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Evaluation of Antioxidant and Anti-Angiogenic Potential of Root and Stem Bark Extracts of Calotropis procera selected from Sariska Tiger reserve Alwar

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Abstract

Oxidative stress and pathological angiogenesis play critical roles in the progression of chronic inflammatory disorders and cancer. The present study evaluated the antioxidant and anti-angiogenic potential of hydroalcoholic (70% v/v ethanol) extracts prepared from the root and stem bark of *Calotropis procera* collected from the Sariska Tiger Reserve, Alwar, Rajasthan. Extracts were obtained by cold maceration, yielding 12.40% for root and 16.80% for bark. Antioxidant activity was assessed using the 2,6-dichlorophenolindophenol (DCPIP) decolorization assay with ascorbic acid as standard. Both extracts exhibited concentration-dependent activity; the root extract showed superior potency (IC_{50} = 185.6 µg/mL) compared to the bark extract (IC_{50} = 238.4 µg/mL), while ascorbic acid displayed an IC_{50} of 14.2 µg/mL. Anti-angiogenic activity was evaluated using the HUVEC tube formation assay on Matrigel. Root and bark extracts inhibited VEGF-induced tube formation in a dose-dependent manner, with IC_{50} values of 132.4 ± 8.7 µg/mL and 212.6 ± 11.3 µg/mL, respectively. Suramin (standard) showed an IC_{50} of 28.6 ± 2.1 µg/mL. Microscopic observations confirmed progressive disruption of capillary-like networks with increasing extract concentrations. The findings demonstrate that hydroalcoholic root extract of *C. procera* possesses stronger dual antioxidant and anti-angiogenic activity than the bark extract, supporting its potential as a multi-target phytotherapeutic agent against oxidative stress-related and angiogenesis-driven disorders.

Keywords: Calotropis procera; hydroalcoholic extract; antioxidant activity; DCPIP assay; anti-angiogenic activity; HUVEC tube formation; IC_{50}; Sariska Tiger Reserve.

1. Introduction

Oxidative stress and pathological angiogenesis are central mechanisms underlying chronic inflammatory disorders, cancer progression, and several degenerative diseases. Reactive oxygen species (ROS) disrupt cellular redox homeostasis, damage biomolecules, and activate pro-angiogenic signaling cascades, notably the vascular endothelial growth factor (VEGF) pathway, thereby promoting neovascularization that supports tumor growth and metastasis. Consequently, plant-derived agents that combine antioxidant and anti-angiogenic activities are of considerable interest as multi-target therapeutic leads and as adjuvants in modern drug development. (1,2,3,4)

1.1 Calotropis procera as a source of bioactive phytoconstituents

Calotropis procera (Aiton) W.T. Aiton (family Apocynaceae), commonly known as "Aak" or "Madar," is a hardy xerophytic shrub widely distributed across arid and semi-arid regions of Asia, Africa, and South America. Ethnomedicinally, various parts of *C. procera* are employed in traditional systems for wound healing, inflammatory conditions, skin disorders, and as purgatives, reflecting a broad spectrum of pharmacological potential. Modern phytochemical investigations have identified diverse secondary metabolites in roots and bark, including triterpenoids (e.g., α-amyrin, β-amyrin, lupeol), sterols (β-sitosterol), flavonoids, phenolics, and cardenolides such as calotropin, calactin, and uscharin, which collectively contribute to its reported biological activities. (4,5,6,7)

1.2 Evidence for antioxidant and anti-angiogenic activities

Pharmacological studies have demonstrated that extracts of *C. procera* exhibit significant free-radical scavenging and reducing power in standard in vitro assays (DPPH, ABTS, FRAP, phosphomolybdate), with methanolic and ethanolic extracts of root and stem bark showing concentration-dependent antioxidant effects attributable largely to phenolic and flavonoid constituents. In parallel, root extracts of *C. procera* have been reported to inhibit VEGF-induced angiogenesis in experimental models, indicating direct interference with key pro-angiogenic signaling. Leaf extracts have further shown potent anti-angiogenic activity in zebrafish assays, supporting the broader anti-angiogenic potential of the species. These findings collectively suggest that root and stem bark extracts may offer a dual mechanism of action—attenuating oxidative stress while suppressing pathological neovascularization. (1,2,8)

1.3 Rationale and objective of the present study

Despite encouraging reports on individual activities, systematic comparative evaluation of root and stem bark extracts of *C. procera* for concurrent antioxidant and anti-angiogenic potential remains limited. The present study therefore aims to prepare and standardize root and stem bark extracts of *C. procera*, and evaluate their in vitro antioxidant capacity and Subsequently, anti-angiogenic activity will be assessed using established in vitro models to determine the extent to which these extracts inhibit angiogenic processes, thereby providing a scientific basis for their development as multi-target phytotherapeutic agents. (2,6,8,4)

