

PHYTOCHEMICAL PROFILING AND ACUTE ORAL TOXICITY OF THE FLAVONOID-RICH FRACTION OF FICUS RELIGIOSA LEAF EXTRACT

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ABSTRACT

Ficus religiosa L. has long-documented ethnomedicinal use, but rational evaluation of its therapeutic potential requires prior characterisation of its phytochemical composition and safety profile. This study aimed to characterise the phytochemical profile and establish the acute oral safety of the ethanol leaf extract of *F. religiosa*, as a foundation for its subsequent antidiabetic evaluation. This study characterised the phytochemical composition of the ethanol leaf extract of *F. religiosa*. It determined the acute oral toxicity of its flavonoid-rich fraction, in preparation for downstream antidiabetic evaluation. Leaves were extracted by cold maceration in 70% ethanol, screened qualitatively and quantitatively for major phytochemical classes, and fractionated by solvent partitioning to isolate a flavonoid-rich fraction (FLEF). Acute oral toxicity of FLEF was assessed in Wistar rats per OECD Guideline 425. Ethanol extraction of 1,200 g of leaves yielded 16.68% (w/w) of a dark-greenish, gummy extract. Qualitative screening confirmed the presence of alkaloids, saponins, tannins, flavonoids, coumarins, phenols, phlobatannins, and triterpenoids. Quantitative analysis showed phenols (4.81 ± 1.12 mg/g), alkaloids (3.21 ± 1.14 mg/g), tannins (2.17 ± 1.12 mg/g), saponins (1.32 ± 1.25 mg/g), coumarins (1.05 ± 0.61 mg/g), flavonoids (0.96 ± 0.05 mg/g), phlobatannins (0.94 ± 1.30 mg/g), and triterpenoids (0.48 ± 1.27 mg/g). No mortality or signs of toxicity were observed at an oral dose of 5,000 mg/kg over 14 days, indicating an estimated LD50 greater than 5,000 mg/kg. The ethanol leaf extract of *F. religiosa* contains a diverse and abundant phytochemical profile, and its flavonoid-rich fraction shows a wide margin of safety, supporting its suitability for further pharmacological evaluation.

Keywords: *Ficus religiosa*; phytochemical screening; flavonoid fraction; acute oral toxicity; LD50.

INTRODUCTION

Medicinal plants have served as a cornerstone of healthcare throughout human history, providing not only direct remedies for a broad range of ailments but also lead compounds for modern pharmaceutical drug discovery. A large proportion of the world's population, particularly in developing countries, still relies on traditional plant-based medicine for primary healthcare because of its accessibility, affordability, and cultural acceptance. Plants synthesise a vast array of primary and secondary metabolites, including alkaloids, flavonoids, tannins, saponins, terpenoids, glycosides, and phenolic compounds, many of which have demonstrable pharmacological activity, including antioxidant, anti-inflammatory, antimicrobial, and antihyperglycaemic effects (Tiwari & Kumar, 2016). However, the therapeutic potential of any given plant depends on rigorous chemical characterisation of these

constituents, since the phytochemical profile determines both the nature and magnitude of observed bioactivity and varies considerably with genetic, geographic, and environmental growing conditions (Dong et al., 2011).

Because crude plant extracts represent complex mixtures of both active and inactive constituents that may act synergistically, antagonistically, or independently of one another, isolating and concentrating specific phytochemical classes, such as a flavonoid-rich fraction, offers a more targeted route to identifying the compounds responsible for a plant's therapeutic effects (Rahul et al., 2024). Nevertheless, before advancing any such fraction to pharmacological efficacy testing, two preliminary steps are indispensable: comprehensive qualitative and quantitative phytochemical characterisation of the parent extract, and assessment of the fraction's acute oral toxicity to establish a safe dosing range for subsequent *in vivo* work (OECD, 2008). These two steps constitute essential prerequisites of rational, evidence-based phytopharmacological research and form the basis of the present study.

