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Original Article

Isolation of Bioactive Metabolites from *Tephrosia maxima* and *Tephrosia callophylla*: A Phytochemical and Functional Study

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ABSTRACT

Tephrosia maxima and *Tephrosia callophylla*, two lesser-studied members of the Fabaceae family, were evaluated for their phytochemical, pharmacological, and toxicological profiles in this study. Aqueous extracts from shade-dried leaves were subjected to SEM analysis, hemocompatibility testing, and assays for anti-bacterial, anti-cancer, anti-inflammatory, antioxidant, and general toxicity. SEM revealed irregular crystalline morphologies, indicative of diverse bioactive constituents. Both extracts exhibited <2% hemolysis, confirming systemic biocompatibility. Antibacterial activity against *E. coli* and *S. aureus* was evident, along with potent cytotoxicity against MCF-7 breast cancer cells. Anti-inflammatory and antioxidant assays demonstrated dose-dependent efficacy, with up to 80% nitric oxide inhibition and 90% DPPH radical scavenging. Toxicity studies using brine shrimp and zebrafish embryos showed high viability and minimal adverse effects. These findings highlight the therapeutic promise and biosafety of *T. maxima* and *T. callophylla*, supporting their potential for drug discovery and biomedical applications. Further studies are needed to isolate active compounds and elucidate molecular mechanisms.

Keywords: Tephrosia, Photochemical, Anti-microbial, Anti-cancer.

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INTRODUCTION

The genus *Tephrosia* (family Fabaceae) consists of various group of flowering plants dispersed widely across tropical and subtropical regions. Various *Tephrosia* species have been conventionally used in managing ailments owing to their ethnomedicinal value like inflammation, microbial infections, and disorders associated with oxidative stress.[1,2] This therapeutic potential is widely attributed to the complicated phytochemical constituents present in the plant, especially secondary metabolites including flavonoids, alkaloids, tannins, and phenolics.[3-5] These compounds have been widely studied for their broad-spectrum pharmacological properties like antioxidant, anti-inflammatory, anti-microbial, and anti-cancer effects.

Tephrosia purpurea has been extensively documented for its specific pharmacological actions. Several *in vitro* and *in vivo* studies have established its potent antioxidant and cytotoxic actions against different cancer cell lines which further substantiates the therapeutic relevance of this genus.[3,6] There are less-explored species such as *Tephrosia maxima* and *Tephrosia callophylla* which offer valuable prospects for pharmaceutical exploration thus providing their traditional medicinal usage and the chances of harboring similar bioactive compounds. The pharmacological diversity observed among *Tephrosia* species may also be attributed to the influence of local environmental conditions on phytochemical synthesis, leading to chemotypic variation within the same species across regions.[7,8] For instance, differences in flavonoid content and biological activity have been reported in different plant parts and ecological settings, further highlighting the necessity for complete biogeographical assessments.[9] Moreover, their promising antioxidant, anti-inflammatory, and cytotoxic activities suggest a potential for multitarget drug development, especially when integrated with models such as brine shrimp lethality and hemolytic assays to screen for general toxicity and biocompatibility.[10,11] In the modern era, advances in analytical techniques have enabled more complete and inclusive investigations about the morphology and bioactivity of plant extracts. Scanning Electron Microscopy (SEM) has enabled academicians and researchers to investigate the surface features and microstructural intricacy of phytochemical residues.[7-9] Safety of medicinal plant extracts can be evaluated through biocompatibility tests like hemolysis assays, following ASTM guidelines.[10-12] In addition, their therapeutic

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potential can be assessed using various pharmacological tests such as antibacterial activity against *E. coli* and *S. aureus*, anticancer effects on cell lines, anti-inflammatory and antioxidant assays, as well as basic toxicity studies using brine shrimp and zebrafish models.[13-18] Despite the growing interest in natural products, research on lesser-known *Tephrosia Species* remains sparse. There is limited experimental evidence that systematically evaluates their pharmacological efficacy using modern bioassays. Therefore, screening under standardized laboratory conditions becomes essential to uncover novel bioactive compounds that could contribute to future therapeutic innovations.[13-15]

The present Phase 1 study was conducted to assess the bioactivity, biocompatibility, and safety profile of aqueous extracts derived from shade-dried leaves of *Tephrosia maxima* and *Tephrosia callophylla*. The rationale of this study lies in generating foundational evidence to support their potential pharmacological applications. The primary aim was to assess the phytochemical surface morphology, hemocompatibility, antimicrobial and cytotoxic properties, anti-inflammatory and antioxidant effects, and general toxicity of these extracts using validated *in vitro* and *in vivo* models.