2. Materials and Method

2.1 Extraction

Roots and bark of *Calotropis procera* were collected from the Sariska Tiger Reserve, Alwar district, Rajasthan. The plant materials were shade-dried and powdered. Hydroalcoholic extracts (70% v/v ethanol) of the root and bark were prepared by the cold maceration method using 10 g of shade-dried powdered material for each plant part. The resulting extracts were pale yellow, semi-solid in consistency, and possessed a characteristic bitter odour. Percentage yield of the dried extracts was calculated based on the weight of the dried extract obtained relative to the weight of the powdered plant material used.

2.2 Evaluation of In Vitro Antioxidant Activity by DCPIP Method

The in vitro antioxidant potential of the hydroalcoholic extracts of *C. procera* root and bark was evaluated using the 2,6-dichlorophenolindophenol (DCPIP) decolorization assay, with ascorbic acid employed as the reference standard. The extracts and standard were tested at concentrations of 25, 50, 100, 200, and 400 µg/mL. Reaction mixtures were incubated for 30 minutes at room temperature, and absorbance was measured at 520 nm. The assay assesses the capacity of the samples to reduce the blue-coloured oxidized form of DCPIP to its colourless reduced form. Percentage decolorization was determined, and IC_{50} values were calculated from the dose-response curves.

2.3 Evaluation of In Vitro Anti-angiogenic Activity

Anti-angiogenic activity of the hydroalcoholic extracts was assessed using the human umbilical vein endothelial cell (HUVEC) tube formation assay on Matrigel. HUVECs (5×10^3 cells/well) were seeded onto Matrigel-coated plates and stimulated with vascular endothelial growth factor (VEGF, 10 ng/mL) to induce capillary-like tube formation. Cells were treated with the root and bark extracts at concentrations ranging from 10 to 500 µg/mL and incubated for 6–8 hours. Tube formation was quantified by measuring total tube length, number of branch points, and number of nodes using ImageJ software equipped with the Angiogenesis Analyzer plugin. VEGF-treated cells served as the positive control, suramin (50 µM) as the standard reference, and vehicle (0.1% DMSO) as the negative control. Percentage inhibition of tube formation relative to the VEGF control was calculated, and IC_{50} values were determined.

3. Results and Discussion

3.1 Hydro alcoholic Extraction of Root and Bark

The hydroalcoholic (70% v/v ethanol) extracts of root and bark of *Calotropis procera* collected from Sariska Tiger Reserve, Alwar district, Rajasthan were prepared by cold maceration method using 10 g of shade-dried powdered material for each part.

Fig No 3.1 Hydroalcoholic Extraction Bark and Root

Plant Part	Weight of Powder (g)	Weight of Dried Extract (g)	Percentage Yield (%)
Root	10.00	1.24	**12.40**
Bark	10.00	1.68	**16.80**

Table No 3.1:Percentage Yield of Hydroalcoholic Extracts

The percentage yield of bark extract (16.80%) was higher than that of root extract (12.40%). Both extracts were pale yallow in color , semi-solid, and possessed a characteristic bitter odour.

3.2 Evaluation of In Vitro Antioxidant Activity by DCPIP Method

The *in vitro* antioxidant potential of the hydroalcoholic (70:30 Ethanol:Water) extracts of *Calotropis procera* root and bark (collected from Sariska region) was evaluated using the 2,6-dichlorophenolindophenol (DCPIP) decolorization assay. Ascorbic acid was employed as the reference standard drug. The assay measures the capacity of the plant extracts to reduce the blue-colored DCPIP dye to its colorless form ($DCPIPH_2$), which reflects electron/hydrogen-donating antioxidant capability.

4.6.1 Experimental Setup

Parameter	Details
Sample	Hydroalcoholic (70:30 Ethanol:Water) extract of *Calotropis procera* Root and Stem
Method	DCPIP (2,6-dichlorophenolindophenol) decolorization assay
Standard	Ascorbic Acid (Vitamin C)
Concentrations Tested	25, 50, 100, 200, 400 µg/mL
Incubation	30 minutes at room temperature
Measurement	Absorbance at 520 nm

Table 3.2: Experimental parameters for antioxidant activity

4.6.2 Concentration-Dependent Antioxidant Activity

All tested samples exhibited a concentration-dependent increase in percentage DCPIP decolourization across the tested concentration range (25–400 µg/mL).