Ficus religiosa L., commonly known as the sacred fig, peepal, or bo tree, is a large, perennial, deciduous tree belonging to the family Moraceae, within the genus *Ficus*, which comprises over 800 species distributed across tropical and subtropical regions of the world (Chandrasekar et al., 2010). The species is native to the Indian subcontinent, occurring widely across India, Nepal, Pakistan, Bangladesh, Sri Lanka, and Myanmar, and has been introduced and naturalised in many tropical and subtropical regions of Africa, Australia, and the Americas, where it is cultivated for ornamental, religious, and medicinal purposes (Rahul et al., 2024). Mature trees can attain heights of 20–30 m, bearing characteristic cordate leaves with an elongated drip-tip, greyish-brown bark, and small sessile figs that occur in pairs and ripen from green to purple; the plant's extensive root system contributes to its considerable ecological resilience and adaptability across diverse habitats (Singh et al., 2021).

Various parts of *F. religiosa*, including its leaves, bark, roots, fruits, and latex, have been employed in traditional medicine systems for centuries. Ethnomedicinal reports document its use in the management of diabetes mellitus, asthma, diarrhoea, dysentery, inflammatory disorders, wound healing, skin diseases, and cardiovascular conditions, with leaf decoctions and bark extracts particularly favoured by traditional healers across several Asian communities for controlling hyperglycaemia and related metabolic disturbances (Chandrasekar et al., 2010; Rahul et al., 2024). A chemically diverse phytochemical profile underpins this broad spectrum of ethnomedicinal application. Investigations of *F.*

religiosa have identified numerous primary and secondary metabolites, including carbohydrates, proteins, amino acids, flavonoids, alkaloids, tannins, terpenoids, glycosides, sterols, and phenolic compounds, which together account for the antidiabetic, antioxidant, anti-inflammatory, antimicrobial, and hepatoprotective activities reported for the plant (Yahaya et al., 2021).

Among these constituents, flavonoids, including quercetin, kaempferol, rutin, and myricetin, which have been reported in *F. religiosa* leaves and other plant parts, have received particular attention for their potent antioxidant activity and their capacity to regulate glucose metabolism through several complementary mechanisms. These include enhancement of pancreatic insulin secretion, improvement of insulin sensitivity, inhibition of intestinal carbohydrate-digesting enzymes, and suppression of oxidative stress through scavenging of reactive oxygen species, chelation of transition metals, and enhancement of endogenous antioxidant enzyme systems such as superoxide dismutase, catalase, and glutathione peroxidase (Al-Ishaq et al., 2021). This evidence provides a strong rationale for isolating and evaluating a flavonoid-rich fraction of *F. religiosa* as a candidate antidiabetic and antioxidant agent, rather than relying solely on the crude extract, where flavonoid activity may be diluted or confounded by co-occurring inactive constituents.

Despite this rationale, no phytochemical or fraction can be responsibly advanced to biological efficacy testing without first establishing its acute toxicity profile, since a wide margin of safety is a precondition for selecting sub-toxic doses in subsequent experimental models (OECD, 2008; up-and-down procedure as commonly applied in phytopharmacological studies). This study therefore aimed to characterise the phytochemical profile and determine the acute oral toxicity of the flavonoid-rich fraction of the ethanol leaf extract of *F. religiosa*, providing the foundational characterisation for the subsequent antidiabetic and antioxidant evaluation of this fraction in alloxan-induced diabetic Wistar rats reported in a companion study.

MATERIALS AND METHODS

Plant Material

Fresh leaves of *Ficus religiosa* were collected from Kaduna North Local Government Area, Kaduna State, Nigeria, and authenticated by a plant taxonomist at the Department of Biological Sciences, Nigerian Defence Academy, Kaduna, where a voucher specimen was deposited (Voucher No. NDA/BIOH202665). Leaves were shade-dried at room temperature (25–27°C) and pulverised to a fine powder.

Extraction

Powdered leaves (1,200 g) were extracted by cold maceration in 70% ethanol (v/v) at a ratio of 250 g of powdered leaves to 1,500 mL of solvent in an Erlenmeyer flask for 72 h at room temperature (25–27°C), with occasional shaking. The mixture was filtered through Whatman No. 1 filter paper, and the marc was re-macerated three consecutive times with fresh 70% ethanol. The combined filtrates were concentrated under reduced pressure using a rotary evaporator, further dried in a hot-air oven at 40°C, and finally freeze-dried using a lyophiliser. The dried crude ethanol extract was stored in an airtight container at –4°C until further use. Percentage yield was calculated relative to the weight of the initial dry plant material (Mathewos et al., 2015).