MATERIALS AND METHODS

Preparation of Extracts of *T. maxima* and *T. callophylla*

Fresh leaves of *Tephrosia maxima* and *Tephrosia callophylla* were collected and washed thoroughly under running tap water to remove surface impurities such as dust and microbial contaminants. The leaves were shade-dried for 7-10 days to retain their phytochemical integrity. After drying, the leaves were cut and ground into fine powder. From each plant, 25.0 g of dried powder was measured, and 5.0 g was extracted with 100 mL of distilled water by heating at 60-70°C until boiling. The resulting aqueous extracts were filtered through Whatman filter paper to obtain crude filtrates used for subsequent analyses.

Scanning Electron Microscopy (SEM) Analysis

SEM analysis was performed to observe the surface morphology of the lyophilized plant extracts. The samples were mounted on stubs, coated with gold, and visualized under the electron microscope to study their microstructural features.

Hemocompatibility Assay

Hemolysis assays were conducted according to ASTM guidelines. Human blood samples treated with EDTA were centrifuged at 2000 rpm for 10 min at 4°C to isolate Red Blood Cells (RBCs), which were then washed with phosphate-buffered saline (PBS, pH 7.4). The RBC suspensions were incubated with different concentrations of the extracts at 37°C for 1 hr. The percentage of hemolysis was determined by measuring the absorbance of the supernatant at 540 nm using a UV-vis spectrophotometer.

Anti-bacterial Activity

The antibacterial potential of the extracts was assessed using the agar well diffusion method against *Escherichia coli* and *Staphylococcus aureus*. Mueller-Hinton agar plates were inoculated with bacterial cultures and wells were loaded with varying concentrations of the extracts. Amoxicillin (10 µg/mL) served as a positive control. Plates were incubated at 37°C for 24 hr and zones of inhibition were measured.

Anti-cancer Activity

MTT cytotoxicity assay was employed using MCF-7 breast cancer cell lines. Cells were cultured in 96-well plates and treated with increasing concentrations of the extracts for 24 and 48 hr. Post-treatment, MTT reagent was added and incubated for 4 hr. The resulting formazan crystals were solubilized in DMSO, and absorbance was measured at 570 nm.

Anti-inflammatory Activity

Nitric Oxide (NO) inhibition assay was used to evaluate anti-inflammatory effects, with ascorbic acid as the standard. Extracts were incubated with sodium nitroprusside, and NO production was estimated using the Griess reagent. Albumin denaturation assay was also conducted by incubating reaction mixtures at 37°C followed by heating at 57°C. Absorbance was recorded at 660 nm to assess inhibition levels.

Antioxidant Activity: DPPH radical scavenging assay was conducted to evaluate antioxidant potential. The extracts were mixed with DPPH in methanol and incubated for 30 min in the dark. Absorbance was measured at 517 nm to determine the percentage inhibition.

Brine Shrimp Lethality Assay: Brine shrimp nauplii were exposed to various concentrations of the extracts for 24 hr. Survival rates were recorded post-incubation to determine cytotoxicity.

In vivo Zebrafish Embryo Toxicity

Zebrafish embryos were treated with different extract concentrations and monitored under a stereomicroscope at regular 24-hr intervals. Viability, hatching rate, and morphological development were recorded to assess potential embryotoxic effects.

RESULTS

Phytochemical Characterization and Biocompatibility

The aqueous extracts of *Tephrosia maxima* and *Tephrosia callophylla* were successfully obtained from shade-dried leaf powders. The extraction process yielded crude dark-colored filtrates, and these were used for subsequent bioactivity analyses. Scanning Electron Microscopy (SEM) revealed irregular, crystalline surface morphology of the extracts, indicating a

heterogeneous phytochemical composition and potential presence of active compounds (Figure 1A). The biocompatibility of the extracts was assessed using hemolysis assays in accordance with ASTM standards. Both T. maxima and T. callophylla exhibited less than 2% hemolysis, confirming their hemocompatibility and suggesting minimal risk of erythrocyte lysis upon contact. Quantitative data supporting this observation is shown in Figure 1B, highlighting their suitability for biomedical applications.