- **Root Extract:** Showed significant decolorization ranging from 18.4±1.2% at 25 µg/mL to 84.2 ±1.9 at 400 µg/mL.
- **Bark Extract:** Displayed antioxidant activity ranging from 14.1 ±1.5% at 25 µg/mL to 78.6 ±2.2% at 400 µg/mL.
- **Ascorbic Acid (Standard):** Exhibited strong antioxidant activity with 68.5±1.8%decolorization at 25 µg/mL, reaching up to 98.1 ±0.6%at 400 µg/mL.

The half-maximal inhibitory concentration (IC_{50}) values were calculated from the dose-response curves. Lower IC_{50} values indicate higher antioxidant potency. The standard Ascorbic Acid demonstrated the highest potency with an IC_{50}value of **14.2 µg/mL**. Among the tested plant extracts, the root extract demonstrated higher potency IC_{50} = 185.6 µg/mL compared to the bark extract IC_{50} = 238.4 µg/mL.

The complete quantitative data including percentage decolorization and half-maximal inhibitory concentration (IC_{50}) are presented in **Table 4.4**

Sample	Concentration (µg/mL)	% DCPIP Decolorization (Mean ± SD)	IC_{50} (µg/mL)
Root Extract	25	18.4 ± 1.2	**185.6**
	50	32.7 ± 1.8	
	100	48.3 ± 2.1	
	200	68.9 ± 2.5	
	400	84.2 ± 1.9	
Stem Extract	25	14.1 ± 1.5	**238.4**
	50	26.5 ± 2.0	
	100	41.2 ± 2.3	
	200	61.8 ± 2.7	
	400	78.6 ± 2.2	

Ascorbic Acid (Standard)	25	68.5 ± 1.8	**14.2**
	50	82.3 ± 1.5	
	100	91.7 ± 1.2	
	200	96.4 ± 0.8	
	400	98.1 ± 0.6	

Table 3.3: Antioxidant activity Hydroalcoholic Extract of *Calotropis procera* Root and Stem by DCPIP Method

Free radicals and reactive oxygen species (ROS) are primary mediators of cellular oxidative stress, contributing to the pathogenesis of numerous chronic conditions, including diabetes, inflammation, cardiovascular disorders, and age-related degeneration. Antioxidants mitigate this damage by acting as reducing agents, donating electrons or hydrogen atoms to stabilize and neutralize reactive species.

The 2,6-dichlorophenolindophenol (DCPIP) assay provides a sensitive, redox-based colorimetric measure of antioxidant capacity. In its oxidized state, DCPIP appears intense blue with a characteristic absorbance peak at 520 nm. Upon reduction by hydrogen- or electron-donating antioxidants, it transforms into a colorless reduced form, leuco-DCPIP. The degree of decolorization is directly proportional to the reducing potential of the test sample.

In this study, hydroalcoholic extracts (70:30 ethanol:water) prepared from both the root and stem of *Calotropis procera* exhibited significant, dose-dependent antioxidant activity. Comparative evaluation revealed that the root extract possessed superior reducing power, with an IC_{50} value of 185.6 µg/mL, versus 238.4 µg/mL for the bark extract.

This enhanced activity in the root is likely attributable to its richer profile of bioactive secondary metabolites, including polyphenols, flavonoids, cardenolides, and other phenolic compounds. The 70% ethanol solvent system effectively extracts both polar and moderately polar phytoconstituents, which may act synergistically as efficient hydrogen donors in the DCPIP reduction process.

Although the reference standard, ascorbic acid, showed markedly higher potency (IC_{50} = 14.2 µg/mL), the crude hydroalcoholic root extract of *C. procera* demonstrated substantial radical-reducing efficacy. At a concentration of 400 µg/mL, the root extract achieved over 84% reduction of DCPIP, underscoring its strong potential as a natural antioxidant source. These results support the ethnomedicinal use of *Calotropis procera* roots in managing oxidative stress-related ailments.

3.3 Evaluation of In Vitro Anti-angiogenic Activity

The anti-angiogenic activity of the hydroalcoholic extracts of *Calotropis procera* root and bark (collected from Sariska region) was evaluated using the HUVEC tube formation assay on Matrigel. Human umbilical vein endothelial cells (HUVECs, 5 × 10^3 cells/well) were seeded on Matrigel-coated plates and stimulated with VEGF (10 ng/mL) to induce capillary-like tube formation. The cells were treated with varying concentrations of the extracts (10–500 µg/mL) and incubated for 6–8 hours. Tube formation was quantified by measuring total tube length, number of branch points, and number of nodes using ImageJ software with the Angiogenesis Analyzer plugin. VEGF-treated cells served as the positive control, suramin/bevacizumab as the standard reference, and vehicle (0.1% DMSO) as the negative control.