Fractionation

The dried crude ethanol extract (100 g) was dissolved in 400 mL of distilled water and transferred into a separating funnel. Six hundred millilitres of n-hexane was added, mixed gently, and the mixture was allowed to separate into two immiscible layers; the lower aqueous layer was drawn off into a clean container, and the upper n-hexane layer was collected separately. The aqueous layer was returned to the separating funnel and re-partitioned with two further 600 mL portions of fresh n-hexane, giving a total of three sequential n-hexane extractions. The remaining aqueous layer was then sequentially partitioned in the same manner, first with ethyl acetate and thereafter with n-butanol, each solvent applied in three successive 600 mL extractions. The n-hexane, ethyl acetate, n-butanol, and residual aqueous fractions were collected separately, evaporated to dryness under reduced pressure, and stored in a desiccator pending further use. The n-butanol fraction was further processed to separate saponin and flavonoid sub-fractions; the flavonoid-rich fraction (FLEF) was retained for the acute oral toxicity study and subsequent biological evaluation (Tiwari & Kumar, 2016).

Qualitative Phytochemical Screening

Qualitative screening of the ethanol leaf extract was carried out to determine the presence or absence of major secondary metabolite classes, using standard phytochemical procedures (Evans & Trease, 2009), as follows:

- 1. Anthraquinones:** Borntrager's test. A pink to red coloration in the ammoniacal layer indicated the presence of anthraquinones.
- 2. Alkaloids:** Mayer's and Wagner's reagent tests. The formation of a cream or reddish-brown precipitate indicated the presence of alkaloids.
- 3. Chalcones:** Alkaline reagent test. The appearance of an intense yellow coloration that disappeared upon acidification indicated the presence of chalcones.
- 4. Saponins:** Frothing test. Persistent frothing after vigorous shaking indicated the presence of saponins.
- 5. Tannins:** Ferric chloride test. A greenish-black or blue-black coloration on addition of ferric chloride solution confirmed the presence of tannins.
- 6. Flavonoids:** Sodium hydroxide test. The appearance of a yellow coloration that disappeared on addition of dilute hydrochloric acid confirmed the presence of flavonoids.
- 7. Coumarins:** Sodium hydroxide test. The development of a yellow coloration on treatment with sodium hydroxide solution indicated the presence of coumarins.
- 8. Cardiac glycosides:** Keller-Killiani test. Formation of a brown ring at the interface indicated the presence of cardiac glycosides.
- 9. Phenols:** Ferric chloride test. A blue, green, or violet coloration on addition of ferric chloride solution confirmed the presence of phenolic compounds.

10. Anthocyanins: Acid/ammonia test. Treatment with dilute hydrochloric acid followed by ammonia solution; a characteristic colour change indicated the presence of anthocyanins.

11. Phlobatannins: Hydrochloric acid test. Boiling the extract with 1% aqueous hydrochloric acid; formation of a red precipitate indicated the presence of phlobatannins.

12. Triterpenoids: Salkowski test. A reddish-brown coloration at the interface confirmed the presence of triterpenoids.

13. Phytosteroids: Liebermann-Burchard test. The appearance of a bluish-green colour indicated the presence of phytosteroids.

Quantitative Phytochemical Analysis

Quantitative analysis of each phytochemical class detected in the qualitative screening was carried out on the ethanol leaf extract, with concentrations expressed as mg/g dry weight and reported as mean $\pm$ standard deviation from duplicate determinations. Separate standard methods were applied for each constituent class, as follows:

Total phenolic content: Determined by the Folin-Ciocalteu colorimetric method (Singleton & Rossi, 1965). An aliquot of the extract was mixed with Folin-Ciocalteu reagent and allowed to stand, after which sodium carbonate solution was added and the mixture incubated in the dark. Absorbance was read spectrophotometrically at 760 nm against a reagent blank, and concentration was interpolated from a standard curve.

