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UNIVERSITY OF MEDICINE,
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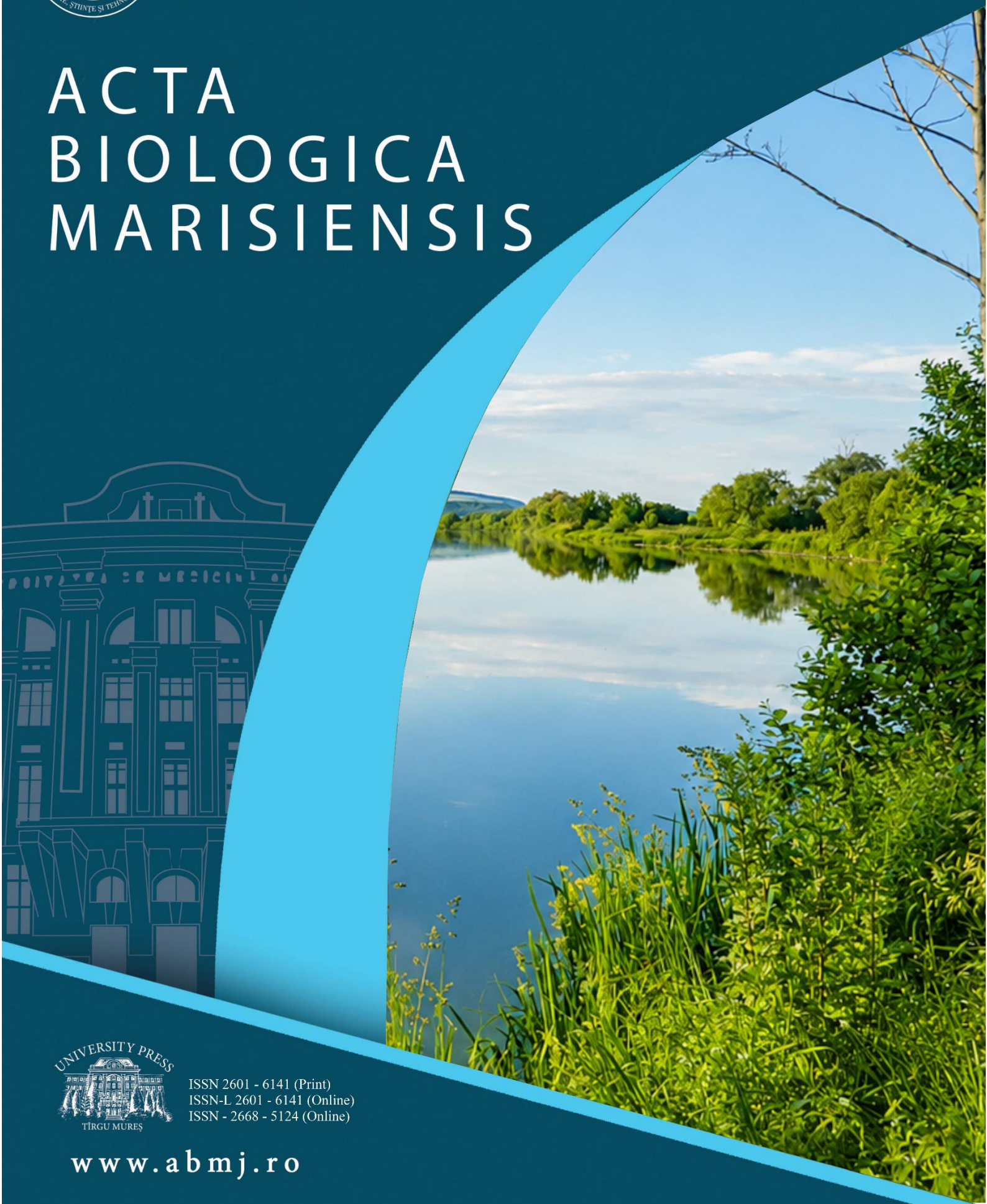
OF TÂRGU MUREŞ

# ACTA BIOLOGICA MARISIENSIS



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# ACTA BIOLOGICA MARISIENSIS

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# TANNINS IN *HEINSIA CRINITA* EXTRACT INHIBIT CARDIAC ARGINASE *IN SILICO* AND *IN VITRO*: POSSIBLE ROLE IN HYPERTENSION

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**Abstract:** Hypertension is afflicting millions worldwide. The present study investigated *in vitro* anti-hypertension of *Heinsia crinita* targeting cardiac arginase in experimental and *in silico* analysis. The methanolic extract of *H. crinita* was subjected to gas chromatography flame ionization detection (GC-FID); Total antioxidant capacity (TAC), DPPH antiradical and inhibition of lipid peroxidation were analyzed. Cardiac arginase inhibitory activity was experimented *in vitro*, also selected tannins from GC-FID chromatography were docked into the active pocket of cardiac arginase PDB: 4HWW. The experimental data reveal tannins, steroids, phenols, flavonoids and saponins. Quantitative phytochemical detected were alkaloids, flavanol, flavonoid, phenols and tannins. Also, the total antioxidant capacity in *H. crinita* was 25.33±0.57 µg ascorbic acid equivalent per gram (AAE/g) the extract of *H. crinita* revealed the following tannins: geranin, epigallocatechin gallate, agrimoniin, punicalagin, praecoxin, amongst others. *H. crinita* displayed antioxidant and antiradical properties. It also inhibited cardiac arginase in dose dependent fashion. The molecular docking results revealed that punicacortein, grenatin a and b, castalin, praecoxin a and punicalagin show better binding affinity of -6.48, -6.29, -4.94, -5.16, -5.53 and -5.16 kcal/mol respectively. In conclusion *H. crinita* extract can be used for the management of cardiac arginase related hypertension.

**Keywords:** tannins, chromatography, molecular docking, hypertension, cardiac arginase

## 1. Introduction

It is statistically proven that by the year 2030 deaths from hypertension, heart failure and atherosclerosis will be more than 23 million (Dumor et al., 2018). Hypertension is a major risk factor for sudden death globally (Agunloye et al., 2023). A lifestyle devoid of light exercise, very low $K^+$ ion-based diet, high table salt intake, and age are some factors that can predispose one to become hypersensitive. Scientific studies have also shown certain enzymatic actions in the progression of hypertension such as adenosine deaminase, arginase, acetyl cholinesterase and angiotensin converting enzyme (Agunloye et al., 2023).

Cardiac arginase metabolizes L-arginine to form two products L-ornithine and urea. The production of urea in mammals is disposed as ammonia which is very toxic (Li et al., 2022). Also, L-ornithine is metabolized by another protein ornithine decarboxylase to form polyamines which are responsible for cell proliferation, growth and transport (Kusano et al., 2008). Again L-ornithine is catabolized by ornithine amino transferase to form L-proline which is important in collagen production (Caldwell et al., 2018). L-arginine is catabolized by a competing enzyme known as nitric oxide synthase converting it into nitric oxide, in the blood vessels functions as regulator of vasodilation, inhibitor of platelet forming plug, adhesion, inhibition of leucocyte adhesion, inflammation and smooth muscle proliferation (Forstermann and Sessa, 2012). The competition of substrate leads to a decrease production of NO and an increase in production of L-ornithine. Polyamines or proline formation from ornithine promotes cell proliferation and collagen formation respectively leading to many health problems like hypertension, heart failure and atherosclerosis (Pudlo et al., 2017).

Also, uncontrolled activity of arginase leads to the deficiency of L-arginine leading to the uncoupling of NOS, where the enzyme produces superoxide ion instead of nitric oxide, this give rise to hypertension among other cardiovascular diseases (Forsterman and Sessa, 2012) Cardiac arginase is a promising target against hypertension (Caldwell et al., 2015). Two synthetic arginase inhibitors in market are derivatives of boronic acid and hydoxy-nor-L-arginine which are quite effective, but their toxicity and poor absorption, metabolism, excretion and distribution profile, makes researchers to look for alternatives which are plant rich polyphenols (Ivanen Kov and Chufarova, 2014; Girard-Thernier et al., 2015).

*Heinsia crinita* is a greenish vegetable mostly found in southern part of Nigeria, it is of the family Rubiaceae. Commonly called bush apple and in southern Nigeria known as “atama”. *H. crinita* leaves and fruits are edible, while the leaves mostly serve as vegetable in local cuisines. *H. crinita* is abound with many bioactives such as polyphenols, flavonoids, and alkaloids (Ozçelik et al., 2011). These compounds are responsible partly to the pharmacological activity of *H. crinita*. The leaves in alcohol extract are used for the management of many diseases like infertility, diabetes, bacterial disease and hypertension (Vladimir-Kneevuc et al., 2004, Ebong et al., 2014). Oboh et al. (2021) reported the anticholinesterase and antioxidant properties of *H. crinita* in *Drosophila melanogaster*. Okokon et al., 2009 also reported the antiplasmodial and antidiabetic effects of *H. crinita* extract. Recent studies have shown that *H. crinita* possesses anti-hyperglycemic, anti-cancer, anti-microbial, anti-inflammatory and antioxidant properties (Mgbeje et al., 2016; Boumba et al., 2022; Iwara et al., 2023). There are very few research reports on *H. crinita* activity against cardiac arginase related to hypertension, lipid peroxidation, phytochemicals and antioxidant properties that is why this research work was carried out. Despite promising and appealing pharmacological activities in *H. crinita*, elaborate analysis of molecular interaction between active constituents of *H. crinita* like tannins against cardiac arginase particularly with respect to amino acid on cardiac arginase interaction and inhibitory mechanisms remains to be elucidated. Many studies only involved preliminary screening solely on identifying inhibitory compounds. However, the present study utilizes an integrative approach that links computational studies with in vitro biochemical validation. In this context, the present study was undertaken to evaluate the cardiac

arginase-inhibitory potential of *H. crinita* extract.

## 2. Materials and Methods

### 2.1. Chemicals

Sodium dodecyl sulphate, ferrous sulphate, ascorbic acid, sodium acetate, acetic acid, 1, 10-phenanthroline, ferrous chloride ($FeCl_2$), sodium phosphate ($Na_3PO_4$) hydrogen peroxide ($H_2O_2$), sodium bicarbonate ($NaHCO_3$), manganese chloride ($MnCl_2$), arginine, perchloric acid ($HClO_4$), Folin-Ciocalteu reagent, sodium carbonate ($Na_2CO_3$), quercetin, gallic acid, sodium nitrite ($NaNO_2$), aluminium chloride ($AlCl_3$), sodium hydroxide (NaOH), ammonium molybdate, Tris-HCl buffer, methanol, thiobarbituric acid (TBA), disodium hydrogen phosphate ($Na_2HPO4$), sodium dihydrogen phosphate ($NaH_2PO4$), trichloroacetic acid (TCA) were purchased from Sigma Aldrich (China) and Urea kit (Randox, UK).

### 2.2. *Heinsia crinita* collection and preparation

*Heinsia crinita* leaves (fresh) were obtained from Swali market in Yenegoa, Bayelsa State. The leaves were identified in Botany Department, Niger Delta University and deposited with the herbarium number NDUP/040. The leaves of *H. crinita* were washed clean and dried for 7 days; the dried leaves were grounded into fine powder. The grounded leaves were weighed 206.26 g and were transferred into 1000 ml amber colored jar holding 500 ml of methanol (Mlozi et al. 2022). The jar was agitated occasionally for 4 days. The extract was filtered, centrifuged at 4000 rpm; the clear supernatant was concentrated into paste. The paste weighed 13.5 g and was stored for further use.

### 2.3. Qualitative Phytochemical Test

The presence of phenols, flavonoids, alkaloids, tannins, steroids, and saponins were qualitatively determined based on the methods of Harbone (1973) and Nabi and Shrivastava (2017).

### 2.4. Quantitative Phytochemical Analysis

#### Estimation of phenol

The amounts of phenols in *Heinsia crinita* extract was according to the described method of Singleton et al. (1999) and Demiray et al. (2009). The plant extract was mixed with diluted (1:10) Folin and kept for 5 minutes, afterwards 4 ml of sodium carbonate (7.5%w/v) was added and the solution incubated at 25 ℃ for 60 minutes with intermittent shaking. The color was read at 765 nm. Total phenol was calculated as milligram GAE/g extract.

#### 2.4.1. Flavonoid content

The spectrophotometric procedure of Zhishen et al. (1999) was used to determine flavonoid content in extract of *Heinsia crinita*. An aliquot of filtrate 0.4 ml was added to 0.6 ml $H_2O$ and 0.06 ml of 5% $NaNO_2$. The mixture was kept at 25 $^0$C for 5 minutes with occasional agitation. Later 10% $AlCl_3$ was added followed by 0.4 ml of 1M NaOH and 0.4 ml of $H_2O$ sequentially. This mixture stood for 30 mins and thereafter absorbance read at 510 nm. Quercetin was used for the calibration of standard curve. Values of total flavonoid were calculated and reported as milligram quercetin equivalent per gram (mgQE/g).

#### 2.4.2. Flavonol content

The procedure of Millauskas et al. (2004) was adopted in evaluating flavonol, in *Heinsia crinita*. Both rutin and *H. crinita* were prepared and were added to 1 ml of $AlCl_3$ (20g/L) and 6 ml of Sodium acetate (50g/L). The mixture was

incubated at 25 ℃ for 4 hours. Thereafter optical density was measured at 440 nm. Total flavonol was extrapolated and reported as mg rutin equivalent/g (mgRE/g).

#### 2.4.3. Alkaloid content

The procedure of Unuofin et al. (2017) was applied in the determination of alkaloids in *Heinsia crinita*. The grounded leaves (5g) were dissolved in 10% acetic acid (0.1 L). The mixture was kept at 25 ºC for 4 hours. The mixture was concentrated at 55 ºC to one fourth of the volume. Then concentrated $NH_4OH$ was added in drops until alkaloid were precipitated. Alkaloids were filtered and dried; the dried weight was calculated and reported in %.

### 2.5. Total condensed tannins

Condensed tannins were evaluated in line with the procedure of Sun et al. (1998). Diluted sample of *Heinsia crinita* was mixed with 3 ml of 4% vanillin prepared in methanol and 1.5 ml of hydrochloric acid was added. The solution was incubated for 15 min at 25 ºC and absorbance was read at 500 nm using methanol as blank. The concentration of condensed tannins in *H. crinita* was expressed as milligram tannic acid equivalent per gram extract (mgTAE/g).

### 2.6. Gas chromatography flame ionization detection (GCFID) for quantification of tannins in *Heinsia crinita* extract

The quantification of tannins in *Heinsia crinita* was carried out on an Agilent 6890 Gas chromatography equipped with a flame ionization detector. The injector temperature was 280 ºC and a velocity of 30 $cms^{-1}$ and Helium was the carrier gas with 2 µl of sample. Tannins were determined by the ratio between the area and mass of internal standards injected into the GC-FID (punicalagin, praecoxin a, castalin, granatin a, granatin b, punicacortein c) and the area of the identified phytochemicals. The concentrations of tannins were express as µg/ml (Kumar and Rajakumar, 2016).

### 2.7. Total antioxidant capacity (TAC)

The phosphomolybdenum procedure of Prieto et al. (1999) was adopted. Graded concentrations of ascorbic acid were mixed with the solution of 0.6 M $H_2SO_4$, 28mM $Na_3PO_4$ and 4mM ammonium molybdate. The tubes were corked and heated at 95 ºC for 90 min. The tubes were cooled and the absorbance was determined at 695 nm using a spectrophotometer. Total antioxidant of *Heinsia crinita* extract was also determined accordingly and calculated from a standard curve of ascorbic acid. The TAC was calculated and reported as µgAAE/g extract.

### 2.8. DPPH antiradical assay

Diluted extract of *Heinsia crinita* was added to 3 ml of ethanolic solution of DPPH (0.9mg/10ml). The reacting solution was incubated at 25 ºC in the dark accompanied by intermittent shaking. After 20 minutes absorbance was read at 517nm. Ascorbic acid served as reference antioxidant, results were calculated as percentages and $IC_{50}$ (Sanchez-Moreno et al, 1998).

### 2.9. Hydrogen peroxide scavenging

*Heinsia crinita*'s ability to scavenge $H_2O_2$ was evaluated according to Nabavi et al. (2009). Hydrogen peroxide 0.04 M was prepared in phosphate buffer pH 7.4. The extract at different concentration was incubated with 600 µl of $H_2O_2$ at 25 ºC in the dark. The ability of *H. crinita* to scavenge $H_2O_2$ was read at 230nm using UV-Vis spectrophotomer. Values were reported as percentages and $IC_{50}$.

### 2.10. Hydroxyl radical scavenging

The ability of *Heinsia crinita* extract to scavenge hydroxyl radicals was evaluated in line with Yu et al. (2004). In a reaction

containing 0.06 ml, 1.0 mM ferrous chloride, 0.09 ml of 1 mM 1, 10-phenanthroline, 2.4 ml of 200 mM phosphate buffer (pH 7.8) 0.15ml of 170 mM hydrogen peroxide and 1 ml of different concentrations of *H. crinita* extract. The reaction was incubated for 5 minutes at 25 ℃ and absorbance was read at 560 nm. Results were calculated and reported as percentages and $IC_{50}$.

### 2.11. Lipid peroxidation assay

The anti-lipid peroxidation property of *Heinsia crinita* was carried out by the procedure of Sato and Bremner (1993). 1ml of ten percent cardiac homogenate was mixed with Tris-Cl buffer 0.15 M, pH 7.2 (0.1ml), ascorbic acid 50 µl, 1% (w/v), 0.07 M $FeSO_4$ (0.05 ml) and varying concentration of the extract. The mixture was incubated at 37 ℃ for 60 minutes. Thereafter 500 µL of 0.1 M HCl, 200µL of 9.8% SDS, 0.9 ml of $H_2O$ and 2 mL of 0.67% TBA were added in sequence, later the mixture was heated at boiling temperature for 30 minutes, cooled and butanol was added (2 ml) and centrifuged at 3000 rpm for 10 minutes. The absorbance of the supernatant was read at 532 nm. Quercetin served as reference.

### 2.12. Cardiac arginase *in vitro*

Wistar albino rats were obtained from the Department of Pharmacology; Niger Delta University. The rats (N=5) were maintained for two weeks according to standard guidelines and procedures. After two weeks of acclimatization rats were sacrificed to extract cardiac arginase for *in vitro* inhibitory studies. The heart tissues were dissected out cleaned in rinsing buffer (KCl) and were weighed. The cardiac tissues were homogenized in cold phosphate buffer. The homogenate was later centrifuged at 4 ºC at 1000 x g for 20 minutes, supernatant used as enzyme source and for anti-lipid peroxidation (Bordage et al. 2017).

### 2.13. Inhibition of *Heinsia crinita* against cardiac arginase

In a reaction mixture consisting of 0.05 mM $NaHCO_3$ buffer pH 9.5, substrate (arginine) 0.02 mM, 0.0005 mM manganese chloride, 0.2 ml of *Heinsia crinita* at different concentrations and 0.79 ml of enzymes source (arginase). The solution was incubated for 1 hour at 37 ℃. At the expiration of 1 hour 0.5 M $HClO_4$ was added to halt the enzymatic activity of arginase. The solution was centrifuged and the supernatant containing urea was determined using urea kits according to Campbell (1961). The results of % inhibition of cardiac arginase were presented as percentages.

### 2.14. Molecular docking of bioactives in *Heinsia crinita* against cardiac arginase (PDB ID:4HWW)

Cardiac arginase protein (PDB ID: (4HWW) with very high percentage homology to that of rat (Ohtake et al. 1998) was downloaded from (www.rcsb.org) and also the six selected bioactives from *Heinsia crinite:* punicacortein, granatin b, granatin a, castalin, praecoxin a and punicalagin, were downloaded from PubChem website. Afterwards the protein cardiac arginase (PDB ID: 4HWW) was prepared by deleting unwanted steric hindrances using the Maestro Suite (Maestro 2023). Molecular docking of cardiac arginase protein (PDB ID: 4HWW) and punicacortein, granatin b, granatin a, castalin, praecoxin a and punicalagin were done using the Maestro software of OPLS3, 2018 Force field (Maestro 2023) and Pymol software (Seeliger and de Groot, 2010). The best docked positions were selected and results presented in tables and figures.

### 2.15. Statistical analysis

All experimental data were carried out in triplicate. Data are presented as mean ± standard deviation (SD). SPSS version 17.0

(New York, USA) was utilized, running on Windows. Differences were considered statistically significant at $p < 0.05$.

## 3. Results and Discussions

Percentage yield of *Heinsia crinita* was 6.58%. Hypertension is a cardinal risk factor for all diseases linked to the cardiovascular system, such as stroke, and a major cause of death (Whelton, 1994). Secondary metabolites are major constituents of plants and they can be revealed through screening and quantitative analysis. The qualitative phytochemical detection of *Heinsia crinita* revealed the presence of phenols, saponins, alkaloids, flavonoids, and steroids (**Table 1**). These metabolites act as antioxidant, antilipid peroxidation and protection of DNA damage (Kumar et al., 2013) antimicrobial (Thompson et al., 2024) and antihypertensive (Ademiluyi et al., 2016).