Antimicrobial, Anticancer, and Anti-inflammatory Activities

Antibacterial activity was tested against *Escherichia coli* and *Staphylococcus aureus* using the agar well diffusion method. Notable zones of inhibition were observed, even at lower extract concentrations (10 mg and 20 mg), indicating effective antimicrobial potential comparable to the standard antibiotic control (Figure 2A). The anticancer efficacy of the extracts was evaluated on MCF-7 breast cancer cells using the MTT assay. A dose-dependent cytotoxic effect was evident, with significant reduction in cell viability observed at concentrations as low as 12.5 µg. Higher extract doses resulted in increased cytotoxicity, highlighting promising anticancer activity (Figure 2B). Anti-inflammatory properties were demonstrated through Nitric Oxide (NO) inhibition and albumin denaturation assays. The NO assay showed inhibition up to 80% at 500 µg/mL, while lower concentrations exhibited proportionate reduction in NO production (Figure 3B). The albumin denaturation assay further supported anti-inflammatory effects, showing a marked reduction in protein denaturation.

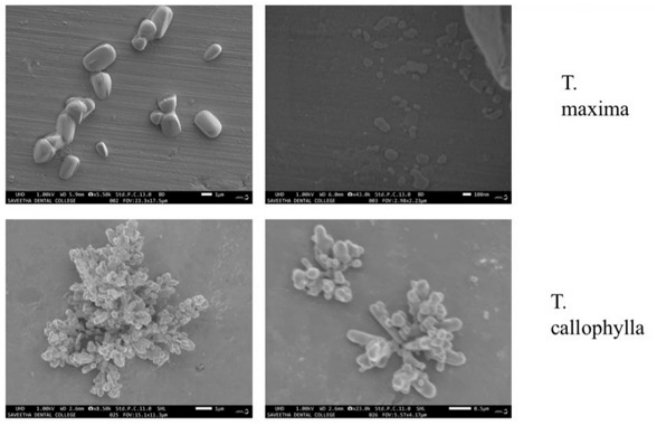
Antioxidant and Toxicity Evaluations

The antioxidant capacity of the extracts was determined using the DPPH radical scavenging assay. Both extracts demonstrated strong free radical scavenging activity, reaching nearly 90% inhibition at higher concentrations (500 µg/mL), while 30-40% inhibition was observed at 100 µg/mL, suggesting potent antioxidant properties (Figure 3A). Safety assessments using the brine shrimp lethality assay revealed low toxicity, with approximately 85% nauplii survival after 24-hr exposure, indicating minimal cytotoxicity and a favorable safety profile (Figure 4A). Further *in vivo* analysis using zebrafish embryo toxicity testing showed approximately 70% viability, with minimal developmental abnormalities observed at varying extract concentrations, suggesting low embryotoxicity and overall biocompatibility (Figure 4B).

DISCUSSION

The present study systematically investigated the extraction, characterization, and *in vitro* bioactivities of *T. maxima* and *T. calophylla*. The results confirm the previous findings on the genus and reveal strong antibacterial, anticancer, anti-inflammatory, and antioxidant properties, indicating promising potential for future therapeutic development with minimal toxicity concerns.

The extraction approach used in this study involved shade drying, pulverization, and hot aqueous extraction of leaves, simulating traditional preparation methods. This method preserved thermally sensitive bioactive compounds, thereby maintaining the therapeutic efficacy of the extracts.[7] The choice of water as a primary solvent aligns with ethnopharmacological practices and sustainable drug discovery approaches. However, the ethanolic extracts demonstrated superior pharmacological activity, particularly in antioxidant and cytotoxic assays, possibly due to their enhanced ability to solubilize semi-polar compounds such as flavonoids, isoflavones, and alkaloids.[5-7,13] SEM revealed irregular crystal morphologies indicative of a complex matrix of phytochemicals, corroborating previous findings by Reddy