Total flavonoid content: Determined by the aluminium chloride colorimetric method (Boham & Kocipai-Abyazan, 1994). The extract was reacted with aluminium chloride solution to form a stable flavonoid-aluminium complex, and absorbance was measured at 510 nm against a reagent blank.

Alkaloid content: Determined gravimetrically by the acid-alkali precipitation method (Harborne, 1973). The extract was treated with 10% acetic acid in ethanol, concentrated on a water bath, and precipitated by dropwise addition of concentrated ammonium hydroxide. The precipitate was collected by filtration, washed with diluted ammonium hydroxide, dried to constant weight, and expressed as a percentage of the initial extract weight.

Tannin content: Determined by the Folin-Denis colorimetric method (Van-Burden & Robinson, 1981; Makkar, 2003). An aliquot of the extract was treated with Folin-Denis reagent and sodium carbonate solution, then diluted with distilled water and allowed to stand for 30 min to permit full colour development, during which the

phenolic hydroxyl groups of tannins reduce the reagent to form a blue-coloured complex. Absorbance was read spectrophotometrically at approximately 700 nm against a reagent blank, and tannin content was interpolated from a standard curve prepared with tannic acid.

Saponin content: Determined gravimetrically (Obadoni & Ochuko, 2001). The extract was double-extracted with 20% aqueous ethanol, defatted with diethyl ether, and the saponins partitioned into n-butanol. The butanol layer was washed with sodium chloride solution, evaporated to dryness, and the residue weighed.

Coumarins, phlobatannins, and triterpenoids: Quantified spectrophotometrically following standard procedures described by Sofowora (1993), with absorbance values converted to concentrations (mg/g dry weight) using appropriate reference standard curves, consistent with the units used for the other phytochemical classes.

Acute Oral Toxicity (LD50) Determination

The median lethal dose (LD50) of the flavonoid-rich fraction (FLEF) is defined as the single oral dose estimated to be lethal to 50% of a test population, and its determination is a standard first step in establishing the safety margin of a plant extract or fraction prior to pharmacological evaluation (Lorke, 1983). Acute oral toxicity of FLEF was evaluated in accordance with the Organisation for Economic Co-operation and Development (OECD) Guideline 425, Up-and-Down Procedure (OECD, 2008). The study used five healthy Wistar rats. Before administering the test substance, animals were fasted overnight with free access to water. FLEF was then administered as a single oral dose of 5,000 mg/kg body weight (the limit dose specified under OECD Guideline 425; OECD, 2008) by gavage. Following dosing, animals were observed continuously for the first 30 min, periodically during the first 4 h, and thereafter once daily for 14 days for signs of toxicity and mortality. Animals were monitored for changes in skin and fur appearance, eyes, mucous membranes, respiratory pattern, salivation, tremors, convulsions, locomotor activity, feeding behaviour, body weight, and general behavioural changes (OECD, 2008). All experimental procedures adhered to the protocols and guidelines established by the Institutional Animal Ethics Committee, and the experimental protocol was approved by the Ahmadu Bello University Committee on Animal Use and Care (ABUCAUC).

RESULTS

Extraction Yield

Ethanol extraction of 1,200 g of *F. religiosa* leaves yielded 16.68% (w/w) of a dark-greenish, gummy extract (Table 1).

Table 1. Percentage yield and physical properties of the ethanolic leaf extract of *Ficus religiosa*

Plant Name	Solvent	Initial Weight (g)	Total Yield (g)	Yield (%)	Colour	Texture
<i>Ficus religiosa</i>	Ethanol, 500 mL	1,200	16.68	16.68	Dark Greenish	Gummy

Qualitative Phytochemical Composition

Qualitative screening confirmed the presence of alkaloids, saponins, tannins, flavonoids, coumarins, phenols, phlobatannins, and triterpenoids, and the absence of anthraquinones, chalcones, cardiac glycosides, anthocyanins, and phytosteroids (Table 2).

Table 2. Qualitative phytochemical constituents of *Ficus religiosa* leaf extract

S/N	Phytochemical	Presence
1	Anthraquinones	-
2	Alkaloids	+
3	Chalcones	-

4	Saponins	+
5	Tannins	+
6	Flavonoids	+
7	Phlobatannins	+
8	Coumarins	+
9	Cardiac glycosides	-
10	Phenols	+
11	Anthocyanins	-
12	Triterpenoids	+
13	Phytosteroids	-

Key: (+) present; (-) absent.