Phenolics, flavonoids tannins and flavanols are potent antioxidants, anti-inflammatory, antidiabetic, antitumor, and antiaging properties (Zhang et al., 2017). The antioxidant mechanisms of secondary metabolites include neutralization of radical and transfer of hydrogen atom to a radical (Valko et al., 2007). Flavonols and flavonoids are aromatic secondary metabolites with a ketone group. In our present investigation the presence of flavonol was 22.0±0.3 mgRE/g while of flavonoid was 18.6±0.5 mgQE/g which act as antioxidant and anti-inflammatory molecules. Flavonoids structurally are $C_3$–$C_6$ bridge linked to a phenol with hydroxyl group (Cowan, 1999 and Lafay and Gil-Izquierdo, 2008). Phenolics present in *H. crinita* was 10.0±0.9 mgGAE/g as depicted in **figure 1**.

**Table 1.** Qualitative phytochemical screening in *Heinsia crinita*

| Phytochemicals | Result |
|---|---|
| Flavonoids | +++ |
| Saponins | + |
| Phenols | +++ |
| Steroids | ++ |
| Tannins | ++ |
| Alkaloids | + |

Key, + = present, - = not present

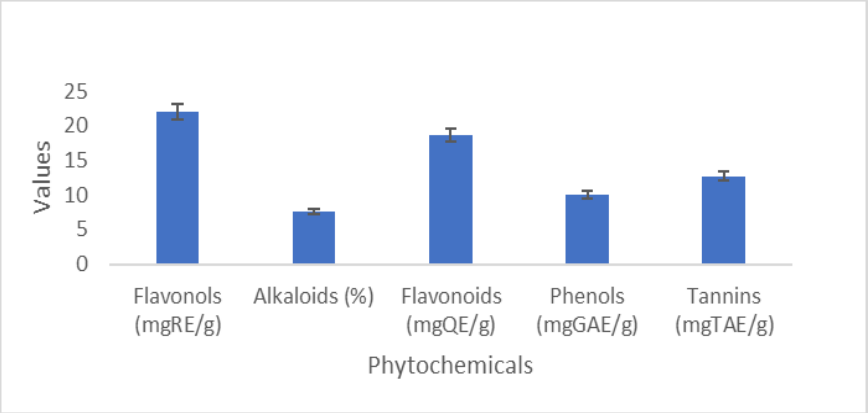


**Fig. 1.** The amounts of phytochemicals in *Heinsia crinita* determined by various methods: Flavonol milligram rutin equivalent per gram extract, alkaloids %, flavonoids milligram quercetin equivalent per gram extract, phenols milligram gallic acid equivalent per gram extract and tannins milligram tannic acid equivalent per gram extract, n = 3 determinations

Tannins are compounds described as polymers of phenols (hydrolysable and condensed). Gallic acid linked to D-glucose forms the monomers of hydrolyzable tannins and flavonoids served as monomers of condensed tannins or proanthocyanidins (Cowan, 1999). Tannins found in *H. crinita* was 12.7±0.8mgTAE/g. Alkaloids are nitrogen containing compounds found in plants (Cushine et al., 2014). The quantitative results revealed the presence of alkaloid 7.64±0.5%. The report of quantitative phytochemicals is similar to that reported by Kumar et al. (2013) and Eboh et al. (2024).

The gas chromatography flame ionization detection analysis of tannins revealed a total of seventeen tannins according to the table and the chromatogram depicted in **figure 2** and **table 2**.

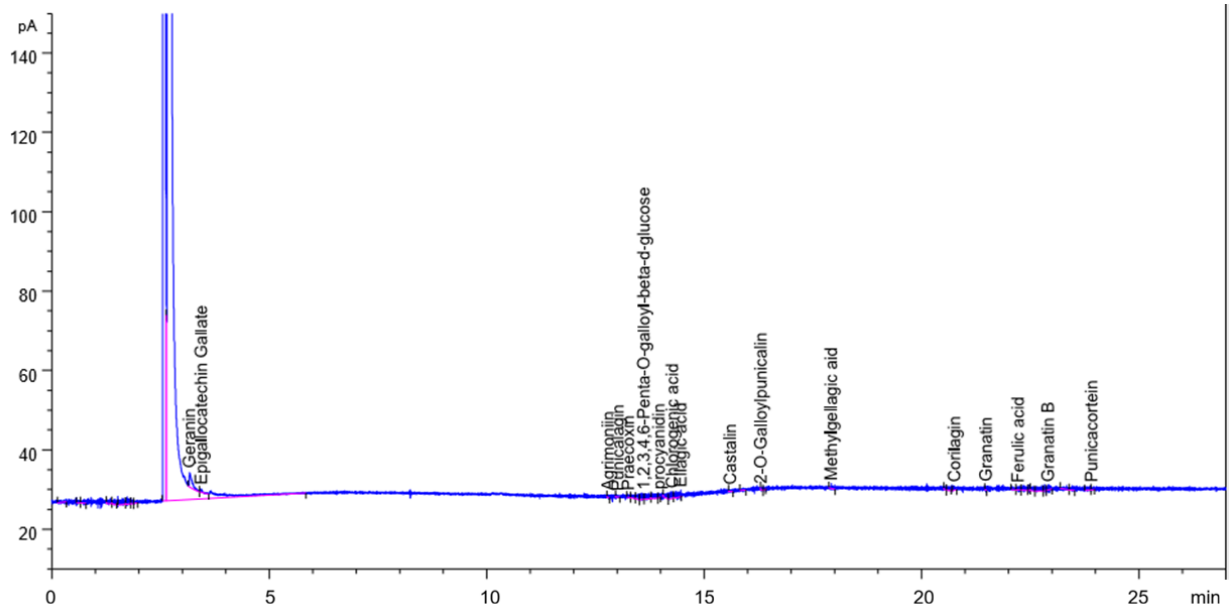


**Fig. 2.** The GCFID chromatogram of *Heinsia crinita* extract revealing different tannins

**Table 2.** The retention time, area, amount and names of tannins detected in *Heinsia crinita*

| S/N | Ret Time (min) | Area (pA∗s) | Amount (µg/ml) | Name |
|---|---|---|---|---|
| 1 | 3.166 | 16.39 | 4.44 | Geranin |
| 2 | 3.433 | 2.36 | $4.82 \times 10^{-1}$ | Epigallocatechin gallate |
| 3 | 12.79 | 1.10 | $2.15 \times 10^{-1}$ | Agrimoniin |
| 4 | 13.0 | 1.14 | $1.99 \times 10^{-1}$ | Punicalagin |
| 5 | 13.25 | 1.26 | $2.46 \times 10^{-1}$ | Praecoxin |
| 6 | 13.60 | 5.07 | 2.05 | 1,2,3,4,6-penta-o-galloyl-β-D-glucose |
| 7 | 13.94 | 1.46 | $2.8 \times 10^{-1}$ | Procyanidin |
| 8 | 14.26 | 5.92 | 2.22 | Chlorogenic acid |
| 9 | 14.46 | 6.65 | 2.38 | Ellagic acid |
| 10 | 15.60 | 1.18 | $2.29 \times 10^{-1}$ | Castalin |
| 11 | 16.3 | 1.52 | $2.79 \times 10^{-1}$ | 2-O-galloyl-punicalin |
| 12 | 17.93 | 2.47 | 5.01 | Methylellagic acid |
| 13 | 20.74 | 2.92 | $5.96 \times 10^{-1}$ | Corilagin |
| 14 | 21.47 | 1.09 | $2.09 \times 10^{-1}$ | Granatin A |
| 15 | 22.22 | 2.48 | $5.03 \times 10^{-1}$ | Ferulic acid |
| 16 | 22.90 | 2.22 | $4.49 \times 10^{-1}$ | Granatin B |
| 17 | 23.90 | 1.67 | $3.16 \times 10^{-1}$ | Punicacortein |

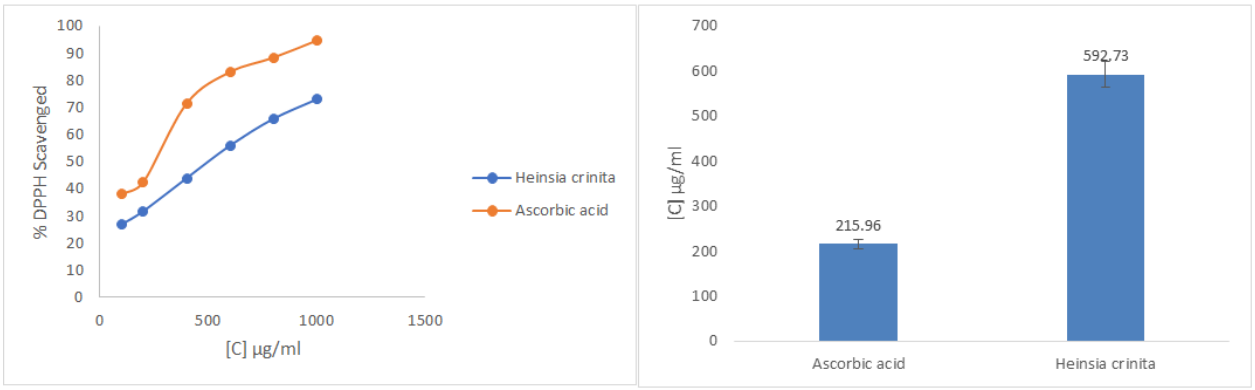


**Fig. 3**. Percentage inhibition of DPPH and $IC_{50}$ values of ascorbic acid and *Heinsia crinita* extract, values are mean±S.D, triplicate determination

Out of these six (punicacortein c, granatin b, granatin a, castalin, praecoxin a, and punicalagin) were selected for the molecular docking studies against cardiac arginase.

DPPH is a non-biological stable free radical that is used to detect the radical scavenging ability of antioxidants. In the present study *H. crinita* at concentrations of 100-1000 µg/ml inhibited DPPH as compared to ascorbic acid. However, the $IC_{50}$ values of *H. crinita* and ascorbic acid are depicted in **figure 3**. Our report is in line with the works of Atere et al. (2018) and Eboh et al. (2024).

The TAC assay is based on the plant extract reducing molybdenum (VI) to (V) which is greenish with maximum absorption of 695nm. The antioxidant capacity of *H. crinita* was 25.33 ± 0.57 µgAAE/g extract. Hypertension is linked to lower levels of antioxidants in the system or increased oxidative stress. In this study the total antioxidant of *H. crinita* shows that it is due to the availability of reducing agents like flavonoids and phenols present as secondary metabolites. Our findings are in consonant with the works of Attia et al. (2019) and Atere et al. (2018).

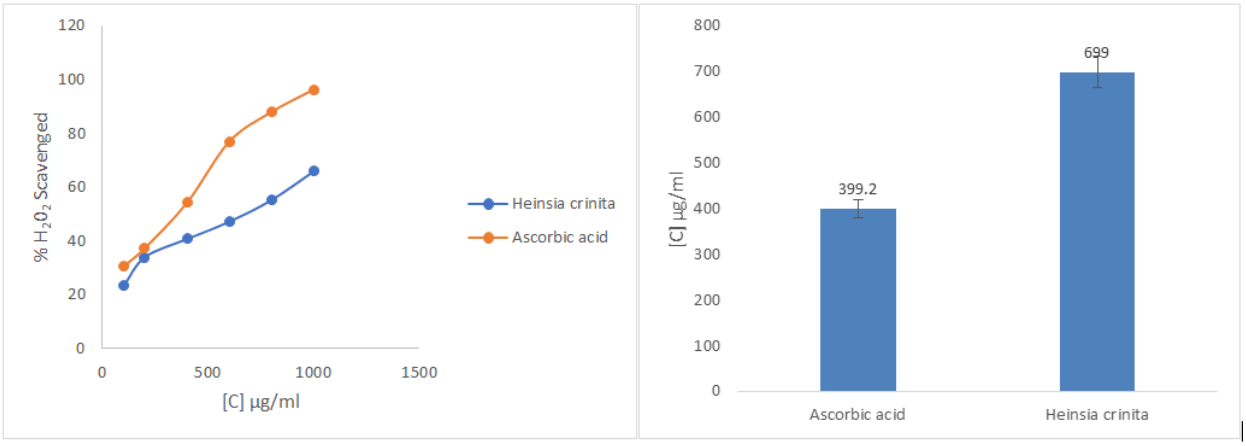


**Fig. 4.** Percentage inhibition of $H_2O_2$ and $IC_{50}$ values of ascorbic acid and *Heinsia crinita* extract, values are mean±S.D, triplicate determination

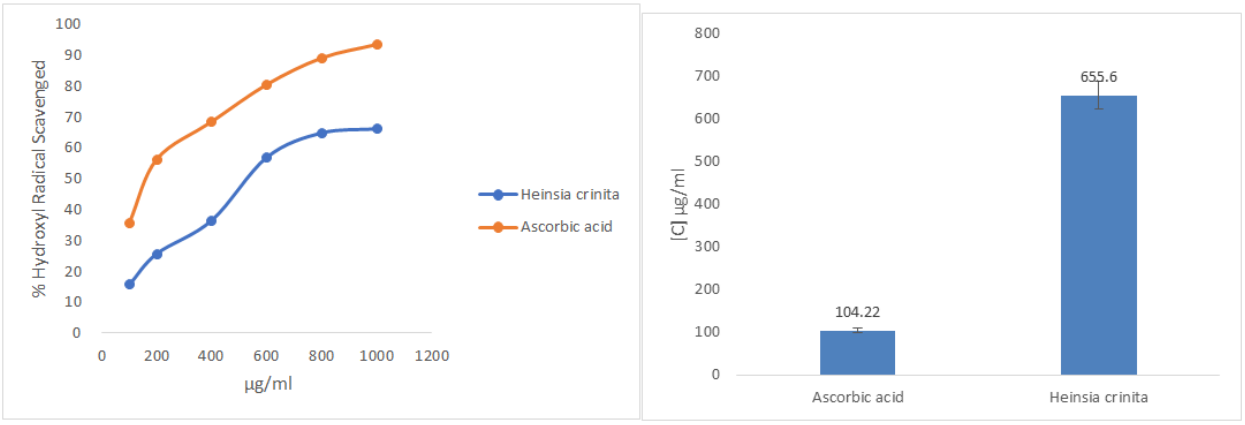


**Fig. 5.** Percentage inhibition of ˙OH radical and $IC_{50}$ values of ascorbic acid and *Heinsia crinita* extract, values are mean±S.D, triplicate determination

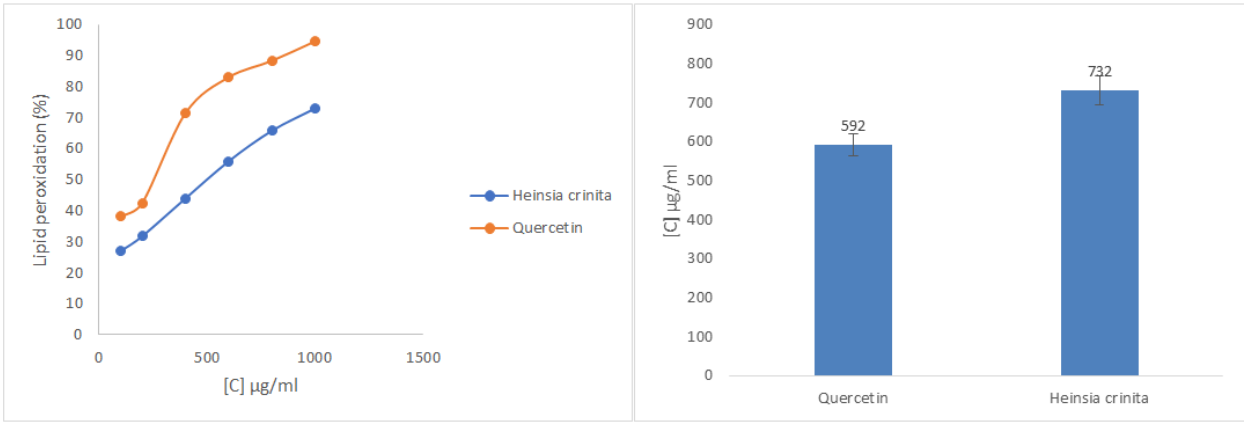


**Fig. 6.** Percentage inhibition of lipid peroxide and $IC_{50}$ values of ascorbic acid and *Heinsia crinita* extract, values are mean±S.D, triplicate determination

**Table 3.** The percentage of *Heinsia crinita* inhibition of cardiac arginase

| **Conc. (mg/mL)** | **% Inhibition** |
|---|---|
| **0.1** | 26.17± 0.98 |
| **0.2** | 39.8± 0.81 |
| **0.4** | 47.73± 0.34 |
| **0.6** | 61.9± 0.76 |
| **0.8** | 77.5± 0.79 |
| **1** | 80.2± 1.18 |
| **$IC_{50}$** | 0.41±0.78 µg/ml |

Values are mean±S.D, triplicate determination

When a radical reacts with hydrogen atom from unsaturated lipid, the lipid becomes a free radical which later reacts with oxygen to forming peroxyl. Upon rearrangement peroxyl radical break down to form malondialdehyde (MDA) and 4-hydroxynonenal (HNE), which cause damage to macromolecules (Marnett, 1999). This process can worsen hypertensive case. Therefore, our report reveals that *H. crinita* inhibits lipid peroxidation in a concentration dependent pattern as shown in **figure 6**.

Hydrogen peroxide present in biological systems can generate free radicals through the enzyme myeloperoxidase/chloride/$H_2O_2$ reaction. Myeloperoxidase in neutrophils converts $H_2O_2$ and $Cl^-$ ions into hypochlorous acid which is an oxidant. Hydroxyl radical can also be generated from $O_2^{-\cdot}$ and $H_2O_2$ or $H_2O_2$ and $Fe^{2+}$ given rise to $^{\cdot}OH$ radical (Nimse and Palb, 2015). This dangerous radical hydroxyl radical is better prevented by neutralizing $H_2O_2$. The results of the present study show that *Heinsia crinita* inhibited $H_2O_2$ in a concentration dependent fashion as shown in **figure 4**.

Cardiac arginase competes with the substrate L-arginine with nitric oxide synthase. Elevated levels of cardiac arginase are leading to endothelial nitric oxide synthase uncoupling, followed by very low levels of NO and increased formation of ONOO. This imbalance in low levels of NO plays a role in endothelial dysfunction leading to hypertension (Mahdi et al., 2020). Our results (**Table 3**) show that *H. crinita* inhibited cardiac arginase in vitro in a concentration dependent manner, this report is similar to the works of Attia et al. (2019) and Oboh et al. (2021).

The hydroxyl radical generated is very reactive can attack DNA, protein and lipids leading to hypertension (Touyz, 2004a) because free radicals like $^{\cdot}OH$, $O_2^{\cdot-}$ and lipid peroxidation products react with NO forming ONOO, thereby reducing its availability for vasorelaxation and myocardial contractility resulting in hypertension (Touyz, 2004a). Depletion of NO and the presence of various ROS are seen in hypertensive patients (Touyz, 2004b). The reports of our study show how *H. crinita* scavenged hydroxyl radical in a concentration dependent way as depicted in **figure 5**.

Molecular docking is a technique used for screening potential druggable candidates carried out by modeling and simulation of the interactions between a protein's receptor and a ligand. In the present study selected bioactives punicacortein c, granatin b, granatin a,castalin, praecoxin a and punicalagin in *Heinsia crinita* extract were docked into the active site of cardiac arginase (PDB ID: 4HWW). These compounds interacted favorably with the active site of cardiac arginase (PDB ID: 4HWW) thereby affording binding affinities of -6.48, -6.29, -4.94, -5.16, -5.53 and -5.16 kcal/mol for punicacortein c, granatin b, granatin a,castalin, praecoxin a and punicalagin respectively. These favorable interactions were made possible through H-bonding, hydrophobic, polar, pi-cation and salt bridge interaction between the ligands and cardiac arginase (PDB ID: 4HWW). These results (**Table 4, figure 7 and 8**) reveal that *Heinsia crinita* extract inhibits cardiac arginase and reduces hypertension *in vitro*. Therefore, all the bioactives docked against cardiac arginase (PDB ID: 4HWW), punicacortein c, granatin b, granatin a,castalin, praecoxin a and punicalagin possess atomic properties favorable to interact with the active site of cardiac arginase inhibiting and lowering the activity. The report is similar to the works of Eboh et al. (2025) and Mahdi et al. (2024).