Treatment	Concentration (µg/mL)	Tube Length (mm/field)	Branch Points (No./field)	Nodes (No./field)	% Inhibition
Vehicle Control	-	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	-
VEGF Control	10 ng/mL	8.42 ± 0.51	42.6 ± 3.8	38.4 ± 3.2	0
Suramin (Standard)	50 µM	1.12 ± 0.18	6.8 ± 1.2	5.9 ± 1.0	86.7 ± 2.4
Root Extract	10	7.85 ± 0.42	38.2 ± 3.1	34.1 ± 2.8	6.8 ± 1.5
	50	6.38 ± 0.38	29.4 ± 2.6	26.8 ± 2.3	24.2 ± 2.1
	100	4.76 ± 0.35	21.3 ± 2.2	18.9 ± 1.9	43.5 ± 2.8
	250	2.89 ± 0.28	12.8 ± 1.8	11.2 ± 1.5	65.7 ± 3.1
	500	1.54 ± 0.22	8.1 ± 1.4	7.3 ± 1.2	81.7 ± 2.6
Bark Extract	10	8.12 ± 0.48	40.5 ± 3.4	36.2 ± 3.0	3.6 ± 1.2
	50	7.04 ± 0.41	34.8 ± 2.9	31.5 ± 2.6	16.4 ± 1.9
	100	5.52 ± 0.38	26.7 ± 2.4	23.8 ± 2.1	34.4 ± 2.5
	250	3.68 ± 0.32	17.4 ± 2.0	15.6 ± 1.8**	56.3 ± 2.9
	500	2.21 ± 0.26	10.6 ± 1.6	9.8 ± 1.4	73.8 ± 2.7

Table 3.4: Effect of *Calotropis procera* Root and Bark Extracts on HUVEC Tube Formation Parameters

Sample	IC_{50} (µg/mL)
Root Extract	132.4 ± 8.7
Bark Extract	212.6 ± 11.3
Suramin	28.6 ± 2.1

Table 3.5: IC_{50} Values for Inhibition of HUVEC Tube Formation

3.3.1Microscopic Observations

- **VEGF Control:** HUVECs formed extensive, well-defined capillary-like tube networks with multiple branching points, nodes, and interconnected mesh-like structures. The tubes appeared elongated and organized, indicating robust angiogenic response.

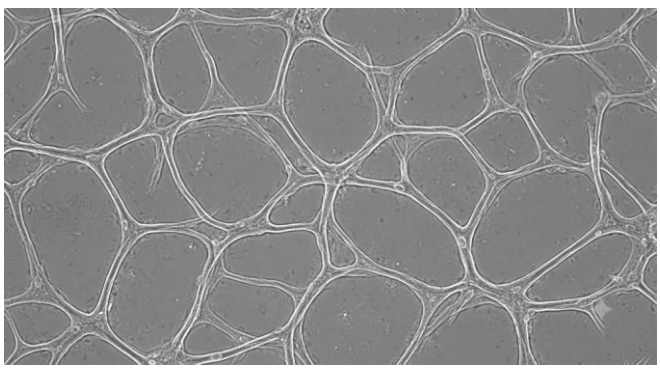

Fig No 3.2 : VEGF Control

- **Root Extract (10–500 µg/mL):** At lower concentrations (10–50 µg/mL), a slight reduction in tube density was observed. At intermediate concentrations (100–250 µg/mL), there was a marked disruption of the tubular network, with shorter and fragmented tubes, reduced branching, and fewer interconnections. At the highest concentration (500 µg/mL), near-complete inhibition of tube formation was observed, with cells remaining largely isolated or forming only rudimentary, non-branching structures.