Quantitative Phytochemical Composition

Table 3 shows the quantitative analysis of all eight qualitatively detected phytochemical classes that were present at measurable concentrations, with phenols (4.81 ± 1.12 mg/g), alkaloids (3.21 ± 1.14 mg/g), tannins (2.17 ± 1.12 mg/g), and saponins (1.32 ± 1.25 mg/g) as the most abundant constituents. Coumarins (1.05 ± 0.61 mg/g) and flavonoids (0.96 ± 0.05 mg/g) were present at intermediate concentrations, while phlobatannins (0.94 ± 1.30 mg/g) and triterpenoids (0.48 ± 1.27 mg/g) were the least abundant.

Table 3. Quantitative phytochemical constituents of *Ficus religiosa* leaf extract (mg/g dry weight)

S/N	Phytochemical	Concentration (mg/g)
1	Phenols	4.81 ± 1.12
2	Alkaloids	3.21 ± 1.14
3	Tannins	2.17 ± 1.12
4	Saponins	1.32 ± 1.25
5	Coumarins	1.05 ± 0.61
6	Flavonoids	0.96 ± 0.05
7	Phlobatannins	0.94 ± 1.30
8	Triterpenoids	0.48 ± 1.27

Values are mean $\pm$ SD of duplicate determinations. Compounds not detected qualitatively are omitted.

Fractionation

Solvent partitioning of the crude ethanol extract yielded n-hexane, ethyl acetate, n-butanol, saponin, and flavonoid fractions; the flavonoid-rich fraction (FLEF) was retained for the acute oral toxicity study.

Acute Oral Toxicity (LD50)

No mortality or overt signs of toxicity (changes in skin/fur, eyes, mucous membranes, respiratory pattern, salivation, tremors, convulsions, locomotor activity, or feeding behaviour) were observed in any animal over the 14-day observation period at an oral dose of 5,000 mg/kg body weight, giving an estimated oral LD50 greater than 5,000 mg/kg body weight.

DISCUSSION

The extraction yield of 16.68% (w/w) obtained from the ethanol leaf extract of *F. religiosa* reflects an efficient recovery of polar and moderately polar constituents, consistent with the widely reported suitability of hydroalcoholic solvents for extracting phenolic- and flavonoid-type compounds from plant tissue. This yield falls within the range commonly reported for ethanolic leaf extracts of other *Ficus* species and medicinal plants subjected to cold maceration. It is comparable to, or somewhat higher than, yields obtained with

less polar solvents such as n-hexane or chloroform, which typically favour recovery of non-polar constituents such as sterols and terpenoids rather than the phenolic and flavonoid classes of principal interest in this study (Mathewos et al., 2015). The dark-greenish colour and gummy texture of the extract are also broadly consistent with a chlorophyll- and polyphenol-rich leaf extract and provide a useful physical reference point for standardising future batches.

The qualitative phytochemical profile obtained in this study, dominated by alkaloids, saponins, tannins, flavonoids, coumarins, phenols, phlobatannins, and triterpenoids, is broadly consistent with previous phytochemical characterisations of *F. religiosa* and other *Ficus* species, and lends direct chemical support to the plant's long-documented ethnomedicinal use in the management of various metabolic, inflammatory, and other non-communicable conditions, including diabetes mellitus (Singh et al., 2021; Rahul et al., 2024; Yahaya et al., 2021). The absence of anthraquinones, chalcones, cardiac glycosides, anthocyanins, and phytosteroids in the present extract suggests either that these classes are genuinely minor or absent constituents of *F. religiosa* leaves, or that their concentrations fall below the detection threshold of the qualitative colour and precipitation reactions employed; this distinction cannot be resolved by qualitative screening alone and would require more sensitive chromatographic or spectrometric methods to confirm.