**Table 4.** Docking score and protein residues-ligand interactions: PDB ID: 4HWW

| S/N | Name | | Type of Protein-Ligand Interaction | | | | |
|---|---|---|---|---|---|---|---|
| | | (kcal/ mol) | H-bonding | Hydrophobic | Polar | Pi-cation | Salt bridge |
| 1 | Punicacortein_C | -6.48 | Asn-139, Hip-141, Asp-181, Asp-128, Glu-186 | Pro-20, Ile-129, Pro-184, Val-182, Val-248 | Thr-136, Ser-137, Asn-130, Asn-139, Thr-246 | Arg-21 | Hip-126, |
| 2 | Granatin_B | -6.29 | Asn-139, Hip-141, Asp-181, Asp-183, Arg-21 | Pro-20 | Asn-139, Ser-137, Asn-130, Thr-136, Thr-246 | - | - |
| 3 | Granatin_A | -4.94 | Asp-128, Arg-21, Asn-139, Thr-136, Glu-186, Asp-181 | Pro-20, Pro-184, Val-182 | Asn-130, Thr-246, Asn-139, Ser-137, Thr-136 | Hip-126 | - |
| 4 | Castalin | -5.16 | Asn-139, Hip-141, Gly-245, Asp-183, Asp-181 | Pro-20, Pro-247, Val-248 | Asn_130, Ser-137, Asn-139, Thr-126 | Arg-21 | Arg-21 |
| 5 | Praecoxin_A | -5.53 | Asp-183, Asp-128, Hip-141, Thr-136 | Pro-20, Val-248, Pro-247 | Thr-246, Thr-136, Ser-137, Asn-139, Asn-130 | Arg-21 | - |
| 6 | Punicalagin | -5.16 | Asp-183, Asp-128, Hip-141, Arg-21, Thr-136, Lys-68 | Val-182, Pro-20 | Thr-246, Thr-136, Asn-139, Ser-137 | - | Arg-21 |

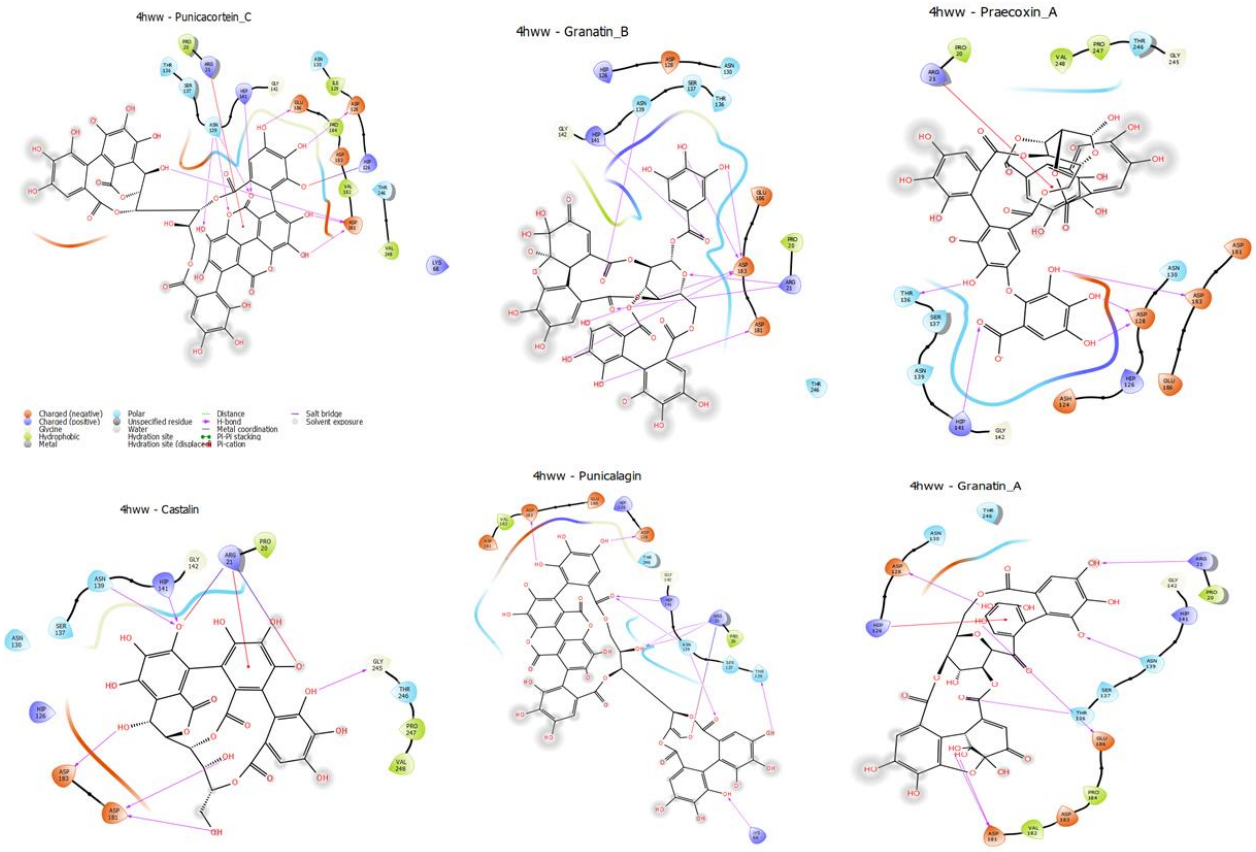


**Fig. 7.** 2D interaction between punicacortein c, granatin b, granatin a,castalin, praecoxin a and punicalagin and the active site of cardiac arginase (PDB ID: 4HWW)

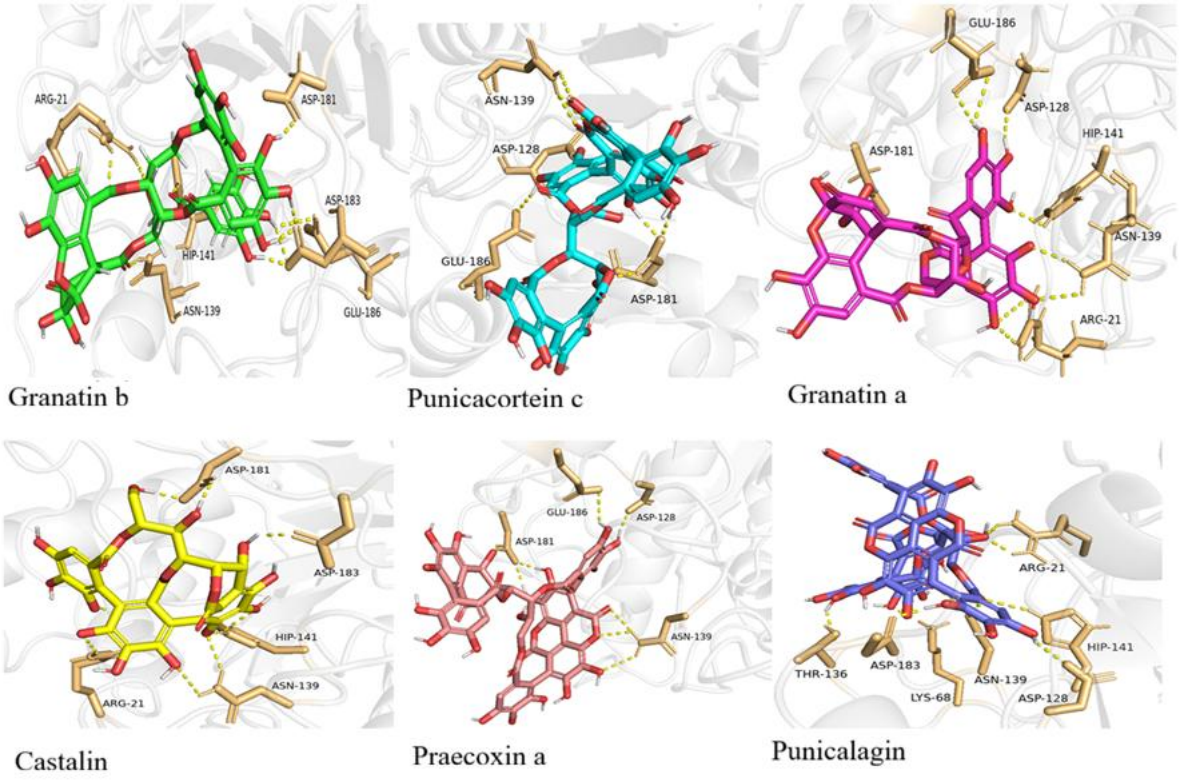


**Fig. 8.** 2D interaction between punicacortein c, granatin b, granatin a, castalin, praecoxin a, and punicalagin and the active site of cardiac arginase (PDB ID: 4HWW)

## Conclusions

Hypertension is a disease that kills silently and has become a problem in the healthcare system. Therefore, our study has revealed that the methanolic extract of *H. crinita* contains phytochemicals that possess diverse roles. Tannins detected in the plant also afforded the plant antioxidant properties by quenching radicals like DPPH, peroxides, $H_2O_2$ and hydroxyl radicals. The extract also inhibited cardiac arginase *in vitro* and *in silico.* Therefore *H. crinita* possess the potential to inhibit cardiac arginase linked hypertension.

## Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

## Author Contributions

Conceptualization: A.S.E and E.W.; Methodology: B.M.A and A.S.E; Software: S.B.; Validation: B.P.S. and E.W.; Formal analysis: B.M.A., A.S.E and E.W; Data curation, writing-original draft preparation: A.S.E; Writing-review and editing, visualization and supervision: A.S.E.

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# PRENATAL BENZYDAMINE EXPOSURE DISRUPTS MATERNAL BEHAVIOR AND POSTNATAL OFFSPRING SURVIVAL IN RATS

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**Abstract:** Maternal behavior is critical for offspring survival. Used for psychotropic effects, benzydamine may influence maternal behavior through its actions on the central nervous system. In this study, 6 pregnant rats received oral benzydamine every 48 hours throughout gestation, while 6 dams served as untreated controls. Maternal behavior and offspring viability were monitored for seven days postpartum, focusing on nursing, pup retrieval, licking/grooming, absence from the nest, and pup-directed aggression or infanticide. Litter size at birth was unaffected by treatment, however, postnatal survival was markedly reduced in benzydamine-exposed dams, with most mortality occurring within the first 24 hours. Treated dams exhibited impaired maternal care, including prolonged absence from the nest, reduced nursing, delayed pup retrieval, and occasional pup-directed aggression. Prenatal benzydamine exposure can impair maternal caregiving and postnatal offspring survival, highlighting the potential neurobehavioral risks of gestational exposure to compounds with central nervous system activity. These results underscore the need for further mechanistic studies to delineate the pathways underlying disrupted maternal behavior and to inform reproductive safety assessments.

**Keywords:** benzydamine, rat, prenatal exposure, maternal behavior, postnatal survival

## 1. Introduction

Postnatal maternal care is a cornerstone of mammalian reproduction, critically influencing offspring survival, growth, and long-term neurobehavioral development. In rodents, maternal behaviors encompass a range of actions, including pup retrieval, nursing, licking and grooming, nest building, and defensive behaviors toward pups. These behaviors serve multiple functions: they maintain pup thermoregulation, ensure adequate nutrition, stimulate physiological processes such as elimination reflexes, and promote social bonding and neural development (Day and Shea, 2025). Maternal care is instinctive, but it is also shaped by neural circuits that integrate sensory input, motivation, and reward, allowing mothers to respond appropriately to the needs of their offspring (Swain et al., 2014).

During pregnancy, exposure to substances that modulate central neurotransmitter systems (dopamine, serotonin, endocannabinoids, or opioids) may lead to neuroadaptive changes (Hess et al., 2002). Sudden withdrawal after parturition can disrupt these adaptations, resulting in dysregulation of mood-related circuits and stress-response systems. This withdrawal-associated neurochemical imbalance may increase vulnerability to depressive symptoms, anxiety, and impaired maternal motivation during the postpartum period (Whiteman et al., 2014). Such effects are particularly relevant given the critical role of dopaminergic reward pathways, serotonergic regulation, and oxytocin signaling in both mood regulation and maternal behavior. Adaptations in reward-related behaviors and mesolimbic dopamine function during motherhood and the postpartum period (Rincón-Cortés et al., 2020). Consequently, postpartum deprivation from psychoactive substances may represent an underappreciated risk factor for postpartum depression and associated disturbances in maternal care.

Pharmacological exposures during gestation can disrupt neural and neuroendocrine systems potentially leading to long-lasting alterations in maternal care (Franks et al., 2020). Benzydamine is a non-steroidal anti-inflammatory drug with a multimodal pharmacological profile, including anti-inflammatory activity, voltage-gated sodium channel blockade, and modulation of dopaminergic, serotonergic, and endocannabinoid signaling. It has also been reported to exhibit psychotropic effects in humans and animal models, likely due to its ability to influence central neurotransmitter systems (Ősz et al., 2023). Given that maternal behavior relies on the precise balance of these systems, prenatal benzydamine exposure may interfere with the development or function of maternal neural circuits, thereby altering postnatal caregiving behaviors.

The present study was designed to investigate the impact of prenatal benzydamine exposure on postnatal maternal behavior in rats. A range of maternal behaviors, including nursing, pup retrieval, licking/grooming, nest attendance, and pup-directed aggression, over the first seven days postpartum, a critical period for offspring survival and maternal-infant bonding were assessed. Litter size at birth, pup viability, and postnatal survival were also monitored to distinguish between effects arising from fetal toxicity and those stemming from disrupted maternal care. Understanding how gestational exposure to pharmacologically active compounds such as benzydamine affects maternal behavior is essential for evaluating reproductive and neurobehavioral safety.

## 2. Materials and Methods

The experiment was performed on white female Wistar rats (n = 12), aged 10-12 weeks at the time of mating. The animals were housed under standard laboratory conditions (temperature 21 ± 2°C, relative humidity 50 ± 10%, 12 h light/12 h dark cycle), with free access to food and water. Following confirmation of pregnancy, the females were randomly assigned to two experimental groups: a treated group (n = 6), which received benzydamine (B1-B6), and a control group (n = 6), which received the vehicle (C1-C6). All experimental procedures were approved by the Scientific Research Ethics Committee of UMFST "G.E. Palade" Târgu Mureș (Approval no. 2073 / 15.02.2023) and were conducted in accordance with Directive 2010/63/EU on the protection of animals used for scientific purposes.

Benzydamine was administered orally by gavage every other day throughout gestation at a dose of 261 mg/kg, calculated based on doses

reportedly used for recreational purposes in humans and calculated using the dose conversion method described by Nair and Jacob, 2016. The control group received an equivalent volume of vehicle.

After parturition, each dam was housed individually with her litter. For each female, the total number of pups at birth and the survival rate of the offspring up to postnatal day 7 were recorded.

Maternal behavior was observed and analyzed over a 7-day postpartum period, corresponding to the phase of maximal parental activity. All observations were conducted under quiet conditions and at the same time each day to minimize circadian influences.

Maternal behavior was assessed by direct observation (2 hours / day, same time) during the first 7 postpartum days. Instances of maternal neglect, offspring rejection (e.g., active pushing away or absence of nursing), and potential episodes of infanticide (defined as active attacks on the offspring) were recorded. Throughout the observation period, the nests were not disturbed to avoid interference with maternal behavior.

On postnatal day 7 (PND7), pup retrieval behavior was assessed. Each dam was briefly removed from the nest, after which the pups were evenly distributed throughout the home cage. The dam was then returned to the cage, and the number of pups retrieved to the nest as well as the latency to retrieve each pup were recorded during a 15-minute observation period.

## 3. Results and Discussions

### Litter size at birth, offspring viability and postnatal survival

As seen in **table 1**, there were no meaningful difference in litter size at birth, suggesting benzydamine did not affect prenatal gestation outcome.

In the benzydamine-treated group, a marked reduction in offspring viability was observed. Complete postnatal loss of all live-born pups within 24 h of birth occurred in 3 out of 6 litters. Consequently, the postnatal viability index (PND0–PND1) showed high inter-litter variability and was substantially lower compared with the control group.

**Table 1.** Neonatal viability and survival in relation to litter size at birth

| Groups | Animals | Total no. of live fetuses | Litter size at birth | | No. of pups after day 1 | Survival (%) | No. of pups after 7 days | Survival (%) |
|---|---|---|---|---|---|---|---|---|
| | | | Total live pups | Mean ± SD per dam | | | | |
| Benzydamine | 1 | 10 | 65 | 10.8 ± 1.7 | 0 | 49.2 % | 0 | 47.7% |
| | 2 | 11 | | | 1 | | 1 | |
| | 3 | 12 | | | 12 | | 12 | |
| | 4 | 13 | | | 13 | | 13 | |
| | 5 | 11 | | | 0 | | 0 | |
| | 6 | 8 | | | 6 | | 5 | |
| Control | 1 | 9 | 63 | 10.5 ± 1.3 | 9 | 98.4% | 8 | 98.4% |
| | 2 | 11 | | | 10 | | 10 | |
| | 3 | 9 | | | 9 | | 9 | |
| | 4 | 12 | | | 12 | | 12 | |
| | 5 | 12 | | | 12 | | 12 | |
| | 6 | 10 | | | 10 | | 10 | |

The postnatal survival index (PND0–PND7) was markedly reduced in the benzydamine-treated group relative to controls. Only minimal additional pup loss was recorded between postnatal Day 1 and Day 7, indicating that most postnatal mortality occurred during the first 24 h after parturition.

**Maternal behaviour during the postnatal period**

Females exposed to benzydamine during gestation exhibited abnormal maternal behavior in the postpartum period compared with controls. A marked reduction in maternal care behaviors was observed, including a decreased frequency of nursing, reduced time spent in direct contact with the pups, and limited engagement in maternal grooming. In addition, prolonged periods of absence from the nest were noted, suggesting diminished maternal interest and impaired motivation to protect the offspring. In some cases, these behavioral alterations were accompanied by aggressive responses toward the pups, culminating in episodes of infanticide (**Table 2**).

Overall, these findings indicate a significant disruption of post-gestational maternal behavior in females prenatally exposed to benzydamine.

Rodents have become a widely used laboratory model for the study of parental care, with maternal behaviors commonly including pup retrieval, nursing, licking and grooming, and defensive responses toward offspring (Rilling and Young, 2014). One of the core maternal behaviors in rodents is pup retrieval, whereby the dam detects the distress vocalizations and locations of displaced pups, retrieves them, and transports them back to the nest. This behavior consists of a sequence of actions requiring intact sensory processing, motor coordination, and motivational drive. Following retrieval, the dam engages in close-contact caregiving behaviors, including nursing and licking/grooming, which serve critical functions such as thermoregulation, maternal-offspring bonding, and stimulation of pup physiological processes, including elimination reflexes (Numan et al., 2009).

**Table 2.** Maternal behavior parameters during the first 7 days postpartum in rats

| Maternal behavior parameter | Observation window | Control | Benzydamine-treated dams during the observation period |
|---|---|---|---|
| Time spent nursing (% of observation time) | PND 0–7 | 80% | 0–68%** |
| Time absent from nest | PND 0–7 | <10–15% vs 35% | 20–35% |
| Active pup rejection (pushing away, refusal to nurse) | PND 0–7 | Rare to absent | 1–7 events |
| Pup retrieval deficits | PND 7 | 0–1 delayed retrieval/day | Frequent delays or failure |
| Infanticidal behavior (active attacks) | PND 0–7 | Absent* | 50% dams |
| Complete litter loss due to maternal behavior | PND 0–7 | 0% | 33 % |

*One control dam exhibited infanticidal behavior, resulting in the death of one pup.

**damns that exhibited infanticidal behavior spent 0% of the observation period nursing their pups. In contrast, the two dams that did not display infanticide spent, on average, 68% of the observation period engaged in nursing and other pup-directed maternal behaviors.

Prenatal pharmacological disruption of any of these systems has the potential to alter maternal motivation, bonding, and defensive or agonistic behaviors toward offspring (Fuentes et al., 2022).

Benzydamine exhibits a multimodal pharmacological profile, including anti-inflammatory activity, blockade of voltage-gated sodium channels, and modulatory effects on the endocannabinoid, dopaminergic, and serotonergic systems (Ősz et al., 2023). During the peri- and postnatal periods, the neural circuits that regulate maternal behavior, such as the medial preoptic area (MPOA), ventral tegmental area (VTA), nucleus accumbens, and the hypothalamic paraventricular nucleus (PVN), critically depend on the coordinated regulation of oxytocin and prolactin signaling and dopaminergic tone.

In the following section, we outline mechanistic considerations that may underlie the altered maternal postnatal behavior observed following prenatal benzydamine exposure and that could inform future research directions.

Our findings indicate that prenatal exposure to benzydamine is associated with alterations in maternal postnatal behavior, including impaired caregiving, prolonged absence from the nest, and, in some cases, infanticide. Given that litter size at birth was not affected, the observed postnatal offspring loss is unlikely to reflect prenatal toxicity or impaired fetal viability; however, assessment of neonatal viability at birth was limited in dams that exhibited immediate pup-directed cannibalism, as offspring were killed shortly after parturition. Instead, the pattern of early, litter-specific mortality suggests a disruption of maternal neurobehavioral regulation. Several interacting neurochemical and neuroendocrine mechanisms may contribute to this phenotype.

Based on its chemical structure and similarities to lysergic acid diethylamide (LSD), benzydamine has been proposed to exhibit agonistic activity at serotonergic 5-HT2A receptors (Balaban et al., 2013), which has been suggested to potentially enhance dopaminergic neurotransmission in the central nervous system (Howell et al., 2015).

Dopamine signaling within the mesolimbic pathway, particularly the VTA to nucleus accumbens (NAc) circuit, is essential to maternal motivation and the rewarding properties of pup-directed behaviors. Activation of this pathway promotes pup retrieval, nursing, and sustained maternal engagement (Day and Shea, 2025). Prenatal exposure to benzydamine may induce transient dopaminergic hyperstimulation (Ősz et al., 2023) that could disrupt the finely tuned dopaminergic balance required for normal maternal motivation, resulting in either diminished caregiving behavior consistent with an anhedonia-like state or maladaptive responses such as hyperactivity or aggression. Behaviorally, this may manifest as reduced nursing postures, delayed pup retrieval, and decreased time spent in contact with offspring (Rincón-Cortés and Grace, 2020).