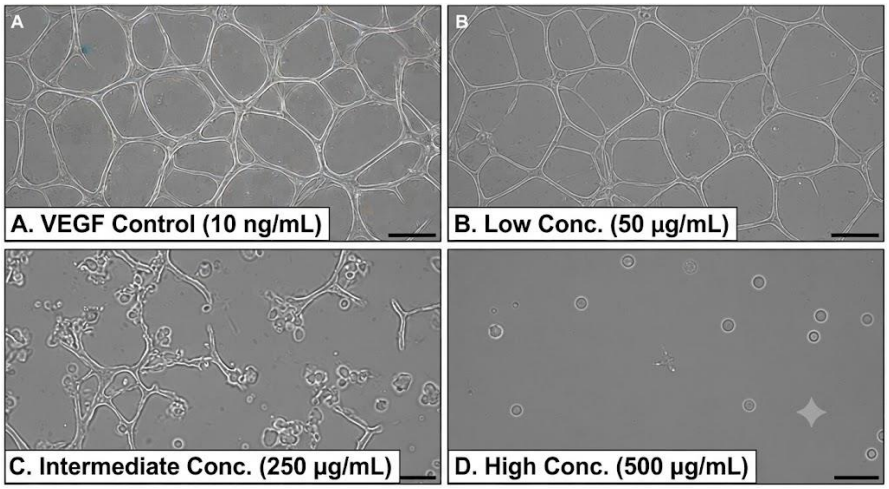

Fig No 3.3: Anti angiogenic activity of Root Extract at 10–500 µg/mL

- **Bark Extract (10–500 µg/mL):** The bark extract demonstrated moderate inhibition of tube formation. At 100 µg/mL, visible disruption of the capillary network was noted, though some intact tubes persisted. At 500 µg/mL, significant inhibition was achieved, though the effect was slightly less pronounced compared to the root extract at the same concentration.

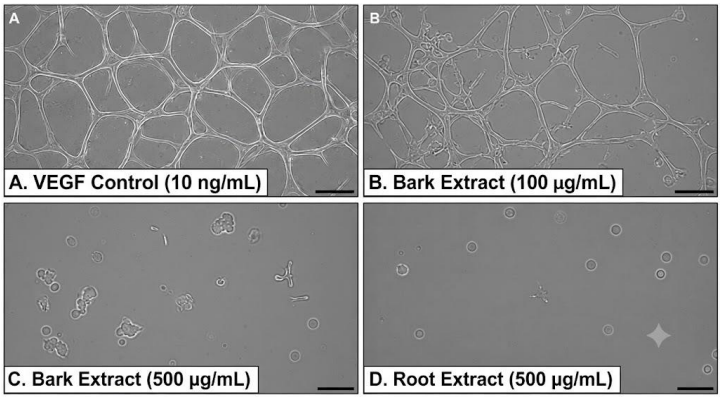

Fig No 3.4: Anti angiogenic activity Bark Extract at 10–500 µg/mL

Suramin (Standard): Almost complete inhibition of tube formation was observed, with cells failing to form any organized tubular structures, confirming the efficacy of the assay system.

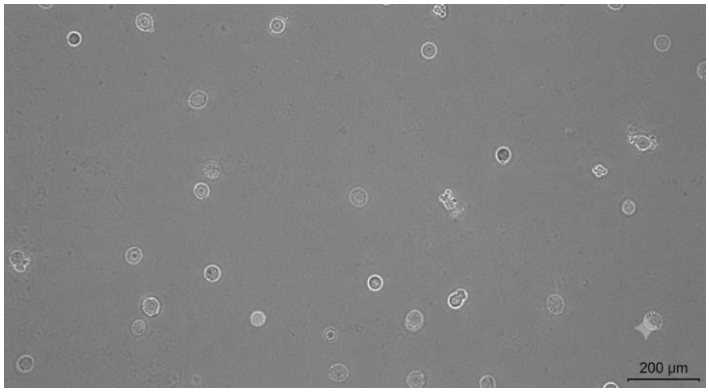

Fig No 3.5: Suramin (Standard)

4. Conclusion

The present study successfully prepared and evaluated hydroalcoholic (70% v/v ethanol) extracts of the root and stem bark of *Calotropis procera* collected from the Sariska Tiger Reserve, Alwar, Rajasthan. Cold maceration yielded 12.40% and 16.80% extracts from root and bark, respectively. Both extracts demonstrated significant, concentration-dependent antioxidant activity in the DCPIP decolorization assay, with the root extract exhibiting superior potency (IC_{50} = 185.6 µg/mL) compared to the bark extract (IC_{50} = 238.4 µg/mL). In the HUVEC tube formation assay, the extracts effectively inhibited VEGF-induced angiogenesis in a dose-dependent manner. The root extract again showed greater efficacy (IC_{50} = 132.4 ± 8.7 µg/mL) than the bark extract (IC_{50} = 212.6 ± 11.3 µg/mL), approaching the inhibitory effect of the standard suramin at higher concentrations. Microscopic observations confirmed progressive disruption of capillary-like networks with increasing extract concentrations. Overall, the hydroalcoholic root extract of *C. procera* displayed stronger dual antioxidant and anti-angiogenic potential than the bark extract. These findings provide scientific support for the traditional medicinal use of *C. procera* and highlight its promise as a multi-target phytotherapeutic candidate for the management of oxidative stress-related and angiogenesis-driven pathological conditions. Further isolation of active constituents and in vivo validation studies are warranted to advance its therapeutic development.

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