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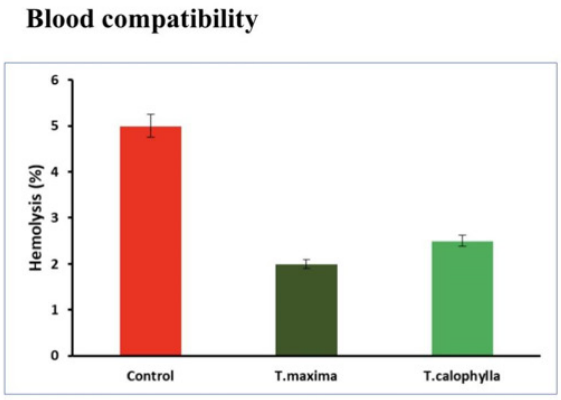

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Figure 1: A) SEM Morphology of *T. maxima* and *T. calophylla* Scanning Electron Microscopy (SEM) images revealing irregular and crystalline surface morphology of both extracts, suggesting heterogeneous phytochemical composition. B) Hemocompatibility Assessment - Hemolysis Assay Bar graph depicting hemolysis percentage for red blood cells treated with *T. maxima* and *T. calophylla* extracts. Both exhibit <2.8% hemolysis, significantly lower than the control (~5%), confirming hemocompatibility.

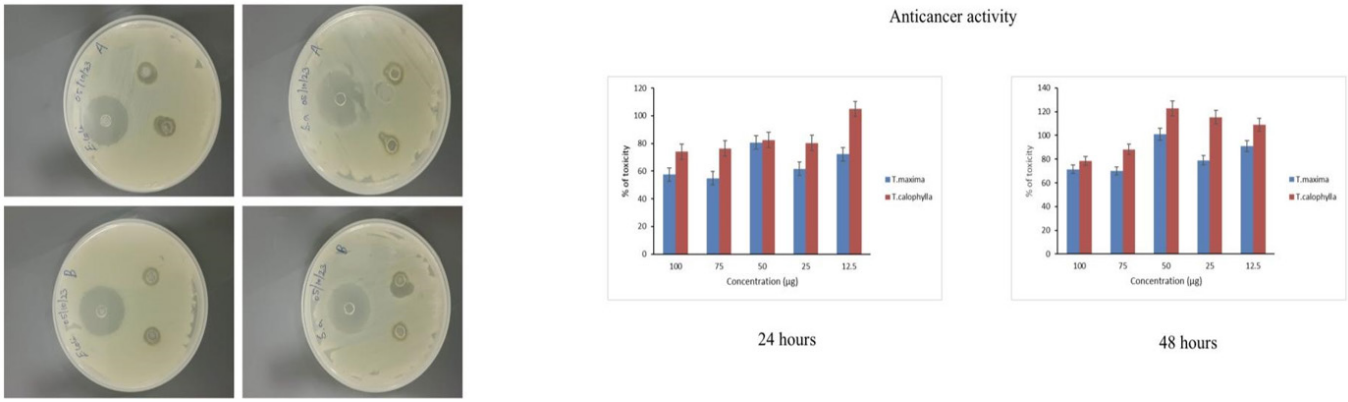

Figure 2: A) Antibacterial Activity Against *E. coli* and *S. aureus* Zones of inhibition from agar well diffusion assay, demonstrating significant antibacterial activity of the extracts at 10 mg and 20 mg concentrations. B) Anticancer Activity on MCF-7 Cells (MTT Assay) MTT assay showing dose-dependent reduction in MCF-7 cell viability following 24 hr and 48 hr treatment with extracts. Strong cytotoxic effect noted even at low concentrations (12.5 µg).