Moreover, 5-HT2A receptors, plays an important role in the regulation of anxiety, impulsivity, and aggressive behavior. Dysregulation of serotonergic tone can lead to behavioral instability and impaired control of emotionally salient responses (Rosell et al., 2010). Prenatal benzydamine exposure may result in abnormal activation of 5-HT2A-mediated signaling, either directly or indirectly, leading to persistent alterations in serotonergic function during the postnatal period. Such dysregulation may increase irritability, behavioral disorganization, and stress reactivity (Jagtap et al., 2023), thereby promoting inappropriate responses to pup-related cues, including neglect or directed aggression.

Stimulation of cannabinoid receptors has been also proposed as an additional mechanism

underlying benzydamine's effects (Avvisati et al., 2018; Howlett et al., 2021). In the adult maternal brain, CB1 receptor signaling modulates reward processing and anxiety, whereas CB2 receptors contribute to immune and neuroinflammatory regulation (Friuli et al., 2025). Prenatal disruption of endocannabinoid signaling by benzydamine may alter the development and later function of neural circuits underlying maternal reward and motivation. Consequently, maternal responsiveness to pup-derived stimuli may be reduced, leading to diminished caregiving behaviors and increased avoidance or disengagement from the litter (Schechter et al., 2013).

Previous studies have shown that disruption of endocannabinoid signaling can modify oxytocin receptor expression (Schechter et al., 2013). Oxytocin and prolactin are central regulators of maternal bonding, nursing, and the inhibition of offspring-directed aggression (Georgescu et al., 2021). High licking/grooming dams exhibit increased VTA oxytocin projections and greater nucleus accumbens dopamine release in response to pups, which is attenuated by VTA oxytocin antagonism (Stolzenberg et al., 2011). Oxytocin regulates social behaviors, including parenting and bonding. Pharmacological or genetic disruption of central oxytocin signaling impairs maternal care, as seen in CD38 knockout mice, whose deficits are rescued by oxytocin administration. Centrally released oxytocin during parturition and nursing facilitates maternal approach behaviors, and rodent dams typically display indiscriminate caregiving toward pups (Ross and Young, 2009).

Numerous studies have documented disruptions of the oxytocin system in depression, suggesting that investigating its role in maternal depression could inform both research and intervention strategies. Plasma oxytocin levels are reduced in individuals with major depression and show an inverse correlation with the severity of depressive symptoms (Frasch et al., 1995; Scantamburlo et al., 2007). Third-trimester plasma oxytocin levels have been shown to predict maternal postpartum depression, while lower first-trimester oxytocin is associated with both postpartum depressive symptoms and reduced maternal attachment behaviors (Skrundz et al., 2011; Feldman, 2012).

Prenatal benzydamine exposure may increase dopaminergic activity, reducing prolactin release (Fitzgerald and Dinan, 2008) and impairing lactation and maternal caregiving behaviors (Kraus et al., 2025).

## Conclusions

Prenatal benzydamine exposure may disrupt the coordinated neurochemical and neuroendocrine regulation of maternal behavior. Rather than reflecting direct fetal toxicity, the observed postnatal offspring loss appears to arise from altered maternal motivation, stress responsiveness, and social behavior. These findings highlight the importance of considering maternal neurobehavioral endpoints when evaluating the developmental and reproductive toxicity profile of pharmacologically active compounds with central nervous system effects.

## Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

## Author Contributions

Conceptualization: Conceptualization, Bianca-Eugenia Ősz; Data curation, Bianca-Eugenia Ősz, George Jîtcă, Andreea Sălcudean,

Camil-Eugen Vari; Formal analysis, Bianca-Eugenia Ősz, George Jîtcă, Andreea Sălcudean, Camil-Eugen Vari; Funding acquisition, Bianca-Eugenia Ősz; Investigation, Bianca-Eugenia Ősz; George Jîtcă, Andreea Sălcudean, Camil-Eugen Vari; Methodology, Bianca-Eugenia Ősz, George Jîtcă; Project administration, Bianca-Eugenia Ősz; Resources, Bianca-Eugenia Ősz, Andreea Sălcudean; Software, George Jîtcă; Supervision, Bianca-Eugenia Ősz, Camil-Eugen Vari; Validation, Bianca-Eugenia Ősz, Camil-Eugen Vari; Visualization, Bianca-Eugenia Ősz, George Jîtcă; Writing – original draft, Bianca-Eugenia Ősz, George Jîtcă; Preparation, Bianca-Eugenia Ősz, George Jîtcă; Writing – review & editing Bianca-Eugenia Ősz, George Jîtcă, Camil-Eugen Vari.

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## Generative AI Statement

During the preparation of this work the author(s) used ChatGPT in order to *improve language and grammar*. After using ChatGPT, the author(s) reviewed and edited the content as needed and are fully responsible for the originality and integrity of the content of the manuscript.

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# VARIATION IN BIOACTIVE, NUTRIENT, AND ANTI-NUTRIENT COMPOSITION OF *GONGRONEMA LATIFOLIUM* PLANT PARTS

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**Abstract:** *Gongronema latifolium* is an important edible plant in West Africa; however, comparative compositional data across its edible parts remain scarce. This study evaluated the phytochemical, proximate, mineral, and vitamin composition of dried leaves, seeds, and fruit of *G. latifolium*. Phytochemicals were quantified using gas chromatography-flame ionization detection (GC-FID), proximate composition was determined with standard AOAC methods, and minerals and vitamins were analyzed using established spectrophotometric and chromatographic techniques. Data were expressed as mean ± SD and analyzed via one-way ANOVA ($p < 0.05$). The results showed significant variation across the different plant parts. *G. latifolium* leaves contained higher levels of kaempferol (22.32 µg/ml) and flavones (17.57 µg/ml), whereas *G. latifolium* seeds exhibited greater phenolic content (22.20 µg/ml) and quercetin (15.49 µg/ml). *G. latifolium* fruit was richer in protein (26.53%), fat (5.09%), and moisture (6.72%), while leaves had higher crude fiber (26.82%) and ash (13.66%). Calcium and potassium were more abundant in seeds, whereas magnesium and sodium were higher in leaves. Vitamin C (205.20 mg/100 g), carotenoids (46.45 mg/100 g), and B vitamins (notably $B_3$ and $B_6$) were more abundant in leaves, while seeds contained higher vitamin E (6.21 mg/g) and $B_1$. Anti-nutritional factors such as phytate and oxalate were present at varying concentrations across plant parts. These findings demonstrate organ-specific biochemical distributions in *G. latifolium*, highlighting complementary nutritional properties among leaves, fruits, and seeds and providing updated compositional data for future nutritional and functional studies.

**Keywords:** *Gongronema latifolium*, bioactive compounds, nutraceutical potential, mineral content, leaves and seeds

## 1. Introduction

Plants are an essential source of nutrients and bioactive compounds that significantly benefit human health and well-being. The medicinal properties of many plant species are strongly linked to their diverse phytochemicals, including flavonoids, alkaloids, phenolic acids, saponins, terpenoids, tannins, and glycosides (Wilson and Roberts, 2014). The therapeutic effects of these plant materials mainly come from interactions among the secondary metabolites they produce. These secondary metabolites possess key properties, including antioxidant, anti-inflammatory, antibacterial, and antidiabetic effects, making them essential

for disease prevention and health promotion (Airaodion et al., 2019; Ohiagu et al., 2021). The rising global interest in medicinal and nutraceutical plants over the past few decades underscores the importance of disease prevention. This trend is driven by the pursuit of safer, more cost-effective alternatives to synthetic medicines, alongside the growing demand for functional foods that provide both nutritional and therapeutic advantages.

*Gongronema latifolium* Benth., commonly known as "Utazi" in southeastern Nigeria, is a perennial climbing shrub in the family Apocynaceae and the genus *Gongronema*. This edible plant features green leaves, yellow flowers, and a milky latex that exudes when cut; it is high in nutritional value. It is characterized by a distinctive, sharp, bitter-sweet flavor, especially when consumed fresh. The leaves are rich in lipids, proteins, vitamins, minerals, and essential amino acids (Okonkwo et al., 2025), making them an important dietary addition. It is often used in soups as a vegetable or dried and ground into powder for use as a spice (Dalziel, 1937). It is also eaten fresh and can be added to salads (Anameze et al., 2023). *G. latifolium* plays a vital role in nutrition and traditional medicine, with its dietary and ethnomedical uses common in eastern Nigeria and West Africa. Traditional healers use the leaves, stems, seeds, and fruits of this plant to treat various conditions, highlighting its cultural significance and the region's rich ethnobotanical heritage (Ojo et al., 2020; Amrelia, 2022).

Most studies on *G. latifolium* have focused on its leaves, which are rich in flavonoids, phenolic acids, alkaloids, saponins, and terpenoids, and are known to have antioxidant, hepatoprotective, and antimicrobial effects (Olufunke, 2021). However, the seeds and fruits have received comparatively less attention. There is a notable research gap, given that secondary metabolites often vary across plant organs, potentially leading to distinct biological functions. Without comparative data across plant parts, it is difficult to fully assess the species' nutraceutical and pharmacological potential.

Members of the Apocynaceae family, including *G. latifolium*, are known to accumulate flavonols, anthocyanins, and saponins in their fruits and seeds, which have significant antioxidant properties (Okonkwo et al., 2025). Although there are some indications, a comprehensive comparison of phytochemical distributions across leaves, fruits, and seeds remains limited, particularly when combined with nutritional profiling of specific organs. Scientific validation of the phytochemical content in the leaves, fruits, and seeds is crucial to support their use in food, nutraceutical, and pharmaceutical industries.

Understanding how bioactive compounds are distributed in different plant parts is essential for validating traditional knowledge and promoting the careful use of *G. latifolium* in modern applications. Comparative studies can identify specific plant parts for targeted medicinal purposes, guide their use in functional foods, and open new avenues for pharmacological research. Additionally, in regions where the plant is cultivated or harvested, gaining detailed knowledge of its bioactive components in various parts could increase its economic value through diverse applications in the nutraceutical, pharmaceutical, and food industries.

This study aimed to compare the bioactive components of *G. latifolium* leaves, fruits, and seeds, and to examine the proximate, mineral, and vitamin contents of the leaves and seeds. The specific goals were to (i) measure major phytochemicals in each part of the plant to identify differences in bioactive compounds, and (ii) analyze the nutritional aspects of the leaves, fruits and seeds, including their proximate makeup, mineral content, and

selected vitamins. By combining phytochemical analysis with nutritional evaluation, this research provides detailed insights into the organ-specific nutritional and medicinal qualities of *G. latifolium*.

## 2. Materials and Methods

### Sample collection and identification

The fruits, seeds, and leaves of *Gongronema latifolium* (**Fig. 1**) were collected from a local farm in Ntezi-Aba, Abakaliki, Ebonyi State, in eastern Nigeria during the early rainy season. The Department of Crop Science at Ebonyi State University, Abakaliki, performed scientific identification of *G. latifolium*.

### Sample preparation

The fresh *Gongronema latifolium* samples (fruit, seeds, and leaves) were thoroughly washed with distilled water to remove dust and debris, then dried in the oven at a temperature of 105 ℃ for 3 hours. The dried samples were cooled in a desiccator, ground individually into a powder, and then stored in airtight containers at room temperature until analysis.


**Fig. 1.** *Gongronema latifolium*: **A.** seeds; **B.** leaves; **C.** fruits.

**Determination of proximate composition**

Using the method specified by AOAC (2023), the crude fiber, crude protein, crude fat, moisture, and ash contents of *Gongronema latifolium* fruit, seed, and leaf samples were measured in triplicate. Ebem et al. (2021) indicated that a different approach was employed to assess carbohydrate content. The carbohydrate amount was calculated using a formula.

**Determination of moisture content**

Approximately 2 g of each powdered sample was dried in a hot air oven at 105 °C until the weight remained constant. Moisture content was calculated as the percentage of weight lost after drying and was expressed as follows:

$$\% \, Moisture = \frac{W \, initial - W \, final}{W \, initial} x \, 100 \quad (1)$$

**Determination of ash content**

Ash was determined by incinerating 2 g of sample in a muffle furnace at 550 °C for 4–6 hours until light grey ash was produced. The ash percentage was calculated based on the initial sample weight as follows:

$$\% \, Ash = \frac{Weight \, of \, ash}{Dry \, weight \, of \, sample} \, x \, 100 \quad (2)$$

**Determination of crude fat content**

Crude fat was determined using Soxhlet extraction. Approximately 2 g of dried sample was extracted with petroleum ether (boiling point 40–60 °C) for 6 hours. The solvent was then evaporated, and the lipid was weighed. The fat content was expressed as a percentage of the dry sample weight.

$$\% \, Crude \, fat = \frac{(Extract \, cup + Residue \, weight) - Extraction \, cup \, weight}{Sample \, dry \, weight} x \, 100 \quad (3)$$

**Determination of crude protein content**

Crude protein was determined using the Kjeldahl method. Samples were digested with concentrated sulfuric acid in the presence of a catalyst, then distilled and titrated. Nitrogen content was multiplied by a conversion factor of 6.25 to compute the crude protein percentage:

$$\% \, Crude \, Nitrogen = \frac{14 \, x \, N \, x \, X \, x \, 100}{1000 \, x \, V \, x \, W} x \, 100 \quad (4)$$

Hence, % protein = % total nitrogen 6.25 (conversion factor). Where, N = normality of $H_2SO_4$, X = ml of standard $H_2SO_4$ required for titration of samples, V = aliquot (ml) of digested extract taken for distillation, W = weight (g) of sample.

**Determination of crude fiber content**

Crude fiber was determined using AOAC Method. The defatted sample was first digested with dilute sulfuric acid and sodium hydroxide, then filtered, dried, weighed, and incinerated. The fiber content was calculated from the difference in weight before and after ashing:

$$\% \, Crude \, fiber = \frac{Weight \, of \, fiber}{Weight \, of \, sample} x \, 100 \quad (5)$$

**Determination of carbohydrate**

Carbohydrate content was determined by subtracting the sum of other proximate parameters, such as Nitrogen Free Extract (NFE), from 100% to find the weight by difference.

$$Carbohydrate \, (\%) = 100 - \% \, Moisture - \% \, Ash - \% \, Crude \, Faber - \% \, Crude \, Protein - \% \, Fat \quad (6)$$

**Extraction of bioactive compounds**

Exactly 20 g of each *Gongronema latifolium* sample was extracted with 100 ml of 95% ethanol using a Soxhlet apparatus for 6 hours. The extracts were filtered through Whatman No. 1 filter paper and concentrated to

dryness under reduced pressure with a rotary evaporator at 40-60 °C. The extracted oil was collected and stored in a reagent bottle for bioactive analysis by gas chromatography.

**Analysis of bioactive compounds by gas chromatography flame ionization detector (GC-FID)**

The bioactive components were analyzed using a BUCK M910 Gas Chromatograph with an FID detector as described by Imo and Uhegbu FO (2015). A RESTEK 15-metre MXT-1 column (15m x 250µm x 0.15µm) was employed. The injector temperature was set to 280°C, with a 2µl splitless injection of the sample and a linear velocity of 30 cm/s. Helium 5.0 purity served as the carrier gas, flowing at 40 ml/min. The oven temperature was set to 200°C and increased at 30°C/min to 330°C, where it was held for 5 minutes. The detector was maintained at 320°C. Exactly 0.5 µL of the sample was injected via the injector, and the analysis of *Gongronema latifolium* components lasted 45 minutes. Identification of bioactive constituents was based on the ratio of the internal standard's area to its mass relative to the detected compounds' areas. The concentrations of the bioactive components are expressed in µg/ml.

**Antinutrient analysis determination**

**Tannin content**

Tannin content was determined following the method outlined by Burns (1971). Exactly 2 g of the sample flour was extracted for 24 hours at room temperature using a mechanical shaker with 10 ml of methanol containing 1% HCl. After centrifuging at 1000 rpm for 5 minutes, 1 ml of the supernatant was collected and combined with 5 ml of vanillin-HCl reagent, prepared by mixing equal volumes of 8% HCl and 4% vanillin in methanol.

Using D-catechin as a standard (0.0–1.4 mg/ml, $R^2 = 0.995$), the absorbance of both the standard and sample solutions was measured after 20 minutes at 500 nm with a UV-Vis spectrophotometer.

**Oxalate content**

The total oxalate was determined using the Munro and Bassir (1969) method, a modification of the Dye (1956) method. Exactly 1 g ground sample was extracted three times by heating and stirring for 1 hour each in 20 $cm^3$ of 0.3 $mol \cdot dm^{-3}$ HCl. The combined extract was then diluted to 100 $cm^3$ with distilled water. From this, 5 $cm^3$ was taken and made alkaline with 1.0 $cm^3$ of 5 $mol \cdot dm^{-3}$ ammonium hydroxide. After adding a few drops of glacial acetic acid and phenolphthalein indicator until the solution turned colorless, 1.0 $cm^3$ of 5% $CaCl_2$ was added. The mixture was left for 3 hours, then centrifuged at 3000 rpm for 15 minutes. The supernatant was discarded, and the precipitate was washed with hot water, then dissolved in 2.0 $cm^3$ of warm 1.5 $mol \cdot dm^{-3}$ $H_2SO_4$ in a water bath. The solution was titrated with freshly prepared 0.01 $mol \cdot dm^{-3}$ $KMnO_4$ at room temperature until it turned pink throughout. After standing until colorless, it was warmed again and titrated until a persistent pink stain lasting at least 30 seconds appeared. These values were used to calculate the sample's oxalate content.

**Phytate determination**

About 0.2 g of the sample was weighed into 250 ml conical flasks. The sample was allowed to soak in 100 ml of 2% concentrated HCl for 3 hours, then filtered. 50 ml of the sample filtrate was placed in a 250 ml beaker, and 100ml of distilled water was added. 10 ml of 0.3% ammonium thiocyanate solution was used as an indicator and was titrated with a standard iron (111) chloride solution containing 0.00195g of iron per ml.

The following formula was used:

$$Phytic\ acid = \frac{Titre\ value\ x\ 0.00195\ x\ 1.19}{Weight\ of\ sample} x\ 100 \quad (7)$$

**Quantification of mineral content**

Mineral elements (Ca, K, Na, Mg, Fe, and Zn) were determined following AOAC (2006) procedures with slight modifications.

Sample Digestion: About 1 g of dried sample was wet-digested with a mix of concentrated nitric and perchloric acids under controlled heating until the solution became clear.

Quantification: Calcium, magnesium, iron, and zinc levels were determined via Atomic Absorption Spectrophotometry (AAS). Sodium and potassium concentrations were assessed with a flame photometer. Calibration curves were prepared using certified standard solutions. All results are reported in mg.

**Determination of vitamin content**

The analysis was conducted according to the Official Methods of Analysis published by the Association of Official Analytical Chemists (AOAC, 2005). Beta-carotene concentrations were determined following Zakaria et al. (1979). The vitamins examined include A, C, E, and the B group vitamins.

***Vitamin A (retinol)***

Exactly 1 g of the sample and the standard were mixed with 30 ml of absolute alcohol. Then, 3 ml of the 50 ml KOH solution was added, and the mixture was gently refluxed for 30 minutes. After washing with distilled water, vitamin A was extracted using 3 × 50 ml of diethyl ether. The extract was evaporated to dryness at low temperature and then dissolved in 10 ml of isopropyl alcohol. About 1 ml of standard vitamin A solution was prepared, and the dissolved extract was transferred to separate cuvettes. Their respective absorbances were measured using a Thermo Fisher Scientific spectrophotometer at 325 nm, with a reagent blank set to zero.

$$Calculation = \frac{Absorbance\ of\ sample\ x\ Concentration\ of\ standard}{Absorbance\ of\ standard} \quad (8)$$

***Vitamin B1 (thiamine) and B2 (riboflavine) determination***

Approximately 1 g of the sample was weighed into a conical flask, dissolved in 100 ml of deionized water, shaken well, heated for 5 minutes, then cooled and filtered. The filtrate was transferred to a cuvette, and the spectrophotometer was set to the specific wavelengths for each vitamin are; 261 nm for B1 and 242 nm for B2 to measure absorbance. Mineral elements (Ca, K, Na, Mg, Fe, and Zn) were determined following AOAC (2006) procedures with slight modifications.

$$Concentration\ (mg\ \%) = \frac{A\ x\ DF\ x\ Volume\ of\ cuvette}{E} \quad (9)$$

Where A = Absorbance, E = extinction coefficient (25 for $B_1$ and $B_2$), and DF = dilution factor (5)

***Vitamin $B_3$ (niacin)***

To determine Vitamin $B_3$ (Niacin), exactly 5 g of the sample was dissolved in 20 ml of anhydrous glacial acetic acid and gently heated. Then, about 5 ml of acetic anhydride was added, and the mixture was stirred until homogeneous. A few drops of crystal violet solution served as an indicator, and titration was performed with 0.1 M perchloric acid until a greenish-blue color appeared. The Vitamin $B_3$ content was calculated using the formula:

$$Vitamin\ B_3(Niacin)\ (g) = \frac{Titre\ value\ x\ 0.0122}{0.1} \quad (10)$$

***Vitamin $B_5$ (pantothiamine)***

Standard Preparation: 0.25 ml of the vitamin B5 working standard was transferred to a 25 ml volumetric flask containing a 1:9 (v/v) mixture of chloroform and methanol. The solution was gently shaken to ensure thorough mixing, then made up to the mark. Sample Preparation: 0.25 ml of the sample was measured into a 25 ml volumetric flask containing the same chloroform-methanol mixture (1:9). The flask was gently shaken to mix, and absorbance was measured at 246 nm against the blank.

