholine. Aortae were deemed denuded if they failed to show significant relaxation to acetylcholine following the denuding procedure.[15] Test concentrations of the samples (0.001, 0.01, 0.1, 1, and 10 mg/ml) were applied cumulatively at 2-minute intervals to KCl-induced and norepinephrine-induced contractions.

Effects of test samples on norepinephrine-induced contractions in aortae: We employed a similar experimental protocol as previously described. To investigate the effect of Cp.Cr on receptor-operated calcium channels, we assessed its ability to inhibit contractions induced by 1 μM norepinephrine in denuded aortic rings. The rationale behind this approach is that the suppression of norepinephrine-induced contractions in aortic tissue provides evidence for the blockade of calcium influx through these channels.[16,17] As endothelial cells are rich with α1 adrenergic receptors, activation of these receptors leads to vasocontraction through the influx of calcium through receptors-operated calcium channels. Therefore, in calcium-free Krebs solution, the NE (1 μM) induced contractions follow the stimulation of α1 adrenergic receptors that facilitate calcium release from internal stores.[17,18] It is important to note that vascular smooth muscle relaxation does not always correlate with the inhibition of voltage-gated calcium channels. Therefore, to confirm the involvement of voltage-gated calcium channels, we tested the Cp.Cr in similar concentrations of 0.001, 0.01, 0.1, 1, and 10mg/ml on 80 mM KCl-induced contractions.[16] Mean Effective Concentrations (EC_{50}) were recorded for test samples and amlodipine.[19,20] The experiments were run four times.

Responses were recorded using Lab Chart 7 which was supplied by Power Lab, AD Instruments Australia.

Antihypertensive activity: As preliminary isolated studies in aortae showed vasorelaxation on contractions induced by 80 mM KCl and on 1 μM Norepinephrine induced contractions, we sought to further investigate its potential via in vivo effects by assessing its impact on the systolic blood pressure of the experimental animals. Non-invasive blood pressure (NIBP) controller system supplied with 4 channels Power Lab (Model No: 4/25T), was used for determining the antihypertensive effect of test samples and amlodipine, a standard CCB. Depomedrol® was used for the induction of hypertension through the subcutaneous route as per our reported procedure.[21,22] Prior to experimentation, rats underwent a 7-10-day acclimation period, including 30 minutes of daily restraint training. The animals were then randomly divided into three groups (n=5/group). The first two groups received oral administrations of Cp.Cr and the aqueous vehicle Cp.Aq, respectively, at doses of 1 and 10 mg/kg. The tail cuff method was used for measuring systolic blood pressure as described.[23] Lab chart 7 was used for recording data using (NIBP) apparatus. Mean systolic blood pressure for treated groups and control hypertensive groups were recorded. Amlodipine in a test dose of 0.0714 mg/kg was used as a standard antihypertensive drug.[21]

Statistical analysis: Response based on isometric tension was plotted as % of control on y-axis versus its respective test concentrations that were plotted on x-axis. Means EC50 were calculated using Graph Pad Prism version 8. Systolic blood pressure (mmHg) was plotted on y-axis versus its respective doses plotted on x-axis. Student 't' test was used to compare the mean of test concentrations with mean of control at 95% CI with 'P' value less or equal to 0.05.

RESULTS

Preliminary phytochemical composition: Cp.Cr was yielded with a quantity of 150 g. Subsequent fractionation resulted in the following yields: ethyl acetate (7.4 g), n-hexane (6.5 g), n-butanol (3.1 g), chloroform (2.06 g), and an aqueous fraction (100g). Phytochemical screening revealed the presence of flavonoids, saponins, cardiac glycosides, and terpenoids. Alkaloids and tannins were absent. The presence of flavonoids and cardiac glycosides was detected at moderate levels. Notably, a strong positive reaction for saponins was observed, with the highest concentration found in the aqueous fraction (100g).

Toxicity profile: Acute oral toxicity studies indicated no mortality at doses up to 1500 mg/kg b.w. in mice. However, a 75% mortality rate was observed with a dose of 2000 mg/kg. The LD50 of the extracts is below 2000 mg/kg.