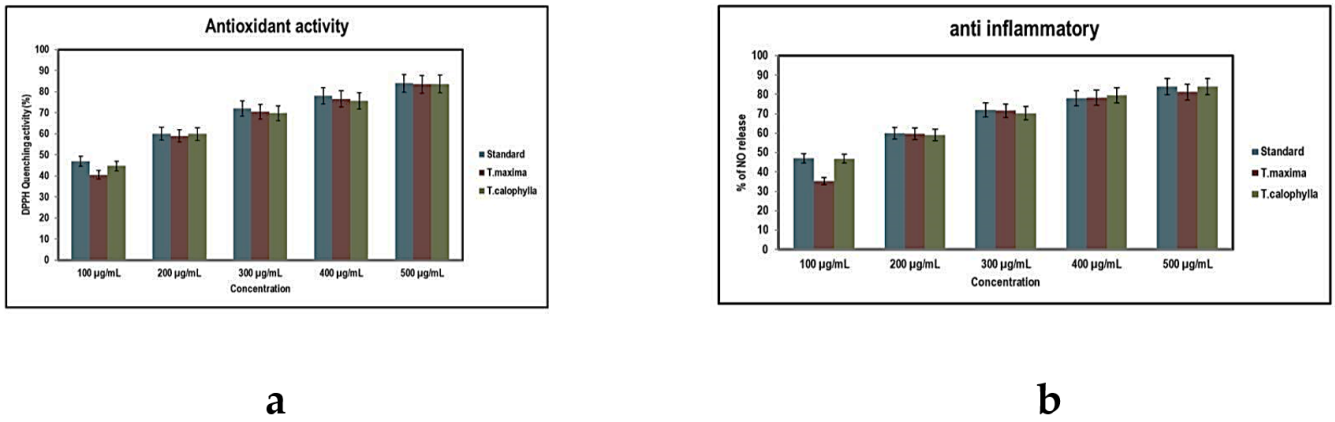

Figure 3: A) Antioxidant Activity - DPPH Radical Scavenging Assay % inhibition of DPPH radicals by the extracts, reaching ~90% at higher concentrations, suggesting potent antioxidant activity. B) Anti-inflammatory Activity - Nitric Oxide Inhibition Graph showing % nitric oxide inhibition by the extracts. Maximum inhibition (~80%) observed at 500 µg/mL, with proportionate decrease at lower concentrations.

et al.,[9] who documented similar crystalline textures in *T. calophylla*. Preliminary phytochemical screening confirmed the presence of key secondary metabolites such as flavonoids, alkaloids, tannins, saponins, phenolics, and terpenoids, which are frequently associated with diverse bioactivities in *Tephrosia* species.[1-3] Touqeer *et al.*, and Sandhya *et al.*, further affirm the therapeutic relevance of these compounds, particularly in oxidative stress modulation, inflammation control, and microbial inhibition.[1,7] Using both polar and semi-polar solvents helps extract more bioactive compounds, as shown in other medicinal plant studies.[5,6,13] Similar broad-spectrum phytochemical profiles have also been observed in *Euphorbia hierosolymitana* and *Trichodesma indicum*, indicating the therapeutic relevance of diverse plant matrices for drug discovery.[19,20,27]

The extracts of both species demonstrated remarkable antioxidant properties, with DPPH radical scavenging reaching up to 90% at 500 µg/mL. This high free radical neutralization potential is attributed to the abundant phenolics and flavonoids, including kaempferol and quercetin derivatives, reported in *T. calophylla* and other *Tephrosia* species.[3,5,6,9] Rizvi *et al.*, confirmed that *T. apollinea* contributes to redox homeostasis through oxidative stress modulation, supporting the antioxidative efficacy observed in the present study.[16] Comparable antioxidant capacities were also reported in *Trichodesma indicum* and *Lotus* species, reinforcing the relevance of flavonoid-rich plant extracts in combating oxidative stress.[20,21] Such activity is significant in the context of chronic diseases like diabetes, cancer, cardiovascular conditions, and neurodegenerative disorders, which are closely linked to oxidative stress.[11] While antioxidant activity at higher concentrations was robust, the moderate activity at lower doses (30-40% at 100 µg/mL) suggests the need for fractionation or purification to isolate the most potent constituents.

The anti-inflammatory potential was evaluated through Nitric Oxide (NO) inhibition and albumin denaturation assays. Both extracts exhibited strong NO inhibition (approximately 80% at 500 µg/mL), which is indicative of their ability to attenuate

inflammatory mediators, consistent with the pharmacodynamics of other *Tephrosia* species such as *T. purpurea* and *T. maxima* pods.[5,8,15,22] Inhibition of albumin denaturation and stabilization of red blood cell membranes also pointed toward lysosomal membrane protection, a key mechanism in anti-inflammatory pathways.[14] These activities were likely mediated by phenolic and flavonoid components that are known to suppress key inflammatory markers such as iNOS and COX enzymes. This is further supported by reports of significant anti-inflammatory efficacy in diverse plant species such as *Euphorbia hierosolymitana*, highlighting the role of polyphenolic constituents in modulating inflammatory responses.[19] While these *in vitro* findings are promising, molecular assays such as qPCR or ELISA targeting inflammatory genes would further confirm the anti-inflammatory mechanisms. Both *T. maxima* and *T. calophylla* extracts showed notable antibacterial efficacy against *Escherichia coli* and *Staphylococcus aureus*, even at low concentrations (10-20 mg/mL). These results align with previous reports on *T. purpurea, T. apollinea*, and other related species, which demonstrated broad-spectrum antibacterial activity.[1,2,6,12] The inhibition is likely due to the disruption of bacterial membranes and metabolic enzymes by bioactive flavonoids and phenolics.[3,9] Although the activity was less potent than standard antibiotics like amoxicillin, the extracts show potential as adjunct therapies, particularly in the context of rising antibiotic resistance. The synergy between flavonoids (e.g., hesperetin and kaempferol) and conventional antibiotics warrants further investigation to enhance antimicrobial efficacy and reduce drug resistance.[23]