***Vitamin $B_6$ (pyridoxine)***

A precise 5 g sample was dissolved in a mixture of 5 ml anhydrous glacial acetic acid and 6 ml mercury (II) acetate solution. Two drops of crystal violet served as an indicator, and titration was performed with 0.1 M perchloric acid until the green endpoint was reached. Calculation: 1 ml of 0.1 M perchloric acid equals 0.02056 g of $C_8H_{11}NO_3 \cdot HCl$.

***Vitamin $B_7$ (biotin)***

Sample Preparation involved taking 0.1 ml of the sample into a separator, adding 5 ml of water, mixing thoroughly, then adding 5 ml of chloroform. The chloroform layer was discarded, and the water layer was transferred to a 50 ml volumetric flask, filtered through anhydrous sodium sulfate, and adjusted to 50 ml with water. Aliquots of 2 ml from both the sample and blank solutions were placed into test tubes. To each, 2 ml of a 0.2% phenylhydrazine solution (in hydrochloric acid and alcohol, 1.5 v/v) was added, and the mixture was mixed well. The mixtures were heated on a water bath until nearly dry, then cooled to room temperature. Approximately 2 ml of a mixture of ammonia and alcohol (1:1 ratio) was added to each test tube, followed by 1 ml of pyridine. Absorbance was measured at 548 nm against the blank. Standard cobalamin was processed and analyzed in a similar manner to the sample.

***Vitamin $B_9$ (folic acid)***

Exactly 0.4 ml of the sample was measured and transferred to a separator. Approximately 5 ml of water was added, mixed thoroughly, followed by 5 ml of chloroform to extract the sample. The water layer was discarded, and the chloroform was collected in a dry 50 ml volumetric flask by passing it through anhydrous sodium sulphate. Then, the flask was adjusted to 50 ml with chloroform. The sample and blank solutions were placed in separate test tubes. Each received exactly 2 ml of a 0.2% phenylhydrazine solution (containing hydrochloric acid and 1.5 v/v alcohol), which was thoroughly mixed. They were heated in a water bath until nearly dry, then cooled to room temperature. About 15 ml of the mixture (ammonia and alcohol in a 1:1 ratio) was added to each test tube. Absorbance was measured at 635 nm against a blank.

***Vitamin $B_{12}$ (cobalamine)***

Exactly 30 mg of the sample was dissolved in 250 ml of deionized water. The absorbance was read at 361nm:

$$Concentration\ (mg\ \%) = \frac{A\ x\ DF\ x\ Volume\ of\ cuvette}{E} \quad (11)$$

Where A = Absorbance, E = Extinction coefficient = 25, DF = dilution factor = 5.

***Vitamin C (ascorbic acid)***

Approximately 3 g of the sample were homogenized in a 6% EDTA/TCA solution. The homogenate was then filtered and analyzed. To the homogenate, 20 ml of 30% KI solution was added, followed by 100 ml of distilled water. Then, 1 ml of a 1% starch solution was added, and titration was performed with 0.1 M $CuSO_4$ solution. The

endpoint was indicated by a black coloration. A reagent blank was also titrated for comparison. The vitamin content was calculated using the following relationship:

$$Vitamin\ C\ (mg/100\ g) = \frac{100\ x\ 0.88\ x\ Titre - Blank}{Weight} \quad (12)$$

***Vitamin E***

Total vitamin E levels were measured according to the AOAC (2023) method.

***Beta-carotene***

Beta-carotene was determined following Zakaria et al. (1979). Samples were extracted with acetone–petroleum ether (1:1 v/v), and absorbance was measured at 450 nm. The beta-carotene content was calculated using the extinction coefficient for β-carotene and expressed as mg/100 g.

**Statistical analysis**

All analyses were conducted in triplicate (n = 3). Results were expressed as mean ± standard deviation (SD). A one-way ANOVA was used to compare means across different plant parts. Differences were considered significant at $p < 0.05$. Statistical analyses were performed using SPSS software (version 23, SPSS Inc., Chicago, IL, USA).

## 3. Results

**Bioactive composition of *Gongronema latifolium* seed, leaf, and fruit**

The results in **table 1** show the concentrations of bioactive compounds in *Gongronema latifolium* seed extract. The seed contains various levels of phenolic and flavonoid compounds. Phenol was present at the highest level (22.20 ± 0.14 µg/ml), followed by gallic acid (17.39 ± 0.02 µg/ml) and quercetin (15.49 ± 0.05 µg/ml). Other significant phytochemicals present in notable amounts include catechin (12.60 ± 0.00 µg/ml) and sapogenin (11.62 ± 0.05 µg/ml). Epicatechin (14.52 ± 0.02 µg/ml) and flavone (8.64 ± 0.01 µg/ml) were also present, although phytate levels were notable (9.68 ± 0.11 µg/ml). Kaempferol and anthocyanin were among the least abundant compounds, at 5.88 ± 0.10 µg/ml and 3.90 ± 0.05 µg/ml, respectively.

The high levels of total phenols and phenolic derivatives (such as phenol, gallic acid, quercetin, catechin, and epicatechin) indicate that *G. latifolium* seed has a strong phenolic profile, which is important because phenols are key contributors to antioxidant capacity. The elevated concentrations of flavonoids (quercetin, catechin, epicatechin) suggest that *G. latifolium* seed may have significant free radical scavenging activity. The presence of sapogenins indicates potential anti-inflammatory and steroidogenic effects, whereas phytate suggests mineral chelating properties. Overall, the composition shows that *G. latifolium* seeds are a rich source of phytochemicals, potentially enhancing their ethnomedicinal significance.

The results from **table 2** show the levels of bioactive compounds in *Gongronema latifolium* leaf extract. The leaf contains various phytochemicals, with kaempferol at the highest level (22.32 ± 0.42 µg/ml), followed by flavone (17.57 ± 0.21 µg/ml) and alkaloids (14.40 ± 0.02 µg/ml). Tannins are present in significant amounts (13.46 ± 0.09 µg/ml), while terpenoids and epicatechin are moderate (8.55 ± 0.13 µg/ml and 5.77 ± 0.03 µg/ml, respectively). Other bioactive compounds detected, including rutin (5.59 ± 0.17 µg/ml), quercetin (4.89 ± 0.03 µg/ml), and phenol (2.81 ± 0.10 µg/ml), are found at lower concentrations.

**Table 1.** Bioactive composition of *Gongronema latifolium* seed

| Bioactive Components | Concentration (ug/ml) |
|---|---|
| Kaempferol | 5.88±0.10 |
| Phenol | 22.20±0.14 |
| Gallic Acid | 17.39±0.02 |
| Flavone | 8.64±0.01 |
| Epicatechin | 14.52±0.02 |
| Sapogenin | 11.62±0.05 |
| Anthocyanin | 3.90±0.05 |
| Quercetin | 15.49±0.05 |
| Catechin | 12.60±0.00 |

Values represent mean ± standard deviation (n = 3).

**Table 2.** Bioactive composition of *Gongronema latifolium* leaf

| Bioactive Components | Concentration (ug/ml) |
|---|---|
| Kaempferol | 22.32±0.42 |
| Anthocyanin | 3.71±0.22 |
| Flavone | 17.57±0.21 |
| Tannin | 13.46±0.09 |
| Rutin | 5.59±0.17 |
| Terpenoid | 8.55±0.13 |
| Phenol | 2.81±0.10 |
| Quercetin | 4.89±0.03 |
| Alkaloid | 14.40±0.02 |
| Epicatechin | 5.77±0.03 |

Values represent mean ± standard deviation (n = 3).

**Table 3.** Bioactive composition of *Gongronema latifolium* fruit

| Bioactive Components | Concentration (ug/ml) |
|---|---|
| Anthocyanin | 7.67±0.02 |
| Gallic Acid | 4.70±0.17 |
| Sapogenin | 18.20±0.06 |
| Epicatechin | 0.94±0.04 |
| Isoflavone | 2.02±0.02 |
| Catechin | 11.81±0.23 |
| **Quercetin** | 25.80±0.19 |
| Kaempferol | 11.78±0.24 |

Values represent mean ± standard deviation (n = 3).

**Table 4.** Proximate composition of *Gongronema latifolium* leaf, fruit and seed (%)

| Parameter | Leaf | Fruit | Seed |
|---|---|---|---|
| Ash | $13.66 \pm 0.22^{a}$ | $5.25 \pm 0.30^{c}$ | $8.57 \pm 0.17^{b}$ |
| Moisture | $1.93 \pm 0.05^{c}$ | $6.72 \pm 0.24^{a}$ | $2.49 \pm 0.16^{b}$ |
| Fat | $1.14 \pm 0.04^{c}$ | $5.09 \pm 0.13^{a}$ | $3.81 \pm 0.15^{b}$ |
| Fiber | $26.82 \pm 0.20^{a}$ | $13.64 \pm 0.62^{c}$ | $18.99 \pm 0.08^{b}$ |
| Protein | $17.04 \pm 0.14^{c}$ | $26.53 \pm 0.31^{a}$ | $21.14 \pm 0.19^{b}$ |
| Carbohydrate | $39.23 \pm 0.09^{c}$ | $42.77 \pm 0.11^{b}$ | $44.76 \pm 0.12^{a}$ |

Values are mean ± SD (n = 3). Different superscript letters within a row indicate significant differences ($p < 0.05$).

Oxalate (3.76 ± 0.12 µg/ml), anthocyanin (3.71 ± 0.22 µg/ml), and phytate (2.79 ± 0.11 µg/ml) are the least abundant components in the leaf sample.

The results from **table 3** show the levels of key bioactive compounds in *G. latifolium* fruit extract. Quercetin was the most abundant compound (25.80 ± 0.19 µg/ml), followed by sapogenin (18.20 ± 0.06 µg/ml). Catechin and kaempferol appeared at moderate concentrations of 11.81 ± 0.23 µg/ml and 11.78 ± 0.24 µg/ml, respectively. Anthocyanin concentration was 7.67 ± 0.02 µg/ml, and gallic acid concentration was 4.70 ± 0.17 µg/ml; both were low. Isoflavone (2.02 ± 0.02 µg/ml) and epicatechin (0.94 ± 0.04 µg/ml) were among the least abundant bioactive compounds.

The presence of quercetin and catechin emphasizes that *G. latifolium* fruit is particularly rich in flavonoids, consistent with the identified antioxidant-related metabolites. The elevated sapogenin levels imply potential steroidogenic or anti-inflammatory effects. The noticeably lower levels of epicatechin and isoflavone, compared to the seed and leaf profiles, indicate that the fruit selectively accumulates certain flavonoid groups. The phytochemical profile reveals that *G. latifolium* fruit possesses significant bioactive potential, primarily driven by its quercetin-related antioxidant activity.

**Proximate composition of *Gongronema latifolium* leaf, fruit and seed**

**Table 4** shows the proximate composition of *Gongronema latifolium* leaves, fruits, and seeds. Significant differences ($p < 0.05$) were observed among the three plant parts analyzed. The leaf exhibited notably higher ash and crude fiber levels, indicating greater mineral and structural carbohydrate content. Conversely, the fruit contained significantly more protein and lipid, while the seed was abundant in carbohydrate fractions, reflecting its role as a nutrient reserve. Moisture content also varied significantly across tissues. These results emphasize a functional nutritional distinction: leaves are richer in minerals and fiber, whereas seeds are more energy-dense in macronutrients.

**Mineral composition of *Gongronema latifolium* leaf, fruit and seed**

The mineral profile (**Fig. 2**) showed statistically significant differences ($p < 0.05$) between the fruits, leaves and seeds.

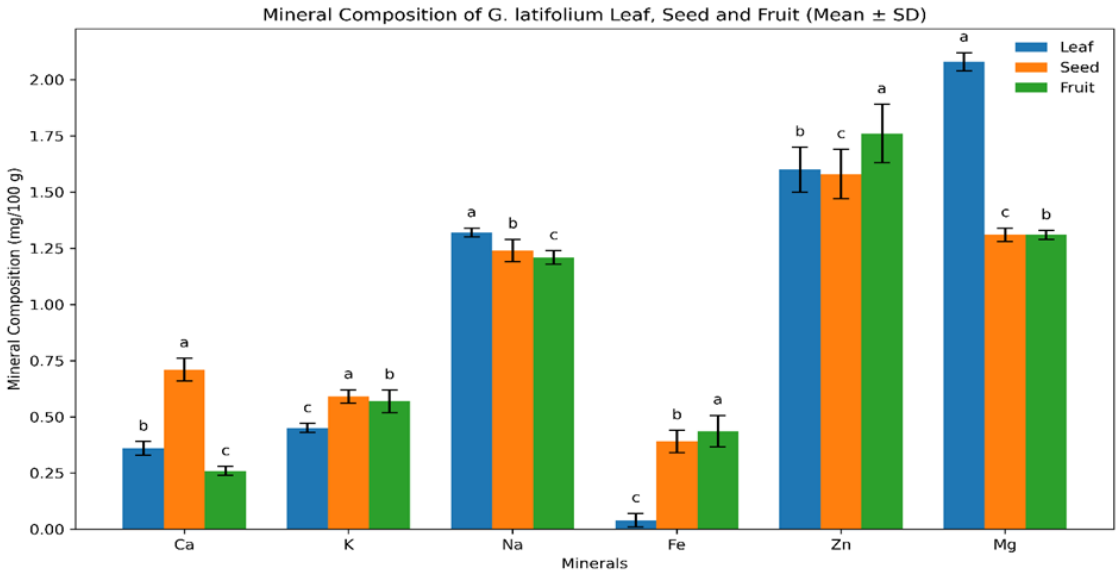


**Fig. 2.** Mineral composition of *Gongronema latifolium*

The leaves had a higher magnesium content, while the seeds contained greater concentrations of calcium, potassium, iron, and zinc. Sodium levels showed minimal variation between the two tissues. The results demonstrate organ-specific mineral distribution within *Gongronema latifolium*.

**Vitamins composition of *Gongronema latifolium* leaf, fruit and seed**

**Table 5** shows a statistically significant difference ($p < 0.05$) in vitamin profiles between leaf, fruits and seed tissues. Leaves contained higher levels of antioxidant vitamins, especially vitamin C and carotenoids, while seeds had comparatively higher amounts of vitamin $B_1$ and vitamin E. Vitamins $B_2$ and $B_{12}$ were similar in both parts. This pattern indicates organ-specific vitamin distribution within *Gongronema latifolium*.

**Phytate and oxalate content**

The results in **table 6** showed the distribution of antinutritional factors. Phytate is mainly present in seeds, which gives them their distinct nutritional properties, while oxalate is confined to leaves and affects their composition. Notably, the levels of these compounds differ significantly ($p < 0.05$) across plant parts, highlighting variation in their accumulation across organs.

**Table 5.** Vitamin composition of *Gongronema latifolium*

| Vitamin Composition | *G. latifolium* Leaf | *G. latifolium* Seed | *G. latifolium* Fruit |
|---|---|---|---|
| Vitamin $B_1$ (mg/g) | $0.42 \pm 0.02^b$ | $0.51 \pm 0.00^a$ | $0.10 \pm 0.00^c$ |
| Vitamin $B_2$ (mg/g) | $0.43 \pm 0.02$ | $0.45 \pm 0.03$ | $0.54 \pm 0.01^a$ |
| Vitamin $B_3$ (mg/g) | $0.68 \pm 0.02^a$ | $0.61 \pm 0.02^b$ | $0.33 \pm 0.03^c$ |
| Vitamin $B_6$ (mg/g) | $0.66 \pm 0.01^a$ | $0.20 \pm 0.00^b$ | $0.05 \pm 0.00^c$ |
| Vitamin $B_{12}$ (mg/g) | $0.31 \pm 0.01$ | $0.33 \pm 0.01$ | $0.11 \pm 0.02^c$ |
| Folic acid (mg/g) | $4.45 \pm 0.11$ | $4.30 \pm 0.12$ | $1.25 \pm 0.12^c$ |
| Biotin (mg/g) | $2.35 \pm 0.15^a$ | $1.46 \pm 0.14^b$ | $0.63 \pm 0.03^c$ |
| Vitamin A (mg/g) | $8.57 \pm 0.08^a$ | $5.11 \pm 0.02^c$ | $7.03 \pm 0.11^b$ |
| Vitamin E (mg/g) | $5.11 \pm 0.02^b$ | $6.21 \pm 0.07^a$ | $3.09 \pm 0.42^c$ |
| Carotenoids (mg/100 g) | $46.45 \pm 0.47^a$ | $36.83 \pm 0.06^c$ | $41.32 \pm 0.64^b$ |
| Vitamin C (mg/100 g) | $205.20 \pm 1.82^a$ | $135.25 \pm 0.40^b$ | $122.65 \pm 1.07^c$ |

Values are mean ± SD (n = 3). Different superscript letters within a row indicate significant differences ($p < 0.05$).

**Table 6.** Antinutritional factors in *Gongronema latifolium* leaf and seed

| Parameter | Leaf | Seed | Fruit |
|---|---|---|---|
| Phytate (µg/ml) | $2.79 \pm 0.00^b$ | $9.68 \pm 0.11^a$ | $0.82 \pm 0.10^c$ |
| Oxalate (mg/100 g) | $3.76 \pm 0.12^a$ | $0.02 \pm 0.00^c$ | $0.03 \pm 0.00^b$ |
| Tannin (%) | $1.59 \pm 0.00^b$ | $3.59 \pm 0.01^a$ | $0.22 \pm 0.00^c$ |

Values are mean ± SD (n = 3). Different superscript letters within a row indicate significant differences ($p < 0.05$).

## 4. Discussions

### Bioactive composition of *Gongronema latifolium*

The study of the bioactive components in *Gongronema latifolium* revealed notable differences in secondary metabolite levels among seed, leaf, and fruit samples. These differences suggest a varied distribution of phytochemicals, likely due to the plant's adaptive metabolism, tissue-specific functions, and growth stages.

### Phenolic compounds and flavonoids

The study revealed distinct organ-specific differences in the distribution of phenolic and flavonoid compounds in *Gongronema latifolium*. The seed exhibited the highest total phenolic content at 22.20 µg/ml, significantly exceeding the leaf's level of 2.81 µg/ml, while the fruit showed moderate gallic acid levels at 4.70 µg/ml. Their variation indicates tissue-specific regulation of the phenylpropanoid pathway, which is vital for phenolic biosynthesis.

The increased phenolic content observed in the seed may contribute to oxidative defence during dormancy and early germination. Seeds are particularly vulnerable to the accumulation of reactive oxygen species (ROS) during desiccation and metabolic reactivation. Phenolic compounds act as hydrogen-donating antioxidants, stabilizing lipid membranes and reducing peroxidative damage (Rajashekar, 2023). Recent studies in seed metabolomics have shown that higher phenolic levels are positively associated with improved seed viability and stress resilience under environmental stresses such as drought and temperature fluctuations (Ware et al., 2024; Dossou et al., 2023). However, the considerable build-up of phenolics in *G. latifolium* seeds may represent an adaptive biochemical strategy to ensure reproductive success.

Kaempferol was the most abundant flavonoid in the leaves at 22.32 µg/ml, while quercetin was the dominant one in the fruit at 25.80 µg/ml. Flavonols, such as kaempferol, tend to accumulate in photosynthetic tissues, where they act as UV-B protectors and help regulate photooxidative stress (Moustaka and Moustakas, 2025). Their presence in leaves helps defend against high light levels and environmental oxidative stress. A recent study shows that increased leaf flavonol levels enhance photoprotection by interacting with ROS generated in chloroplasts and modulating redox signaling pathways (Postiglione et al., 2024). The high levels of quercetin in the fruit may relate to its roles in pigmentation, defence against pathogens, and fruit ripening regulation. Quercetin and its derivatives are linked to fruit protection through antimicrobial effects and modulation of oxidative bursts during ripening (Zheng et al., 2025; Aghababaei and Hadidi, 2023). This variation in flavonoid distribution highlights functional specialization among different plant organs.

Furthermore, the high levels of phenolics and flavonoids across all organs support the traditional use of *G. latifolium* for conditions linked to oxidative stress. The variety of flavonoid subclasses indicates the potential for synergistic antioxidant effects, as polyphenol mixtures generally show stronger radical-scavenging capacity than individual compounds.