Effects of Cp.Cr, Cp.Aq on KCl-induced contractions: The potency of the Cp.Cr is quantified by its EC_{50} value, which is the concentration required to produce 50% of the maximum effect. Effects of Cp.Cr.,Cp.Aq and amlodipine on potassium chloride and norepinephrine-induced contractions

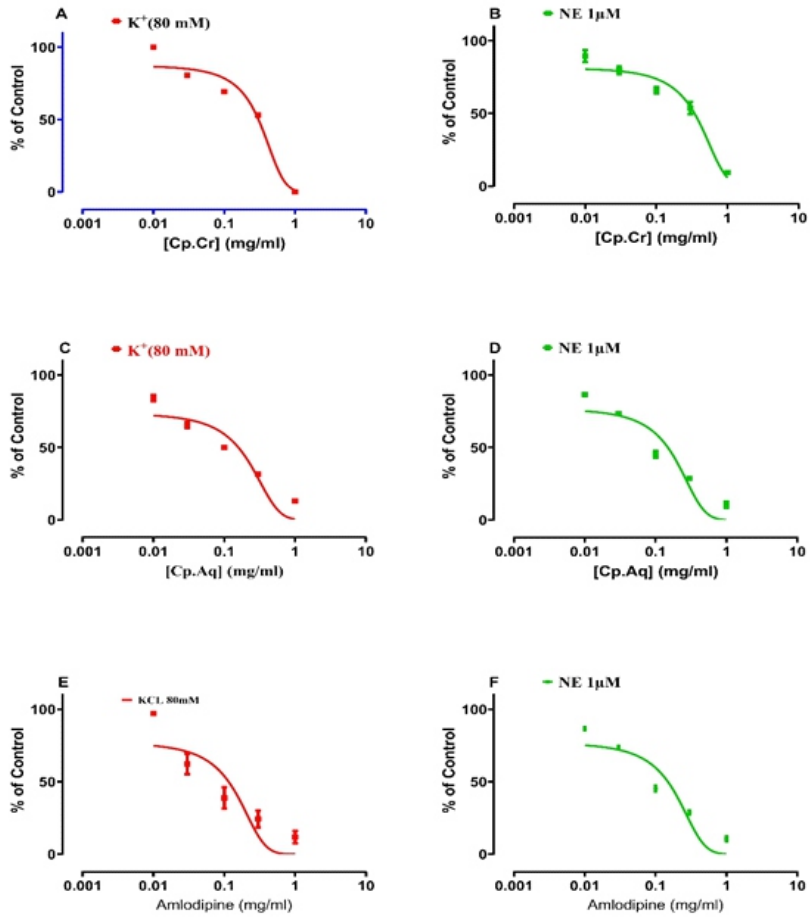

Figure 2: Effects of Cp.Cr, Cp.Aq and amlodipine on contractions induced by potassium chloride (KCl) and norepinephrine (NE) in isolated aortic strips preparations. All values are represented as mean ± SD (n=4).

on rabbits' thoracic aortae are shown in Figure 2. The crude extract demonstrated inhibitory effects on contractions induced by 80 mM KCl (Figure 2A) starting at 0.03 mg/ml and relaxed at 1 mg/ml with mean EC_{50} value of 0.31±0.06 mg/ml (Table I). The crude extract also completely relaxed norepinephrine (NE 1 µM) induced contractions (Figure 2B) at 1 mg/ml while its relaxing effect started at 0.01 mg/ml. A relatively potent and significant effect of Cp.Aq was observed on KCl-induced contractions and 1µM NE-induced contractions with their respective EC_{50} as 0.15±0.04 and 0.13±0.03mg/ml. The EC_{50} of Amlodipine on KCl-induced and 1µM NE-induced contractions were almost superimposable with that of EC_{50} of Cp.Aq fraction.

Effect on systolic blood pressure: Both the extracts Cp.Cr and Cp.Aq exhibited a dose-dependent hypotensive effect. At a dose of 10 mg/kg, both Cp.Cr and Cp.Aq portion significantly reduced systolic blood pressure ($P<0.05$) as illustrated in Figure 3. The mean values for these effects are presented in Table II.

DISCUSSION

The present study demonstrated that the crude (Cp.Cr) and aqueous (Cp.Aq) extracts of *Calotropis procera* possess significant vasorelaxant and hypotensive effects. These findings were supported by in vitro vascular relaxation against KCl- and norepinephrine-induced contractions, as well as dose-dependent reductions in systolic blood pressure in hypertensive rats, with Cp.Aq showing potency comparable to amlodipine.