MTT cytotoxicity assays revealed dose-dependent anticancer activity of both extracts against MCF-7 breast cancer cells. *T. calophylla* ethanolic extract was especially potent, producing significant cytotoxicity at concentrations as low as 12.5 µg/mL. This is consistent with earlier studies on *T. purpurea* and *T. apollinea*, which demonstrated apoptotic and cytostatic effects on various cancer cell lines.[4,13,18,24] The anticancer effects are likely mediated through multiple mechanisms, including ROS-induced apoptosis, inhibition of cell proliferation, and modulation of pathways such as MAPK and PI3K/Akt, although these were not explicitly assessed in the current study. Bioactive compounds such as rotenoids and quinone reductase inducers identified in other *Tephrosia* species may also contribute to these effects.[4] This aligns with evidence from *Euphorbia hierosolymitana* and *Lotus* species, which exhibited potent cytotoxic activity through apoptosis induction and cell cycle arrest mechanisms.[19,21,25] However, limitations include the use of only one cancer cell line (MCF-7), which restricts generalizability. The findings from Mc-Ag-MgO nanoparticles synthesized using *Moringa concanensis* bark further support the therapeutic promise of plant-based nanomaterials.[26] Future investigations should incorporate a broader panel of cancer cell lines, including HeLa, HepG2, and oral squamous cell carcinoma, and apply mechanistic studies such as flow cytometry, caspase assays, and gene expression profiling.

Toxicity evaluation through brine shrimp lethality and zebrafish embryotoxicity assays revealed high survival rates (>85% and >70%, respectively), with no observable developmental abnormalities or deformities. These findings indicate that both extracts possess favorable safety profiles, consistent with reports on *T. purpurea* and other *Tephrosia*species.[1,2,17,18] The hemocompatibility assay showed <2% hemolysis, confirming the biocompatibility of the extracts and their potential for systemic applications. While some variability in toxicity among *Tephrosia* species has been noted,[27] the current data suggest a wide therapeutic window for *T. maxima* and *T. calophylla*. As highlighted in a comprehensive phytochemical screening by Yang and Yue, plant-derived compounds with high bioactivity often demonstrate acceptable biocompatibility and low toxicity, reinforcing their potential for safe therapeutic use.[28,29] However, further toxicological studies including sub-chronic, chronic, and genotoxicity evaluations are necessary to affirm long-term safety, especially in mammalian models.