### Tannins, alkaloids, and terpenoids

The alkaloid concentration in the leaves (14.40 µg/ml) aligns with previous studies on *Gongronema latifolium* leaves by Okochi et al. (2024) and Okonkwo et al. (2025), which reported the presence of nitrogen-based secondary metabolites in these leaves. The recorded alkaloid level slightly exceeds that of various tropical medicinal plants. This difference is likely due to ecological factors

such as soil type, climatic stress, plant age, or extraction methods, as reported by Isah (2019). These environmental and methodological factors significantly influence secondary metabolite production. The detection of both tannins (13.46 µg/ml) and alkaloids supports the Optimal Defence Theory, which proposes that plants mainly allocate defensive compounds to tissues most vulnerable to herbivores and environmental stress (Isah, 2019). *G. latifolium* and other medicinal plants demonstrate similar organ-specific accumulation patterns (Edeoga et al., 2005; Ezeani et al., 2022), indicating adaptive metabolic distribution. Their higher concentrations in leaves probably reflect their role as the primary site for biotic interactions. From an ethnopharmacological perspective, the high alkaloid content may partly explain the traditional use of "Utazi" for hypertension and malaria. Although pharmacological testing was not performed in this study, plant alkaloids are recognized for their antihypertensive properties through calcium channel modulation and for their anti-plasmodial activity via DNA intercalation and disruption of parasitic metabolism (Rajabian et al., 2022). The abundance of alkaloids in the leaves provides a biochemical basis for their medicinal properties. Additionally, terpenoids (8.55 µg/ml) identified in the leaves are known to play roles in defence and anti-inflammatory actions, possibly working synergistically with polyphenols, as suggested by prior phytochemical studies (Ojo et al., 2020). However, further chromatographic and bioassay-guided research is necessary to identify specific active compounds.

**Catechin, epicatechin, and sapogenin**

The distinct patterns of catechin and epicatechin distribution in seeds, fruit, and leaves indicate organ-specific regulation of the phenylpropanoid pathway. The much higher epicatechin level in seeds (14.52 µg/ml) compared to fruit (0.94 µg/ml) suggests its possible role in proanthocyanidin synthesis, which may help reinforce and protect seed coats during dormancy. The fruit exhibited the highest sapogenin concentration (18.20 µg/ml), indicating its potential as a source of triterpenoid derivatives. Since sapogenins are precursors to steroidal compounds and are linked with hypoglycemic activity, this supports the findings of Ogunyemi et al. (2022) and Okonkwo et al. (2025), who reported that triterpenoid saponins from *Gongronema latifolium* inhibited α-amylase and α-glucosidase. Although enzyme assays were not performed in this study, the elevated sapogenin levels could partly explain the fruit's reported antidiabetic properties.

**Phytate, oxalate and tannin**

The high phytate levels in the seeds (9.68 µg/ml) highlight their role as the main phosphorus reserve in reproductive tissues. During germination, phytate provides essential phosphorus and energy, supporting early seedling development (Isah, 2019). This accumulation pattern is common in leguminous and medicinal plant seeds, where phytic acid functions as both a nutrient store and mineral regulator. Although elevated phytate levels can hinder the absorption of minerals such as iron and zinc by forming insoluble complexes, this presents a trade-off between plant health and human nutrition; however, the levels measured are comparable to those in edible tropical plant seeds (Ukorebi, 2021). Traditional processing methods, such as cooking or fermentation, may reduce mineral-binding effects. Oxalate was detected only in the leaves (3.76 µg/ml), likely as calcium oxalate crystals, which protect the plant by deterring herbivores, causing mechanical irritation, and helping to regulate calcium and ion-balance. While moderate, oxalate remains nutritionally significant

because excessive intake can increase the risk of kidney stones in sensitive individuals. However, oxalate levels are similar to those in common leafy vegetables (Okonkwo et al., 2025). The leaves possess notable phytochemical and medicinal properties, and proper processing, along with moderate consumption, can ensure safety. Overall, the distinct distribution of phytate in seeds and oxalate in leaves reflects the plant's specialized functions, balancing growth needs with potential human health risks.

**Proximate composition of *Gongronema latifolium***

The proximate analysis showed distinct nutrient distributions among the leaves, fruits, and seeds of *Gongronema latifolium*. The leaves have a significantly higher ash content (13.66%) than the seeds (8.57%) and the fruits (5.25%), indicating greater mineral accumulation in the leaves. Recent studies report ash contents in *G. latifolium* leaves ranging from 8.5% to 12.4% (Adeyeye & Olaleye, 2020; Nneoyi-Egbe et al., 2024), with the current value at the upper end of this range. Their variation could result from differences in soil, environmental factors, or sample processing techniques. The higher ash content observed in the leaves likely reflects their active metabolism, as minerals are essential for photosynthesis and enzymatic functions.

The fruit samples exhibited significantly higher levels of protein (26.53%), lipids (5.09%), and moisture (6.72%) compared to leaf and seed samples ($p < 0.05$). This elevated protein content indicates that the fruit could serve as a valuable dietary source. While Okochi et al. (2024) reported lipid levels of approximately 8%, the lower lipid content observed in this study may be attributed to varietal differences or the sensitivity of the extraction method. The dominance of macromolecular reserves in seeds reflects typical seed physiology, where storage compounds accumulate to support germination. Therefore, the distribution pattern observed suggests functional specialization rather than random nutrient allocation.

The leaves exhibited significantly higher ash (13.66%) and fiber (26.82%) contents than both fruit and seed samples ($p < 0.05$), indicating a richer mineral and structural carbohydrate profile. Previous research on *G. latifolium* leaves reported lower fiber levels, ranging from 2% to 12% (Adeyeye & Olaleye 2020; Okochi et al., 2024; Joshua Ndukwe et al., 2024). The higher fiber content observed here could be due to genetic differences, environmental factors, or variations in analytical methods. Besides their dietary role, the increased fiber in the leaves likely contributes to structural and protective functions, helping to reinforce vegetative tissues against mechanical stress and environmental challenges.

**Mineral composition of *Gongronema latifolium***

The mineral analysis confirms the organ-specific distribution of nutrients. Magnesium levels were significantly higher in leaves (2.08 mg/100 g) compared to seeds (1.31 mg/100 g) and fruit (1.37 mg/100 g). Recent studies on tropical leafy vegetables (Alabi et al., 2022; Nneoyi-Egbe et al., 2024) have also reported similar values. Since magnesium is essential for chlorophyll formation and ATP-dependent processes, its higher concentration in leaves reflects its vital role in photosynthesis.

*Gongronema latifolium* seeds contained higher levels of calcium (0.71 mg/100 g) and potassium (0.59 mg/100 g). While earlier studies reported higher iron content in *G. latifolium* leaves (Edeoga et al., 2005), the current results indicate that iron is more abundant in fruits (0.44 mg/100 g). This discrepancy could be due to differences in soil

mineral composition or regional factors. The elevated iron in seeds likely supports mitochondrial respiration during embryo development.

The similar zinc levels in both plant parts examined suggest a regulated overall distribution rather than localized accumulation. The zinc content, 1.60 mg/100 g in leaves and 1.58 mg/100 g in seed, reported in this study, differs markedly from the estimated average daily dietary zinc intake range of 5.6 to 13 mg/day in infants and children and 8.8 to 14.4 mg/day in adults aged 20 to 50 years (FAO, 1990). Recent phytochemical studies (Okochi et al., 2024; Oko et al., 2018) also noted minimal variation in zinc across different plant organs, supporting the idea of strict homeostatic regulation. Understanding these mineral concentrations requires caution. Although the differences are statistically significant, the actual values are relatively low compared to dietary recommendations. Therefore, while *G. latifolium* can contribute to mineral intake, it should not be regarded as a primary mineral source without considering bioavailability.

**Vitamins composition of *Gongronema latifolium***

The vitamin profile of *Gongronema latifolium* varies across plant parts (leaves, fruits, and seeds), suggesting specialized metabolism rather than a consistent distribution. The leaves have higher levels of water-soluble vitamins such as $B_3$, $B_6$, and vitamin C, along with carotenoids, while the seeds are comparatively richer in vitamin E and show a slight increase in vitamin $B_1$.

**Vitamins B Complex**

The high levels of vitamins $B_3$ (niacin) and $B_6$ (pyridoxine) in the leaves are probably related to their functions in primary metabolism and enzymatic redox reactions within photosynthetically active tissues. Recent research on tropical leafy vegetables has also noted similar increases in B-complex vitamins (Nneoyi-Egbe et al., 2024; Okochi et al., 2024). The variation in $B_6$ levels observed in this study exceeds that previously reported, indicating that environmental factors or different varieties may affect $B_6$ concentrations.

The levels of vitamins $B_1$ (thiamine) and $B_2$ (riboflavin) were similar in both leaves and seeds of the plant. However, the seed had slightly higher $B_1$ (0.51 mg/g) than the leaf (0.42 mg/g). The marginal increase in vitamin $B_1$ in seeds may suggest its role in carbohydrate metabolism during germination. Thiamine-dependent enzymes are essential for glycolysis and the pentose phosphate pathway, both active during early seed development. The consistent riboflavin ($B_2$) levels across tissues, especially in the fruit, indicate uniform distribution, underscoring its importance in oxidative metabolism (Aminul et al., 2025). Vitamin $B_{12}$ levels were low and stable in both plant parts. As higher plants do not synthesize cobalamin, its presence may result from microbial associations or environmental contamination; therefore, these findings should be interpreted with caution.

**Biotin and folic acid**

Folate and biotin levels were slightly higher in the leaves compared to the fruits and seeds. This pattern indicates a greater need for folate in rapidly dividing and metabolically active tissues, especially for nucleic acid synthesis. While previous studies on *Gongronema latifolium* confirmed the presence of folate (Balogun et al., 2016; Edelman and Colt, 2016), recent research has provided few direct quantitative comparisons, highlighting a gap in comprehensive micronutrient data. Although the differences observed are statistically significant, they are relatively minor in practical terms. While *G. latifolium*

leaves may contribute to dietary folate intake, further studies on bioavailability are necessary before conclusive nutritional claims can be made.

**Carotenoids and fat-soluble vitamins A and E**

Significant levels of carotenoids, vitamins A and E were found in the plant parts, highlighting the role *of Gongronema latifolium* as a natural antioxidant source. The leaves exhibited higher concentrations of carotenoids (46.45 mg/100 g) and vitamin A (8.57 mg/g) compared to the seeds, which contained 5.11 mg/g and 36.83 mg/100 g for leaves and seeds, respectively. Vitamin A and its precursors, the carotenoids, are essential for vision, immune system function, and epithelial tissue health (Maqsood et al., 2020; Okochi et al., 2024). The reported values are among the highest for tropical medicinal plants, suggesting vigorous pigment production in *G. latifolium* leaves. However, *G. latifolium* seeds showed higher vitamin E (tocopherol) levels, which is consistent with their physiological role in protecting polyunsaturated fatty acids in oil-rich tissues (Okonkwo et al., 2025). The increased vitamin E in seeds aligns with their higher lipid content, as confirmed by proximate analysis, suggesting a coordinated antioxidant response in storage tissues.

**Vitamin C (ascorbic acid)**

Vitamin C levels were significantly higher in the leaves compared to the seeds. However, high levels of ascorbic acid have been reported in fresh *Gongronema latifolium* leaves (Nneoyi-Egbe et al., 2024; Alabi et al., 2022), although the exact values vary depending on processing and testing methods. The increased ascorbate levels in leaves likely play a role in reducing photooxidative stress during photosynthesis. However, high vitamin C levels can enhance antioxidant capacity; this study did not specifically assess its bioactivity or therapeutic effects.

## Conclusions

This study highlights notable differences in the nutritional content and bioactive compounds among dried *Gongronema latifolium* leaves, seeds, and fruit. The leaves contain higher levels of vitamins, crude fiber, and certain phytochemicals, whereas the seeds are richer in protein, carbohydrates, and specific minerals. This demonstrates the functional specialization within the plant. These findings deepen our understanding about the biochemical composition of *G. latifolium* and offer valuable insights into its potential health benefits. However, it is essential to acknowledge the study's limitations, particularly the lack of information on bioavailability and therapeutic efficacy. Therefore, further research is necessary to explore how these compositional differences influence dietary and therapeutic applications. The comparative data presented support more detailed investigations into the nutritional and pharmacological significance of *G. latifolium*, paving the way for its potential use in dietary and medicinal contexts.

## Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

## Author Contributions

Conceptualization: Emmanuel C. Ebem and Joshua Ndukwe; Data analysis: Emmanuel C. Ebem and Joshua Ndukwe; Interpretation of data: Emmanuel C. Ebem and Joshua Ndukwe; Drafting the original manuscript: Emmanuel C. Ebem; Review and editing: Emmanuel C. Ebem and Joshua Ndukwe. All authors

contributed to the article and approved the submission.

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# EXPLORATORY ASSESSMENT OF BEHAVIORAL ALTERATIONS IN YOUNG RATS AFTER INTRAUTERINE BENZYDAMINE EXPOSURE

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**Abstract:** Substance use is a major global public health issue with important medical, social, and economic consequences. Drug use during pregnancy is particularly concerning, as this period involves increased biological vulnerability for both mother and fetus. Intrauterine exposure to psychoactive substances may lead to severe and sometimes irreversible effects. Benzydamine misuse for recreational purposes, often referred to as "benzydamine trips," has been documented globally, particularly among adolescents. It was hypothesized that intrauterine benzydamine exposure would lead to sex-specific deficits in cognitive and exploratory behavior in adolescence. In this study, 40 female Wistar rats were mated (2:1 ratio) and randomly assigned to two groups: one received recreationally relevant doses of benzydamine during pregnancy. Offspring behavior was assessed using the Novel Object Recognition (NOR) test for memory and the Elevated Plus Maze (EPM) for exploratory and anxiety-related behavior. Biochemical analyses were performed at the end of the experiment. The EPM showed no significant group differences in time spent in open or closed arms. Females displayed slightly higher locomotor activity, and treated females spent more time in open arms compared to males and untreated females. This is the first study evaluating offspring exposed prenatally to recreational doses of benzydamine. The findings suggest that benzydamine may impair memory and alter behavior, with more evident effects in females.

**Keywords:** benzydamine, novel object recognition, discrimination index, elevated plus maze, behavior

## 1. Introduction

Substance use is a major public health problem worldwide, with significant medical, social and economic implications. This category includes both classic illicit substances, such as opioids, cannabis, cocaine or amphetamines, and certain over-the-counter (OTC) medications, which, although perceived as safe, can have psychoactive effects and can be addictive when used inappropriately (Chiappini et al. 2021, Ősz et al. 2023, La Nou et al. 2024). The increased accessibility of these substances, associated with insufficient information for the population, contributes to the trivialization of consumption and the underestimation of short- and long-term risks. In parallel, many OTC drugs, such as those containing codeine, pseudoephedrine or sedative antihistamines, are frequently used outside therapeutic indications, in high doses or for prolonged periods, precisely because of their euphoric or sedative effects (Ősz et al. 2023). This practice is often found among

teenagers and young adults but is not exclusive to these categories.

A particularly worrying aspect is the use of narcotics and potentially abusive drugs during pregnancy. Pregnancy is a period of increased biological vulnerability, for both mother and fetus, and intrauterine exposure to psychoactive substances can have severe and sometimes irreversible consequences (Franks et al. 2020). Narcotic substances easily cross the placental barrier, affecting fetal development, especially during critical stages of organogenesis and maturation of the central nervous system.

A relevant example of an often overlooked OTC drug with narcotic potential is benzydamine, a topical nonsteroidal anti-inflammatory drug commonly used as a solution, spray, or vaginal suppository. Although indicated for its analgesic and antiseptic properties, benzydamine can produce psychoactive effects when administered orally in high doses, including visual and auditory hallucinations, confusion, psychomotor agitation, and delusional behavior (Ősz et al. 2023). These effects are explained by the substance's interaction with serotonergic and dopaminergic receptors.

Benzydamine abuse has been reported particularly among adolescents and young adults, favored by its high accessibility, low cost, and lack of strict release control, compared to other psychoactive substances (Ősz et al. 2023). Epidemiological studies from Central and Eastern Europe and Latin America indicate an increase in the recreational use of benzydamine, which is sometimes perceived as a "legal" alternative to classic illicit drugs (Opaleye et al 2009, Zaprutko et al. 2016).

From an epidemiological perspective, the use of OTC drugs with narcotic potential represents a significant component of substance abuse, especially among young people. Data from the World Health Organization and the European Monitoring Centre for Drugs and Drug Addiction (EMCDDA) suggest that between 3% and 6% of adolescents have experienced at least once the misuse of an OTC drug with psychoactive effects [8]. In women of reproductive age, the exact prevalence is likely underestimated, due to underreporting and low perception of risk.

In the context of pregnancy, epidemiological data on the use of benzydamine is limited, but the existence of maternal neuropsychiatric effects raises questions about fetal safety, especially in the case of uncontrolled systemic administration. Given the lack of robust clinical studies on prenatal exposure, the precautionary principle requires avoiding the use of benzydamine orally or in inappropriate doses during pregnancy (Ősz et al. 2023).

The inclusion of benzydamine in the discussion of "atypical" drugs highlights the need to broaden the classical definition of substances of abuse and strengthen prevention strategies to include not only illicit drugs but also OTC medications. From a public health perspective, recognition and monitoring of this phenomenon are essential to reduce the associated risks, especially in vulnerable populations.

To our knowledge, this is the first study that aims to verify the effects of benzydamine on the central nervous system in the offspring of female rats treated during pregnancy with benzydamine at doses used for recreational purposes.

## 2. Materials and Methods

### Animals and treatment

Benzydamine (Tantum Rosa, powder for solution for vaginal irrigation, Angelini Pharma, Italy) was purchased from the Romanian pharmaceutical market. Forty female Wistar rats (mean body weight: 253 ± 23 g), 6-month-old were obtained from the Animal Facility of George Emil Palade University of

Medicine, Pharmacy, Science and Technology of Târgu Mureș.

Female Wistar rats were housed with males at a ratio of 2:1, and pregnancy was confirmed by the presence of sperm in vaginal smears (gestation day 1 – GD1). The sample size (n=40 females) was chosen based on previous exploratory behavioral studies to ensure adequate power for detecting sex-specific differences in offspring. Pregnant female rats were randomly allocated to two experimental groups: one received benzydamine at a dose representative of recreational human exposure (BZY, 261 mg/kg), while the control (CTRL) group received vehicle only. Medication was included in the food and administered daily, between 8:00 AM and 10:00 AM. This dose represents a recreationally relevant human exposure.

Body weight was recorded once a week for dose adjustment. Offsprings from the two maternal treatment groups were housed in pairs according to their mothers' treatment and gender. Offspring were handled daily from postnatal day 7 onward to minimize stress and reduce the risk of maternal rejection. Animals were maintained under standard environmental conditions (12 h light/dark cycle, ambient temperature 20 ± 2 °C, relative humidity 60% ± 10%), with unrestricted access to standard laboratory rodent pellets and water throughout the study. At the end of the experiment, all animals were decapitated under anesthesia with isoflurane (3%) in order to collect samples of blood for further investigations.

All experimental procedures were conducted in accordance with the European Directive 2010/63/EU on the protection of animals used for scientific purposes. The study protocol was reviewed and approved by the Ethics Committee for Scientific Research of the George Emil Palade University of Medicine, Pharmacy, Science and Technology of Târgu Mureș (approval no. 2073/15.02.2023).

**Behavioral assessment**

Behavioral assessments on offsprings were done in different days when their age corresponded to the adolescence period in humans (4 weeks). Behavioral testing and video analysis were performed by researchers unblinded to the treatment groups.

**Novel Object Recognition**

Novel object recognition test (NOR) was used to evaluate memory. The objects used were about the same height. They were placed at an equal distance of 30 cm from the corners, diametrically opposite and after each analysis were wiped with 70% alcohol to limit the appearance of any olfactory bias.

Initially, two identical objects were placed for each rat for a period of 7 minutes. At the end of this period, they were returned to the personal cage, at which point the box and objects were cleaned. Subsequently, after 4 hours, the procedure was repeated, but one of the familiar objects was replaced with a new one for evaluating the retention process. This test lasted for 5 minutes. Exploratory activity was considered valid when rodents sniff objects or touch them with their front paws but without leaning on or sitting on them. For behavioral evaluation EthoVision XT, Noldus IT, Wageningen, The Netherlands, version 11.5 was used. Discrimination index (DI) represents the difference of exploration time of the new object (EB) compared to the familiar object (EA) compared to the total time spent exploring the two objects in the retention process, DI = (EB - EA) / (EA + EB) (Rajagopal et al. 2014, Antunes and Biala 2012).

**Elevated Plus Maze**

The assessment of the exploratory behavior was performed with the aid of the Elevated Plus Maze test (EPM). This device comprises a plus-shaped maze, with two

opposite open arms (50 × 10 cm) and two closed arms (50 × 10 × 40 cm). The distance from the floor was set at 60 cm height. The rats were placed at the crossroad, facing the open arm. After each rat, the maze was cleaned with 70% alcohol, and the activity was recorded for 5 min. The time spent in open and/or closed arms and in the center zone, rearing, and head dipping were recorded (Kraeuter et al. 2019, Knight et al. 2021).

**Biochemical analysis**

Biochemical analyses were performed using an Element RC Clinical Chemistry Analyzer (Scil), using the General Health Rotor for albumin (ALB), total protein (TP), globulins (GLOB), total bilirubin (TB), aspartate aminotransferase (AST), alanine aminotransferase (ALT), alkaline phosphatase (ALP), creatinine (CREA), Blood Urea Nitrogen (BUN), glucose (GLU), total cholesterol (TC), calcium ($Ca^{2+}$), phosphate (PHOS), potassium ($K^{+}$), and sodium ($Na^{+}$), measurements.

**Statistical analysis**

Statistical analysis was performed using GraphPad Prism software (San Diego, California USA, ver. 9). The Shapiro-Wilk test was performed to test the normality of the data and values were expressed as mean ± SEM or median [min – max] where appropriate. For the statistical analysis a t test or Mann-Whitney test were performed. The significance level was set at *p* value less than 0.05.