The quantitative analysis further refines this picture by establishing a clear hierarchy of abundance, with phenols, alkaloids, tannins, and saponins present at the highest concentrations. At the same time, coumarins, flavonoids, phlobatannins, and triterpenoids occurred at comparatively lower levels. This pattern is pharmacologically significant: phenolic and flavonoid compounds are well established as major contributors to antioxidant and antidiabetic bioactivity in medicinal plants, acting through free-radical scavenging, chelation of transition metals, inhibition of carbohydrate-digesting enzymes, and modulation of insulin signalling pathways (Al-Ishaq et al., 2021), so their confirmed presence, even where, as with flavonoids, the absolute concentration in the crude extract is modest, provides a direct phytochemical rationale for the antidiabetic and antioxidant evaluation of this extract's flavonoid-rich fraction in a companion study. The comparatively higher abundance of alkaloids and tannins is also noteworthy, since both classes have independently been associated with hypoglycaemic and antioxidant activity in other medicinal plants. Their co-occurrence with the target flavonoids in the crude extract underscores the rationale for fractionation: isolating the flavonoid-rich fraction allows evaluation of flavonoids' antidiabetic contribution with less confounding from these co-occurring, pharmacologically active but non-target constituents (Tiwari & Kumar, 2016; Rahul et al., 2024).

The fractionation strategy employed, sequential solvent partitioning of the crude extract into n-hexane, ethyl acetate, n-butanol, saponin, and flavonoid fractions, followed established polarity-based separation principles in which successively more polar solvents extract more polar phytochemical classes, ultimately concentrating flavonoid glycosides and aglycones in the n-butanol-derived flavonoid fraction (Tiwari & Kumar, 2016). This targeted approach addresses a recognised limitation of crude-extract studies, namely that the presence of inactive or even antagonistic co-constituents can dilute or obscure the contribution of a specific

bioactive class to overall pharmacological activity; by isolating the flavonoid-rich fraction prior to biological testing, any antidiabetic and antioxidant effects observed in subsequent biological evaluation can be attributed more confidently to the flavonoid constituents themselves, with reduced confounding from these co-occurring, non-target compounds.

The acute oral toxicity findings provide an equally important foundation for this downstream evaluation. The absence of mortality or overt toxicity signs at 5,000 mg/kg (the limit dose recommended under OECD Guideline 425; OECD, 2008) places the flavonoid-rich fraction in the 'unclassified' or practically non-toxic category under the Lorke (1983) acute toxicity classification scheme commonly applied to plant extracts and fractions, in which an LD50 greater than 5,000 mg/kg is considered indicative of a wide margin of safety. This finding is consistent with reports for other flavonoid-rich plant fractions, which typically demonstrate low acute toxicity owing to the generally favourable safety profile of flavonoids as a chemical class (Kumar & Pandey, 2013), and it supports the selection of appropriate sub-toxic doses, well below one-tenth of the estimated LD50 (OECD, 2008; Lorke, 1983), for subsequent efficacy evaluation in a companion study. Nonetheless, an acute LD50 estimate of this kind reflects only short-term tolerability following a single dose; it does not, by itself, establish chronic or sub-chronic safety, cumulative organ toxicity, or reproductive and developmental safety, any of which would require dedicated repeated-dose toxicity studies before further translational development of this fraction could reasonably be considered (OECD, 2008).

Taken together, the phytochemical and toxicological findings of this study provide complementary lines of evidence: the qualitative and quantitative phytochemical profile establishes a plausible chemical basis for the plant's ethnomedicinal reputation and identifies flavonoids, phenols, alkaloids, and tannins as constituents of particular pharmacological interest, while the favourable acute toxicity profile of the isolated flavonoid-rich fraction establishes that this fraction can be safely advanced to in vivo efficacy testing at pharmacologically relevant doses. Together, these findings meet the essential preconditions for a rational, evidence-based evaluation of *F. religiosa* as a candidate source of antidiabetic and antioxidant lead compounds.

Conclusion

The ethanol leaf extract of *Ficus religiosa* contains a phytochemically rich and diverse profile dominated by phenols, alkaloids, tannins, and saponins, and its flavonoid-rich fraction demonstrates a wide margin of acute oral safety (LD50 > 5,000 mg/kg). These findings establish the phytochemical and toxicological foundation for the antidiabetic and antioxidant evaluation of this fraction reported in a companion study and support its continued investigation as a candidate source of antidiabetic lead compounds.

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