Hypertension, a major global health challenge, significantly increases the risk of cardiovascular diseases. While synthetic antihypertensive drugs such as calcium channel blockers and beta-blockers are widely used, their side effects and cost necessitate the search for safer, affordable alternatives from natural sources. In this context, traditional medicine offers a rich resource, with numerous plants historically employed for managing hypertension. Among them, *Calotropis procera* is a medicinal plant of significant ethnobotanical relevance in treating cardiovascular ailments piqued our interest, prompting a scientific investigation into its antihypertensive potential. This study focuses on evaluating the vasorelaxant and antihypertensive effects of C. *procera* using both methanolic and aqueous extracts, selected for their ability to cover a broad polarity spectrum and efficiently extract diverse phytochemicals.[20-22] Alongside, a toxicity profile was established to ensure its safety for potential therapeutic use. A foundational step in developing any phytotherapeutic agent is a comprehensive safety assessment. In our study, toxicity tests of the crude methanolic extract of C. procera indicated a wide safety margin.[22-25] Animals tolerated doses up to 1500 mg/kg without mortality, achieving 100% survival. However, administering a dose of 2000 mg/kg led to significant mortality (75%), reflecting a steep increase in toxicity beyond the established threshold. Observed adverse effects included respiratory depression and sedation, with mild symptoms at lower doses (10 and 100 mg/kg) escalating to severe respiratory depression at higher doses (1000 and 1500 mg/kg). These findings highlight a therapeutic window, warranting further acute and subacute toxicity studies to optimize dosage and better understand the mechanisms of adverse effects. The vasorelaxant effects of C. *procera* were explored using isolated aortic rings pre-contracted with norepinephrine (NE) and potassium chloride (KCl) (Figure 2, Table I). Both the crude methanolic extract (Cp.Cr) and aqueous extract (Cp.Aq) induced significant relaxation, providing mechanistic insights into their antihypertensive action. Relaxation of NE-induced contractions suggests inhibition of receptor-operated calcium channels (ROCCs), while relaxation of contractions induced by high-

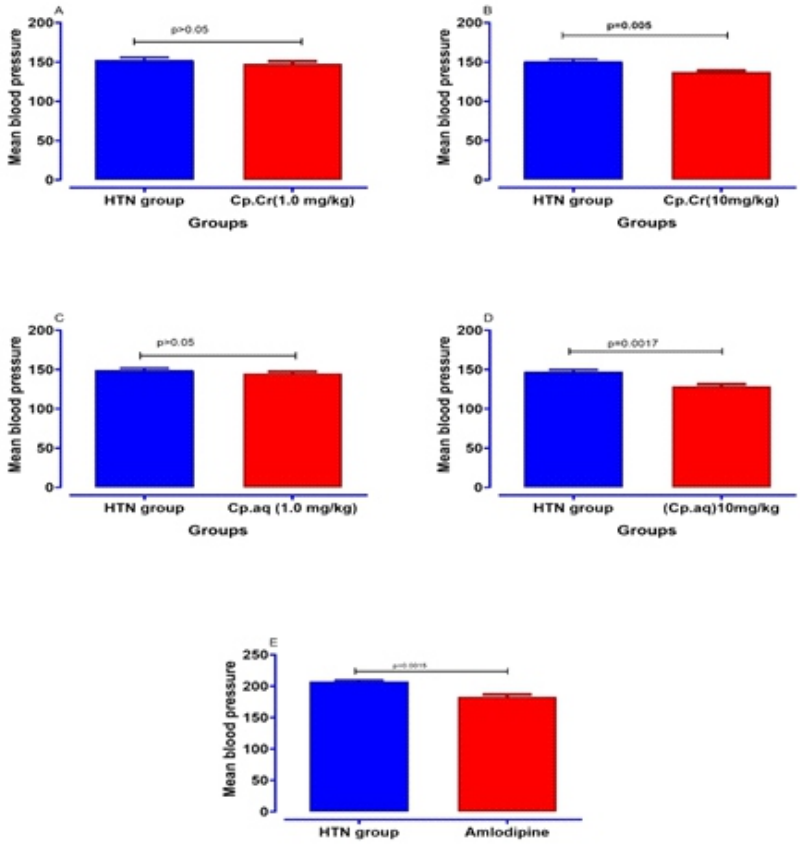

Figure 3: All the values are represented as mean ± standard deviation (n=4). BP: Blood pressure; Cp.Cr: crude extract of flowering twigs of C. *procera*; Cp.Aq: Aqueous fraction of flowering twigs of C. *procera*.