## 3. Results and discussions

To our knowledge, this is the first study to verify the effects of intrauterine exposure to recreational doses of benzydamine on the central nervous system of rat offspring. Our findings suggest that prenatal exposure leads to distinct behavioral alterations in adolescence, characterized by memory deficits and a reduction in anxiety-like inhibitory avoidance, particularly in females.

In the NOR test, differences in discrimination indices were observed, though the statistical significance was not definitive across all groups. To evaluate the motor activity in the retention trial, the t test revealed no significant differences between groups. For better representation we compared control group and experimental groups separately (CTRL F (858.7 ± 42.04 cm) vs EXP F (878.3 ± 77.73 cm), CTRL M (637.4 ± 169.1 cm) vs EXP M (752.9 ± 48.82 cm) as shown in **Figure 1A** and **1B**. Following the determination of DI (**Fig. 1C** and **1D**), no significant differences were found between the groups included in the study. However a better result was observed for the male groups, which may be influenced by the distance traveled (CTRL F (-0.333 ± 0.108) vs EXP F (-0.278 ± 0.0855), CTRL M (0.331 ± 0.41) vs EXP M (0.055 ± 0.118)). The overall trend suggests that benzydamine may alter memory retention processes. This cognitive impairment can be interpreted through benzydamine's ability to stabilize neuronal membranes and inhibit basal excitability. Such alterations in neuronal signaling during critical periods of hippocampal development may disrupt the synaptic plasticity required for long-term object recognition.

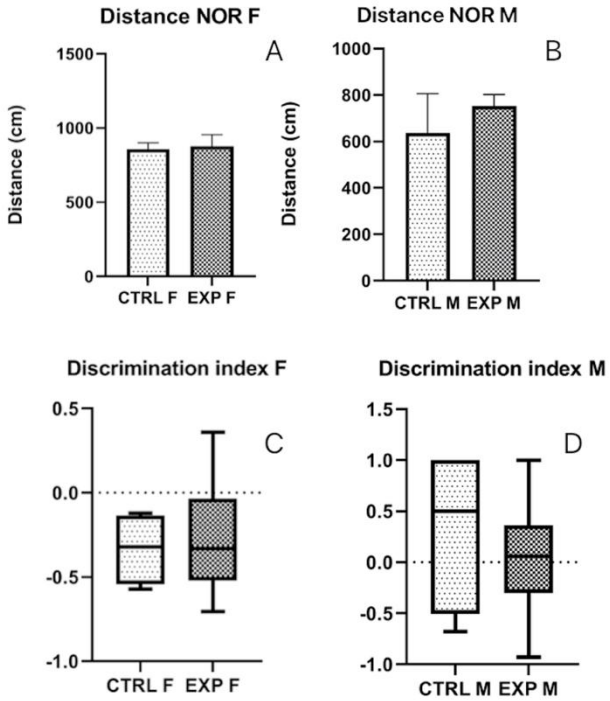


**Fig. 1.** Distance and discrimination index between the offsprings resulted from treated and not treated mothers. CTRL F – female control, EXP F – experimental female, CTRL M – male control, EXP M – experimental male, NOR – novel object recognition.

No experimental studies were found in the literature evaluating the effect of benzydamine in the context of NOR test, but review studies suggest a negative effect on memory (Ősz et al. 2023), and further studies should evaluate whether this is due to memory impairment or impairment of interest in exploration. Regarding motor activity, due to the toxicological profile, moderate doses of benzydamine favor psychomotor agitation, and high doses can lead to an inhibition of motor activity, by inducing a state of confusion and disorientation. The results of the present study do not show significant differences between groups, but it is observed that in both the treated and control groups, the motor activity of females is slightly increased.

The EPM did not reveal any statistically significant difference between the groups for the time spent in open arms, **Figure 2A-D**. However, in both sexes, the time spent in the open arms was lower in the control groups than in the experimental groups, consistent with the distance traveled. The head dipping frequency differed between the CTRL F (4.5 ± 1.258 s) and EXP F (10.83 ± 1.424 s) as can be seen in Figure 2K. It is worth mentioning that rearing time was longer for EXP M than for the CTRL M, Fig. 2N.

Locomotor activity in the EPM is similar to that in the NOR test, with females traveling slightly longer distances. Regarding the time spent in the open arms of the maze, it is greater for treated females, both compared to males and to untreated females. One study from the literature demonstrated that females had a less anxious behavior (Knight et al. 2021). These data should be viewed in correspondence with the activity in the closed arms, where times are similar for all groups, which suggests that these data are not due to hyperactivity, but to exploratory behavior. While this initially appears as an anxiolytic effect, it likely represents behavioral disinhibition. The rearing behavior supports a shift in how these animals process novel environments and risk.

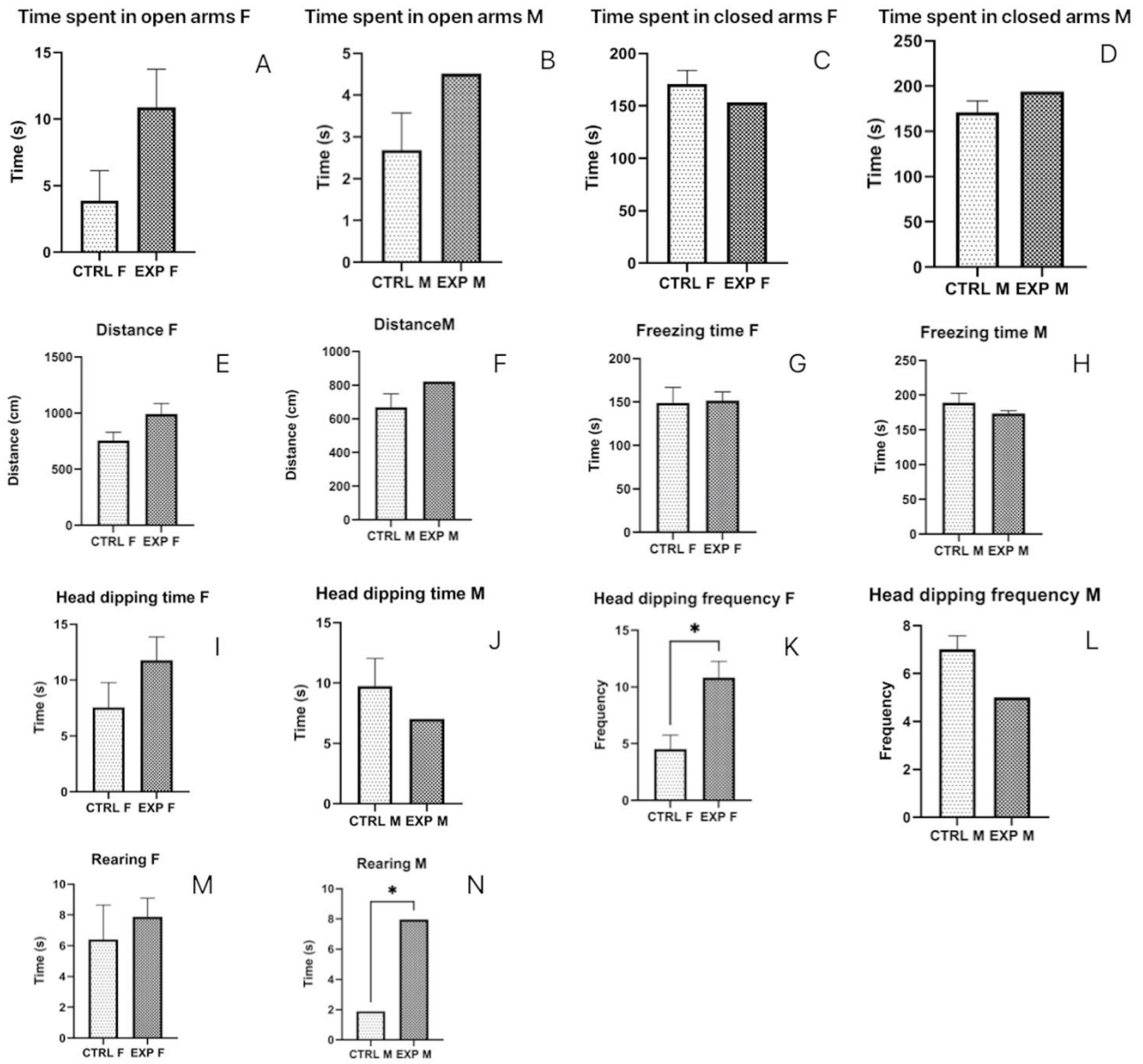


**Fig. 2.** Parameters comparison between between the offsprings resulted from treated and not treated mothers. CTRL F – female control, EXP F – experimental female, CTRL M – male control, EXP M – experimental male. $^{*}p < 0.05$.

The increased exploratory activity and head dipping suggest an interference with the limbic-striatal reward pathways, potentially mediated by benzydamine's structural similarity to indoleamines like serotonin and its reinforcing properties.

Recent studies indicate that benzydamine also acts via a central cannabinoidergic mechanism, specifically interacting with CB1 receptors to modulate glutamatergic transmission in the prefrontal cortex. In rat brain slices, benzydamine-induced longterm depression-like responses in the prelimbic cortex-to-nucleus accumbens circuitry were significantly reduced by the CB1 receptor antagonist AM251 (Avvisati et al. 2018).

A study demonstrated that methadone administration produced effects in offspring of both sexes, whereas buprenorphine administration influenced behavior only in females, as observed in the EPM test (Chen et al. 2015). It has also been reported that exposure to methadone may impair long-term spatial memory in offspring (Kongstorp et al.

2020). Furthermore, prenatal exposure to methadone or buprenorphine has been associated with an increased risk of anxiety-like behaviors in offspring (Nyberg et al. 2024). Similarly, exposure to amphetamine was linked to reduced performance in the Morris Water Maze test, although this effect was observed only in females (Šlamberová et al. 2014).

Biochemical analyses indicated some systemic alterations, most notably the increase in alkaline phosphatase (ALP) in both males and females exposed to benzydamine and a decrease in blood urea nitrogen (BUN) in males. The other parameters were not statistically different (**Table 1**). The lower BUN levels ($p = 0.022$) in treated males might indicate shifts in protein metabolism or renal filtration efficiency following prenatal drug stress.

Higher ALP levels in young rats often reflect altered bone metabolism or hepatic stress during rapid growth phases. Numerous scientific studies highlight the fact that the use of narcotics can have significant toxic effects on the liver. Following hepatic biotransformation processes, many narcotics are transformed into reactive metabolites that may have an increased cytotoxic potential, with changes in biochemical parameters. One mechanism by which these changes in liver function occur is represented by oxidative stress, and long-term exposure can cause alterations in liver function (Atici et al. 2005, Valente et al. 2012). An increasing trend in AST levels was also observed in females exposed to benzydamine, but the ALT levels were not altered.

Consequently, the perception of benzydamine as a "safe" over-the-counter medication contributes to its misuse. However, our results underscore the potential for irreversible behavioral alterations in offspring following recreational use during pregnancy.

This study has several limitations that should be considered when interpreting the findings. First, the work was explicitly designed as an exploratory assessment and as acknowledged in the manuscript, it represents the first attempt to examine behavioral outcomes in offspring after prenatal exposure to recreational doses of benzydamine, which limits direct comparison with previous experimental literature and makes the current results necessarily preliminary. Second, the behavioral assessment was also limited in scope. The study relied on only two tests, NOR for memory-related performance and EPM for exploratory or anxiety-like behavior, both performed during a single developmental window corresponding to adolescence at approximately 4 weeks of age. While these paradigms are established and useful, they capture only a narrow portion of neurobehavioral function and do not allow firm conclusions regarding broader domains such as social behavior, impulsivity, reward sensitivity, attention, sensorimotor gating, spatial learning, or long-term executive function. Third, as was explicitly stated above, behavioral testing and video analysis were performed by researchers unblinded to treatment groups, which can introduce bias. In addition, the use of a single benzydamine dose, the absence of pharmacokinetic confirmation of fetal exposure, the lack of mechanistic brain analyses, and the limited characterization of maternal factors constrains causal interpretation and translational generalization.

**Table 1.** Biochemical parameters

| Parameter | Gender | Control | Experimental | *p* Value [c] |
|---|---|---|---|---|
| ALB | F | 3.900 ± 0.0817 | 3.940 ± 0.0476 | 0.668 |
| | M | 3.700 [3.600 – 3.700] | 3.700 [2.900 – 4.000] | |
| TP | F | 5.500 ± 0.1225 | 5.630 ± 0.07753 | 0.264 |
| | M | 5.450 ± 0.0289 | 5.283 ± 0.0908 | 0.407 |
| GLOB | F | 1.800 [1.800 – 2.000] | 1.600 [1.500 – 2.200] | 0.15 |
| | M | 1.850 [1.800 – 1.900] | 1.600 [1.200 – 2.000] | 0.062 |
| AST | F | 82 [69 – 87] | 88.50 [81 – 141] | 0.056 |
| | M | 96.25 ± 11.64 | 82.54 ± 6.97 | 0.38 |
| ALT | F | 52 [47 – 59] | 52 [47 – 71] | 0.768 |
| | M | 54.5 [47 – 61] | 50.50 [38 – 62] | 0.468 |
| ALP | F | 223.5 [201 – 256] | 303.50 [283 – 425] | 0.002* |
| | M | 358 ± 7.842 | 435.60 ± 18.21 | 0.064 |
| Crea | F | 0.2 [0.1 – 0.2] | 0.250 [0.1 – 0.4] | 0.166 |
| | M | 0.250 [0.2 – 0.3] | 0.2 [0.1 – 0.4] | 0.384 |
| BUN | F | 18.51 [16.20 – 20.69] | 17.48 [14.85 – 19.94] | 0.454 |
| | M | 17.07 ± 0.3572 | 15.06 ± 0.3682 | 0.022* |
| GLU | F | 162.9 ± 1.896 | 167.8 ± 4.947 | 0.651 |
| | M | 162 ± 4.602 | 158 ± 3.764 | 0.564 |
| TC | F | 73.76 ± 3.425 | 82.23 ± 3.091 | 0.143 |
| | M | 47.07 ± 3.723 | 56.81 ± 2.225 | 0.069 |
| $Ca^{2+}$ | F | 10.22 ± 0.228 | 10.32 ± 0.1165 | 0.674 |
| | M | 10.18 [10.18 – 10.31] | 10.21 [7.520 – 11.04] | 0.865 |
| PHOS | F | 8.470 ± 0.3659 | 8.058 ± 0.2456 | 0.381 |
| | M | 10.23 [9.530 – 10.55] | 9.80 [5.980 – 10.79] | 0.187 |
| $K^+$ | F | 5.498 ± 0.3371 | 5.175 ± 0.0938 | 0.222 |
| | M | 5.215 [4.850 – 5.600] | 5.660 [3.690 – 6.810] | 0.173 |
| $Na^+$ | F | 148.9 ± 0.3582 | 148.6 ± 0.0734 | 0.175 |
| | M | 149.1 [148.3 – 149.3] | 148.9 [144.3 – 151.2] | 0.797 |

F – females, M – males, ALB – albumin, TP – total proteins, GLOB – globulin, AST – aspartate aminotransferase, ALT – alanin aminotransferase, ALP – alkaline phosphatase, Crea – creatinine, BUN – blood urea nitrogen, GLU – glucose, TC – total cholesterol, PHOS – phosphate.

## Conclusions

To our knowledge this is the first study in the literature which assessed the behavior of the offsprings resulted from mother which were on treatment with recreational doses of benzydamine during the pregnancy. In conclusion, benzydamine should be considered a substance which is able to alter memory but also the behavior, effects more evident in females. Time spent in open arms in EPM, head dipping frequency and rearing behavior suggest a reduction in anxiety. The exploratory and innovatory character of the present study limits the possibility of direct comparison to data from the literature, highlighting the necessity of future studies.

## Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

## Author Contributions

Conceptualization, GJ and B-EO; Data curation, GJ and ZG; Formal analysis, C-EV and B-EO; Funding acquisition, B-EO; Investigation, GJ and ZG; Methodology, GJ and ZG; Project administration, C-EV and B-

EO; Resources, B-EO; Software, GJ and ZG; Supervision, C-EV and B-EO; Validation, C-EV and B-EO; Visualization, GJ; Writing – original draft, GJ; Writing – review & editing, ZG, C-EV and B-EO.

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## Generative AI Statement:

During the preparation of this work the author(s) used ChatGPT in order to *improve language, grammar*. After using ChatGPT, the author(s) reviewed and edited the content as needed and are fully responsible for the originality and integrity of the content of the manuscript.

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Acta Biologica Marisiensis

# FLUOROQUINOLONES AS ENVIRONMENTAL CONTAMINANTS: OCCURRENCE, FATE, AND PHYTOTOXICOLOGICAL IMPLICATIONS

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**Abstract:** Fluoroquinolones (FQs) are among the most widely used broad-spectrum antibiotics worldwide, valued for their efficacy against both Gram-positive and Gram-negative bacteria. However, their persistence in the environment has raised significant ecological concerns. Due to incomplete absorption and metabolic transformation in humans and animals, large quantities of FQs are excreted unchanged and subsequently enter aquatic and terrestrial ecosystems via wastewater, agricultural runoff, and biosolids application. This review synthesizes current evidence on the environmental fate of FQs, their phytotoxic effects on aquatic and terrestrial plants, and the potential of phytoremediation as a mitigation strategy. A systematic literature search was conducted for studies published between 2014 and 2025 using major scientific databases, including PubMed, ScienceDirect, Web of Science, and Scopus, to ensure comprehensive coverage of the relevant literature. Studies consistently demonstrate that FQs inhibit plant growth, reduce photosynthetic efficiency, and induce oxidative stress in a dose-dependent manner. Furthermore, FQs promote the dissemination of antibiotic resistance genes in microbial communities, posing an indirect but serious threat to human health. The review also highlights knowledge gaps and areas requiring further research, particularly regarding long-term ecological consequences and the optimization of phytoremediation systems.

**Keywords:** fluoroquinolones, ciprofloxacin, phytotoxicity, aquatic plants, terrestrial plants, bioaccumulation, antibiotic resistance, phytoremediation

## 1. Introduction

The global rise in antibiotic consumption has become a growing concern in the scientific community. Residues of antibacterial compounds released into the environment pose significant ecological and public health challenges. Among these, fluoroquinolones (FQs) are of particular interest due to their widespread use in both human medicine and

veterinary practice, their high stability, and their strong adsorption to soil and sediment particles (Bhatt and Chatterjee, 2022).

Antibiotics, particularly FQs, are widely used to treat bacterial infections by targeting highly conserved enzymes, such as DNA gyrase and topoisomerase IV, which are essential for DNA helix unwinding during replication and transcription. Quinolones are synthetic antibiotics introduced into clinical practice approximately 50 years ago and remain widely used today for the treatment of a broad range of infections caused by both Gram-positive and Gram-negative bacteria (Martinez, 2019; Andriule et al., 2023).

FQs constitute the third largest group of antibiotics by global consumption. They are preferred in clinical practice due to their low allergenic potential, high oral bioavailability, and broad-spectrum antibacterial activity. Their use spans the treatment of urinary tract, respiratory, and gastrointestinal infections, as well as sexually transmitted diseases, and includes prophylactic and therapeutic applications in livestock and aquaculture (Bhatt and Chatterjee, 2022; Hamad, 2010; Collinsworth et al., 2022).