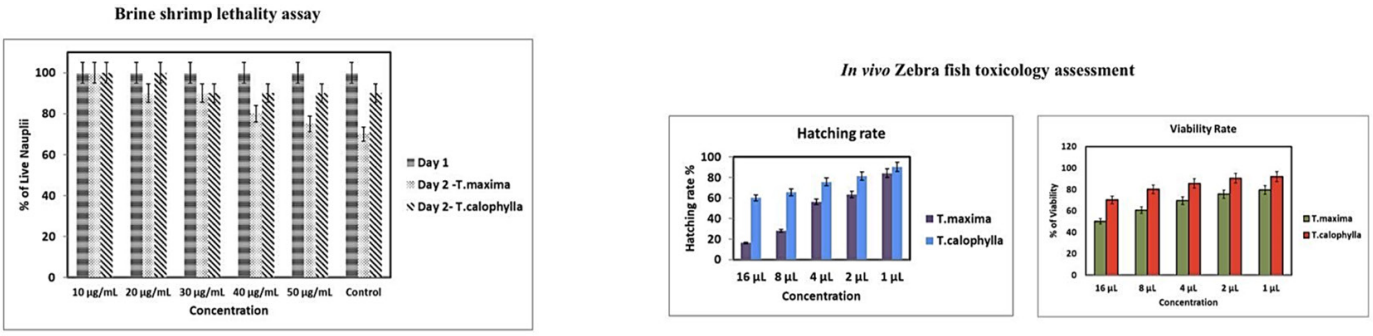

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Figure 4: A) Brine Shrimp Lethality Assay Toxicity assessment showing ~85% survival rate of brine shrimp nauplii after 24 hr exposure to the extracts, confirming low cytotoxicity. B) Zebrafish Embryo Toxicity Assessment Viability and morphological observations of zebrafish embryos exposed to varying concentrations of plant extracts. Approximately 70% viability with minimal abnormalities was noted, indicating favorable biocompatibility.

This study exhibits several strengths, including a comprehensive evaluation of antioxidant, anti-inflammatory, antibacterial, and anticancer properties of *Tephrosia maxima* and *Tephrosia calophylla*. The use of both aqueous and ethanolic extracts allowed optimal recovery of diverse phytochemicals. SEM-based physicochemical characterization provided morphological insights, while early-phase toxicity assessments using brine shrimp and zebrafish models confirmed a favorable safety profile. Hemocompatibility testing further reinforced systemic biocompatibility, supporting the therapeutic promise of these extracts. However, certain limitations constrain the study's translational potential. Advanced analytical techniques such as LC-MS/MS and NMR were not employed, preventing precise identification of active constituents. Mechanistic studies elucidating apoptosis, signaling, or gene modulation were not undertaken, limiting understanding of bioactivity pathways. The anticancer analysis was confined to a single cell line (MCF-7), reducing generalizability. Moreover, the absence of *in vivo* pharmacokinetic and efficacy studies restricts the clinical applicability of the findings. Thus, it is essential to address these gaps to fully validate the biomedical potential of the species.

CONCLUSION

This study highlights *Tephrosia maxima* and *Tephrosia calophylla* as promising medicinal plants with strong antioxidant, anti-inflammatory, antibacterial, and anticancer activities, likely due to their flavonoid and phenolic content. Safety was confirmed through hemolysis, brine shrimp, and zebrafish assays. These findings suggest their potential in managing oxidative stress, inflammation, infections, and cancer. Further studies involving LC-MS/MS, NMR, molecular docking, and gene expression analysis are recommended to identify active compounds and understand their mechanisms of action. *In vivo* validation in rodent models will help establish dosing, efficacy, and pharmacokinetics. Additionally, exploring synergistic combinations with conventional drugs and evaluating potential for nutraceutical or cosmeceutical use could further broaden clinical applicability.

CONFLICT OF INTEREST

The authors declare that there is no conflict of interest.

ETHICAL APPROVAL

Ethical clearance was not required for this study as it involved only *in vitro* experiments and early-stage non-vertebrate models (brine shrimp and zebrafish embryos). No human or vertebrate animal subjects were used.

ABBREVIATIONS

SEM: Scanning Electron Microscopy; **PBS:** Phosphate Buffered Saline; **RBC:** Red Blood Cells; **NO:** Nitric Oxide; **DPPH:** 2,2-Diphenyl-1-picrylhydrazyl; **MTT:** 3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium Bromide; **MCF-7:** Michigan Cancer Foundation-7 (Human Breast Cancer Cell Line); **µg:** Microgram; **mg:** Milligram; **mL:** Milliliter; **EDTA:** Ethylenediaminetetraacetic Acid; **UV-vis:** Ultraviolet Visible; **ASTM:** American Society for Testing and Materials.

SUMMARY

This study evaluates the bioactivity and safety of *Tephrosia maxima* and Tephrosia callophylla extracts using *in vitro* and *in vivo* models. SEM analysis revealed heterogeneous crystalline surfaces indicating phytochemical richness. Hemolysis assays confirmed <2% erythrocyte lysis, suggesting excellent biocompatibility. Both extracts displayed notable antibacterial activity and dose-dependent cytotoxicity on MCF-7 breast cancer cells. Anti-inflammatory action was evidenced by nitric oxide inhibition and albumin stabilization assays. Strong antioxidant properties were observed through DPPH assays. Brine shrimp and zebrafish embryo toxicity tests demonstrated minimal toxicity, establishing a strong safety profile. These findings support the potential of these species in developing natural therapeutic agents.

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