Table I: Mean EC_{50} values of Cp.Cr and Cp.Aq in isolated denuded aortae of rabbits' preparations

Varible	Simple					
	Cp.Cr		Cp.Aq		Amlodipine	
Types of contractions	80 mM KCl-induced	1 μM NE-induced	80 mM KCl-induced	1 μM NE-induced	80 mM KCl-induced	1 μM NE-induced
Mean (EC_{50}±SD) (mg/ml)	0.31±0.06	0.14±0.02	0.15±0.04	0.13±0.03	0.12±0.03	0.13±0.03

All the values are represented as mean ± standard deviation (n=4). NE: norepinephrine; KCl: potassium chloride; Cp.Cr: crude extract of flowering twigs of C. *procera*; Cp.Aq: Aqueous fraction of flowering twigs of C. *procera*.

concentration KCl (80 mM) indicates inhibition of voltage-gated calcium channels (VGCCs), specifically L-type channels.[26-28] Compounds that relax KCl (>30 mM)-induced contractions are generally recognized as VGCC inhibitors, supporting the hypothesis that calcium influx modulation underlies the vasodilatory effect of C. *procera*. Additionally, the potential role of endothelium-mediated mechanisms was considered. Nitric oxide (NO), a key mediator in vascular tone regulation, activates guanylyl cyclase, increasing cyclic guanosine monophosphate (cGMP) levels and promoting smooth muscle relaxation. However, the relaxation response in endothelium-denuded tissues points to an endothelium-independent pathway, reinforcing calcium channel blockade as a primary mechanism.[29,30] The aqueous extract (rich in saponins) exhibited superior activity compared to the crude extract (Figure 3, Table II), suggesting the presence of concentrated active vasorelaxant constituents in the aqueous fraction. The in vivo translation of these findings demonstrated a dose-dependent reduction in both systolic and diastolic blood pressure. The aqueous extract displayed more potent antihypertensive effects than the methanolic extract, achieving a reduction in systolic blood pressure equivalent to 75% of the response elicited by amlodipine, a standard calcium channel blocker. This aligns with in vitro results, confirming the aqueous extract's potential to inhibit both VGCCs and ROCCs, leading to effective vascular relaxation. Overall, C. *procera* holds significant promise as a source of novel antihypertensive agents. However, comprehensive pharmacological profiling and advanced toxicological studies are warranted to advance its development into a safe and effective therapeutic option.

CONCLUSION

The present study demonstrates that Calotropis procera is safe for oral administration at doses up to 1500 mg/kg, confirming its potential for therapeutic use with minimal toxicity at this dosage. The aqueous extract of C. *procera*, which is rich in saponins, exhibits a significant and dose-dependent blood pressure-lowering effect. This hypotensive action appears to be mediated through a vasorelaxation mechanism, primarily involving the inhibition of voltage-gated calcium channels, thereby reducing calcium influx into vascular smooth muscle cells. These findings highlight the plant's potential as a natural antihypertensive agent and provide a foundation for future research into its bioactive compounds and their mechanisms of action. Further studies are warranted to explore its long-term safety, efficacy in clinical settings, and potential interactions with conventional antihypertensive therapies."

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Table II: The effect of crude Cp.Cr, Cp.Aq and amlodipine on systolic blood pressure in hypertensive

Dose (PO)	Baseline Blood Pressure (mm Hg)	After treatment Blood Pressure (mm Hg)	Fall in BP (mm Hg)	Fall in BP as % of Amlodipine
Cp.Cr 1 mg/kg	152.42 ± 8.04	147.8 ± 7.2	4.6± 4.20	19.2
Cp.Cr 10 mg/kg	150.8 ± 6.3	137 ± 5	13.8± 8.40	57.2
Cp.Aq 1 mg/kg	149.2 ± 5.8	145.2 ± 4.5	4 ± 1.4	16.7
Cp.Aq 10 mg/kg	147.2 ± 6.1	128.6 ± 6.5	18.6 ± 4.7	75
Amlodipine 0.0714 mg/kg	207.5±4.2	183±7.8	24±3.5	100

All the values are represented as mean ± standard deviation (n=4). BP: Blood pressure; Cp.Cr: crude extract of flowering twigs of C. *procera*; Cp.Aq: Aqueous fraction of flowering twigs of C. *procera*.

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AUTHORS' CONTRIBUTION

The following authors have made substantial contributions to the manuscript as under:

WUK & MN: Acquisition, analysis and interpretation of data, drafting the manuscript, approval of the final version to be published

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Authors agree to be accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved.

CONFLICT OF INTEREST

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DISCLAIMER

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