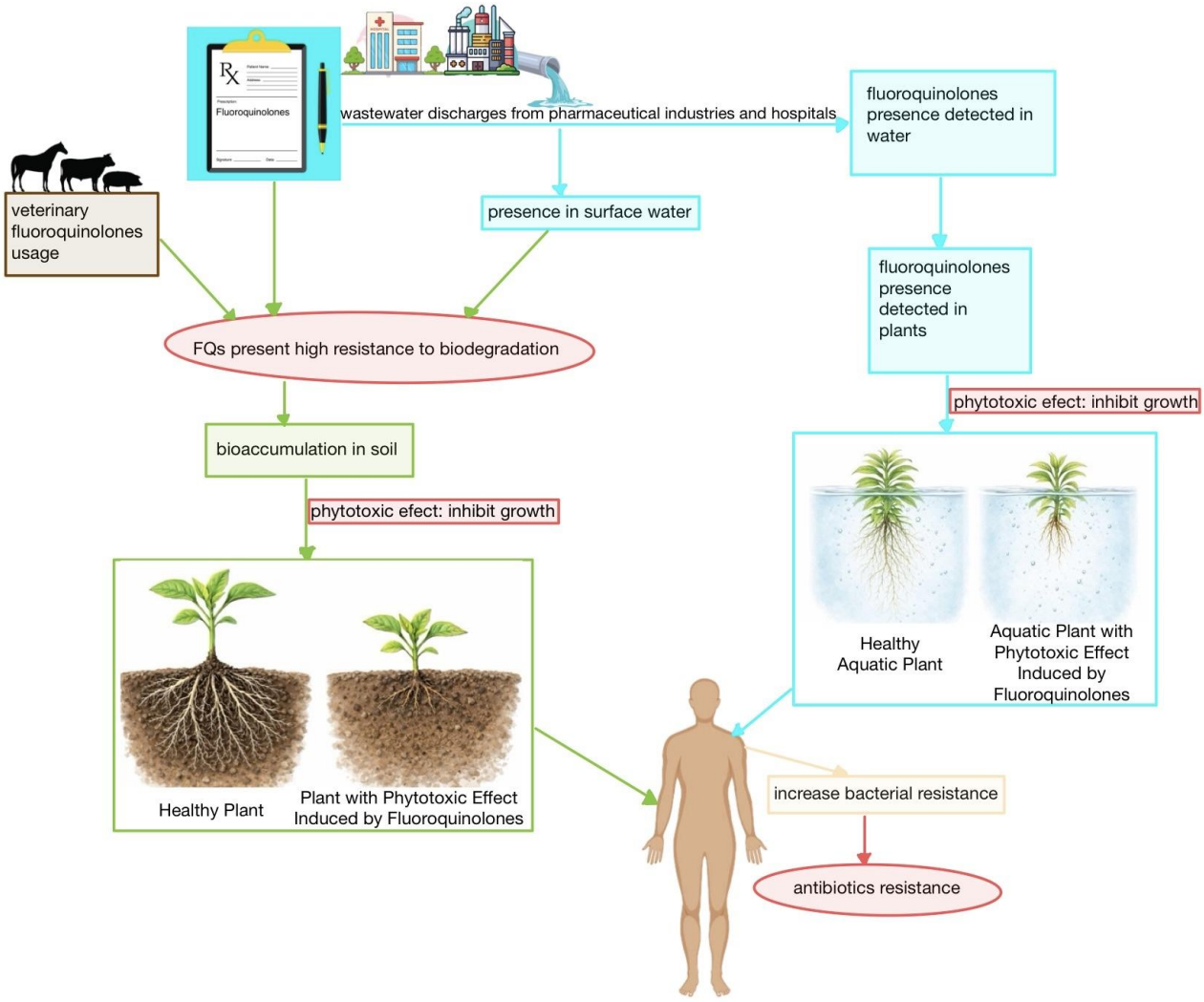


**Fig. 1**. Environmental pathways and phytotoxic effects of fluoroquinolones (FQs) leading to antibiotic resistance

Given their environmental relevance, this study adopts a systematic review approach to critically evaluate the existing literature on fluoroquinolones in environmental systems. This approach ensures a transparent and reproducible synthesis of available evidence regarding their occurrence, fate, and biological effects.

This situation highlights the importance of assessing and characterizing the phytotoxicity of these antibiotics, with particular attention to their effects on both aquatic and terrestrial plant species. Additionally, since FQs can disrupt basic microbial mechanisms, they can alter the natural balance of soil and aquatic ecosystems, promoting the spread of antibiotic resistance genes (Chen et al., 2024; Zhu et al., 2013).

**Figure 1** illustrates the environmental dissemination of FQs from veterinary and pharmaceutical sources, their persistence and bioaccumulation in soil and water systems, and the resulting phytotoxic effects and contribution to the development of bacterial antibiotic resistance.

## 2. Materials and Methods

This study is presented as a systematic literature review to identify and synthesize peer-reviewed research on the environmental fate and phytotoxic effects of fluoroquinolones (FQs). A structured, reproducible search strategy was used to ensure transparency and consistency in study selection.

A comprehensive bibliographic search was conducted from March to April 2026, covering publications from January 2014 to December 2025. Relevant studies were identified in four major international scientific databases: PubMed, ScienceDirect, Web of Science, and Scopus (Page et al., 2021).

The search strategy employed combinations of the following keywords and Boolean operators: “fluoroquinolones” OR “ciprofloxacin” OR “norfloxacin” OR “enrofloxacin” AND “phytotoxicity” OR “aquatic plants” OR “terrestrial plants” OR “plant uptake” OR “bioaccumulation” OR “phytoremediation” OR “oxidative stress” OR “environmental contamination” OR “antibiotic resistance”.

Study selection was performed using predefined eligibility criteria. Inclusion criteria comprised peer-reviewed original research articles, systematic reviews, and meta-analyses reporting quantitative or semi-quantitative data on fluoroquinolone occurrence in environmental matrices, plant uptake, or associated phytotoxicological effects. Studies were excluded if they lacked primary experimental or environmental exposure data, focused exclusively on human pharmacokinetics, or did not include environmental or plant-related endpoints.

The selection process involved an initial screening of titles and abstracts, followed by full-text evaluation of potentially relevant studies. Data extraction was conducted in a standardized manner and included information on study design, environmental compartment, plant species investigated, fluoroquinolone compounds evaluated, exposure concentrations, and reported biological and biochemical endpoints such as growth inhibition, oxidative stress markers, and bioaccumulation patterns.

## 3. Classification and General Properties of FQs

FQs are a subclass of the broader quinolone family, distinguished by the presence of a fluorine atom substituent on the bicyclic ring system. The presence of fluorine in the molecular structure enhances antibacterial potency and broadens the spectrum of activity compared with earlier quinolone antibacterials. Generations of FQs are classified based on their spectrum of

activity and therapeutic use (Hamad, 2010; Collinsworth et al., 2022):

- **first-generation (e.g., norfloxacin),** primarily active against Gram-negative uropathogens, were characterized by limited systemic distribution (Andriule et al., 2023);
- **second generation (e.g., ciprofloxacin, enrofloxacin)** present extended activity against Gram-negative organisms and some Gram-positive cocci and improved systemic bioavailability (Andriule et al., 2023);
- **third-generation (e.g., levofloxacin)** are characterized by an extended antibacterial spectrum compared to the second generation, mainly a broader Gram-positive spectrum and enhanced activity against *Streptococcus pneumoniae* (Andriule et al., 2023);
- **fourth-generation (e.g., moxifloxacin)** is valuable due to the extended anaerobic spectrum, besides broad-spectrum activity (Andriule et al., 2023).

**Table 1.** Chemical structures and IUPAC names of selected fluoroquinolones (FQs) used in human and veterinary medicines (Gao and Pedersen, 2005)

| FQs | IUPAC Name | Therapeutic application |
|---|---|---|
| Ciprofloxacin | 1-cyclopropyl-6-fluoro-4-oxo-7-piperazin-1-ylquinoline-3-carboxylic acid | Broad-spectrum: UTI, respiratory, GI infections |
| Danofloxacin | 1-cyclopropyl-6-fluoro-7-[(1*S*,4*S*)-5-methyl-2,5-diazabicyclo[2.2.1]heptan-2-yl]-4-oxoquinoline-3-carboxylic acid | Veterinary medicine |
| Enrofloxacin | 1-cyclopropyl-7-(4-ethylpiperazin-1-yl)-6-fluoro-4-oxoquinoline-3-carboxylic acid | Veterinary medicine (metabolised to ciprofloxacin) |
| Levofloxacin | (*S*)-9-fluoro-2,3-dihydro-3-methyl-10-(4-methylpiperazin-1-yl)-7-oxo-7H-pyrido[1,2,3-de]-1,4-benzoxazine-6-carboxylic acid | Respiratory infections, community-acquired pneumonia |
| Norfloxacin | 1-ethyl-6-fluoro-4-oxo-7-piperazin-1-ylquinoline-3-carboxylic acid | Urinary tract infections, veterinary use |
| Marbofloxacin | 7-fluoro-2-methyl-6-(4-methylpiperazin-1-yl)-10-oxo-4-oxa-1,2-diazatricyclo[7.3.1.05,13]trideca-5(13),6,8,11-tetraene-11-carboxylic acid | Veterinary medicine |
| Moxifloxacin | 7-[(4a*S*,7a*S*)-1,2,3,4,4a,5,7,7a-octahydropyrrolo[3,4-b]pyridin-6-yl]-1-cyclopropyl-6-fluoro-8-methoxy-4-oxoquinoline-3-carboxylic acid | Respiratory, urogenital, skin and soft-tissue, intra-abdominal, gynaecological, and ocular bacterial infections (Rusu et al., 2021) |
| Orbifloxacin | 1-cyclopropyl-7-[(3*S*,5*R*)-3,5-dimethylpiperazin-1-yl]-5,6,8-trifluoro-4-oxoquinoline-3-carboxylic acid | Veterinary medicine |
| Pradofloxacin | 7-[(4a*S*,7a*S*)-1,2,3,4,4a,5,7,7a-octahydropyrrolo[3,4-b]pyridin-6-yl]-8-cyano-1-cyclopropyl-6-fluoro-4-oxoquinoline-3-carboxylic acid | Veterinary medicine |

All FQs share a zwitterionic character at physiological pH, which influences their solubility, adsorption behavior, and uptake by organisms. Their amphoteric nature, with p$K_a$ values typically between 5.7 and 9.3, results in complex interactions with soil organic matter and mineral surfaces, particularly clay minerals and metal cations such as $Fe^{3+}$, $Al^{3+}$, and $Ca^{2+}$ (Bhatt and Chatterjee, 2022; Gao and Pedersen, 2005) **(Table 1)**.

Currently, systemic FQs are classified as agents requiring restricted use due to their high ecological impact, rising resistance among Gram-negative pathogens, and safety concerns highlighted by international health authorities (Olivieri et al., 2023).

FQs currently used in veterinary practice comprise a limited group of compounds specifically approved for animal use by major regulatory authorities. The veterinary FQs include enrofloxacin, which is widely authorized for use in companion animals and food-producing species; marbofloxacin and orbifloxacin, both commonly employed in small-animal medicine; pradofloxacin, a later-generation FQ approved for cats in the United States and for both dogs and cats in the European Union; and danofloxacin, which is restricted primarily to cattle for defined indications. Notably, many human FQs are not approved for veterinary use and are discouraged due to antimicrobial stewardship and resistance concerns (Papich and Riviere, 2009).

## 4. Environmental Sources and Fate of FQs

The environmental presence of FQs originates from multiple sources. In human medicine, oral FQs are incompletely absorbed, and a significant portion is excreted as unchanged parent compounds or active metabolites through urine and feces. It is estimated that up to 70% of administered FQs may be excreted unchanged and enter municipal wastewater systems. Conventional wastewater treatment plants (WWTPs) are not specifically designed to remove antibiotics and typically achieve only partial elimination of FQs, with removal efficiencies ranging from 14% to 80%, depending on treatment configuration and compound properties (Bhatt and Chatterjee, 2022; Chen et al., 2024).

In veterinary and agricultural settings, FQs are administered to livestock and aquaculture species, and residues enter the environment through manure application, effluent discharge from fish farms, and direct excretion into water bodies. Enrofloxacin, widely used in poultry and swine production, is metabolized in part to ciprofloxacin, extending the range of FQs present in agricultural soils and sediments (Chen et al., 2024; Zhang et al., 2019).

Once in the environment, FQs strongly adsorb to soil particles and sediments due to their zwitterionic character and tendency to form stable complexes with multivalent metal cations. This high sorption capacity, combined with low biodegradability under aerobic conditions, leads to the accumulation of FQs in soils and sediments over time. Studies have detected FQs in soils from Asia, Europe, and the Americas, with concentrations varying greatly by region. For instance, six different FQs were quantified in soils from Beijing and Shanghai at concentrations up to 2160 μg $kg^{-1}$, significantly higher than those recorded in Chennai, India (126.29 μg $kg^{-1}$) (Chen et al., 2024; Gao and Pedersen, 2005).

Photolysis is considered the primary degradation pathway for FQs in aquatic systems under sunlight, generating transformation products that may exhibit antibiotic activity or toxicity. Under anaerobic conditions, such as in waterlogged soils or sediments, biodegradation is even slower, further contributing to environmental persistence. FQs have also been detected in

surface waters and groundwater, posing risks to both aquatic organisms and water quality (Bhatt and Chatterjee, 2022; Van Boeckel et al., 2014).

## 5. Phytotoxic Effects: Dose-Response Relationships

The phytotoxic effects of FQs depend strongly on their concentration in the growth medium or soil. A dose-response framework is essential for understanding the spectrum of responses observed across different plant species and experimental conditions (Martins et al., 2012; Calouro et al., 2020).

At low, environmentally relevant concentrations (typically below 1 $\mu g \cdot L^{-1}$), the effects of FQs on plants are often subtle, primarily involving biochemical and metabolic changes. These include shifts in antioxidant enzyme activity, altered secondary metabolite profiles, and, in some cases, a hormetic response, in which low doses transiently stimulate plant growth or protective enzyme activity. Hormesis has been described in several FQ-plant pairs and reflects the activation of adaptive stress-response mechanisms as a form of plant defense (Martins et al., 2012; Lin et al., 2025).

At moderate concentrations (ranging from 1 to 100 $\mu g \cdot L^{-1}$), the most frequently reported effects include inhibition of seed germination, reduced root and shoot elongation, decreased chlorophyll and carotenoid content, impaired photosynthetic efficiency, and increased markers of oxidative stress such as malondialdehyde (MDA) accumulation and elevated reactive oxygen species (ROS) levels. The degree of inhibition is species-specific and depends on the plant's capacity to accumulate and detoxify FQs (Martins et al., 2012; Nunes et al., 2019; Mravcová et al., 2024).

At high concentrations (above 100 $\mu g \cdot L^{-1}$ or 5 $mg \cdot kg^{-1}$ in soils), ciprofloxacin and other FQs cause significant phytotoxic effects, including severe growth suppression, major metabolic disruptions, structural damage to plant tissues, including root necrosis and chloroplast disorganization, and, in some cases, plant death. At these concentrations, plant antioxidant defense systems are overwhelmed, and cellular damage becomes irreversible (Chen et al., 2024; Zhao et al., 2024).

## 6. Bioaccumulation of FQs and Absorption in Aquatic and Terrestrial Plants

Bioaccumulation refers to the process by which organisms accumulate substances from the environment at concentrations exceeding ambient levels. In the context of FQs, plants can take up these antibiotics from contaminated soil or water through their roots, with subsequent translocation to aboveground tissues. The general pattern of FQ distribution in plant tissues follows the gradient: root > stem > leaf, suggesting that roots are the primary site of accumulation, and translocation to shoots and leaves is more limited (Chen et al., 2024; Chen et al., 2023).

Bioaccumulation in plants has direct consequences for food safety, as residues in edible plant parts may enter the human food chain. Moreover, it contributes to antibiotic resistance in soil microbial communities. The selective pressure exerted by even sub-inhibitory concentrations of antibiotics in soils promotes the selection of resistant bacterial strains and the horizontal transfer of resistance genes, leading to the emergence and spread of multidrug-resistant bacteria that significantly threaten effective treatment of human diseases (Bhatt and Chatterjee, 2022; Martinez, 2019; Zhu et al., 2013).

Compared to other antibiotic classes, FQs exhibit especially strong adsorption and high resistance to biodegradation, leading to their accumulation at elevated concentrations in both

soil and plant tissues. Their amphoteric character allows them to interact with root cell membranes and metal complexes, facilitating uptake even in soils with high organic matter content (Chen et al., 2024; Gao and Pedersen, 2005).

### 6.1. Aquatic Plants

Plants are essential components of freshwater ecosystems, providing oxygen, habitat, and nutrients. They can also serve as biomonitors and potential bioremediators of chemical contaminants. Since not all administered FQs are fully absorbed by target organisms, a significant amount is excreted into wastewater and subsequently detected in coastal, riverine, and lacustrine environments (Chen et al., 2024; Lin et al., 2025).

Macroalgae are among the most sensitive aquatic organisms to FQ toxicity. Their simple morphology and close contact with surrounding water facilitate direct uptake of dissolved contaminants. Studies have shown that macroalgae can accumulate FQs at concentrations several times higher than those in the surrounding medium. For submerged macrophytes, root uptake from sediment is an additional route of exposure, particularly relevant in contaminated aquaculture systems (Chen et al., 2023; Lin et al., 2025).

### 6.2. Terrestrial Plants

When FQs enter the soil, a series of physicochemical and biological processes govern their availability for plant uptake. The fraction that remains dissolved in soil pore water (the bioavailable fraction) is determined by the equilibrium between adsorption to soil particles and dissolution. Root exudates can influence this equilibrium by modifying soil pH or chelating metal ions that form complexes with FQs (Chen et al., 2024; Gao and Pedersen, 2005).

Studies have shown that root uptake of FQs is generally passive and directed by concentration gradients, though active transport mechanisms may also contribute. Translocation from roots to shoots is more limited, particularly for FQs that form stable metal complexes in the apoplast. In hydroponic systems, where FQs are dissolved directly in the nutrient solution, uptake and translocation are typically higher than in soil-based systems, where adsorption reduces bioavailability. Research indicates that FQs can both enhance growth and exhibit toxicity for terrestrial crops at higher concentrations (5 $mg \cdot L^{-1}$), consistent with the hormesis framework described above (Chen et al., 2024; Mravcová et al., 2024).

## 7. Effects of FQs on Aquatic Plants

Multiple studies confirm that FQs can harm a broad range of aquatic organisms, including marine bacteria, algae, crustaceans, and fish. The mechanisms of phytotoxicity in aquatic plants include direct inhibition of prokaryotic-type plastid division (targeting the FtsZ protein, a structural analog of bacterial tubulin), impairment of chloroplast electron transport chains, and induction of oxidative stress through ROS overproduction (Chen et al., 2024; Lu et al., 2025; Sun et al., 2021).

### 7.1. *Gracilariopsis heteroclada*

Recent evidence shows that FQs inhibit the growth of aquatic plants in a dose-dependent manner. Norfloxacin, enrofloxacin, and lomefloxacin have been shown to affect the growth of the red macroalga *Gracilariopsis heteroclada*, a thermotolerant species mainly found in the Northern South China Sea. This alga is commercially important for its high agar content (approximately 29.8% by dry weight) and its applications in the food, cosmetics, and pharmaceutical industries. Other recent studies have also identified its capacity to decrease the relative abundance of antibiotic resistance

genes in its surrounding environment (Lin et al., 2025).

The dose-response pattern of *G. heteroclada* to the three FQs was non-linear. At low concentrations, fluoroquinolones stimulated algal growth, whereas at higher concentrations they exerted inhibitory effects, a pattern consistent with hormesis. Importantly, at most tested concentrations, the FQs did not cause complete inhibition, and a 50% reduction in growth was not observed within 96 hours, suggesting a relatively high tolerance of this species to FQ exposure. Sorption of FQs by algal biomass was also quantified, indicating that *G. heteroclada* can serve as a sorbent for these contaminants (Lin et al., 2025).

### 7.2. *Hydrilla verticillata*

Ciprofloxacin, one of the most frequently detected FQs in aquatic environments, causes phytotoxicity in the submerged macrophyte *Hydrilla verticillata*, a widespread species in tropical and subtropical freshwater systems. The phytotoxic mechanisms include growth inhibition, decreased photosynthetic efficiency (as measured by the Fv/Fm ratio), and induction of oxidative stress. Antibiotics can disrupt photosynthetic processes by impairing electron transport at photosystem II, leading to an overproduction of reactive oxygen species (ROS) that cause lipid peroxidation and cellular membrane damage (Lu et al., 2025).

A notable finding in *H. verticillata* is the significant increase in anthocyanin levels following ciprofloxacin exposure. Anthocyanins, belonging to the flavonoid family, function as non-enzymatic antioxidants and UV-screening pigments. At lower ciprofloxacin concentrations, elevated ROS signaling activated transcriptional pathways for anthocyanin biosynthesis, representing a protective response. At high ciprofloxacin concentrations, this biosynthetic capacity was suppressed, indicating overwhelmed defense mechanisms (Lu et al., 2025).

### 7.3. *Lemna minor* and *Lemna gibba*

The duckweeds *Lemna minor* and *Lemna gibba* are standardized test organisms frequently used in ecotoxicological assessments due to their rapid growth, simple morphology, and sensitivity to environmental contaminants. Studies evaluating the effects of ciprofloxacin on these species measured catalase activity as a marker of antioxidant response and lipid peroxidation as an indicator of oxidative damage (Nunes et al., 2019; Harpaz et al., 2021).

Ciprofloxacin treatment increased catalase activity, indicating an active antioxidant response to oxidative stress. Paradoxically, lipid peroxidation levels decreased, suggesting the antioxidant defense system effectively scavenged ROS before lipid peroxidation could occur. Chlorophyll a and b contents did not differ significantly between treated and control groups. However, carotenoid levels were significantly reduced, potentially impairing the photoprotective function of the photosynthetic apparatus and increasing susceptibility to photoinhibition (Martins et al., 2012).

## 8. Effects of FQs on Terrestrial Plants

Fluoroquinolones (FQs), especially ciprofloxacin, are commonly detected in agricultural soils as antibiotic residues resulting from the application of sewage sludge, animal manure, or irrigation with treated wastewater. In experimental studies, plants are often exposed to concentrations that exceed those typically reported in the environment, which may amplify observed phytotoxic effects compared with environmentally relevant exposure scenarios. Exposure to sub-lethal concentrations of FQs has been shown to induce a range of physiological and biochemical changes in terrestrial plants,

including alterations in germination rate, root growth, photosynthetic pigment content, and the activity of antioxidant enzymes such as superoxide dismutase and catalase (Martins et al., 2012; Carée et al., 2021).

FQs can also interfere with plant secondary metabolism, affecting the biosynthesis of phenolic compounds and flavonoids, which play key roles in plant antioxidant defence and have broader ecological and pharmacological relevance. Consequently, changes in their levels may have implications beyond individual plant physiology, potentially affecting plant–microbe interactions and ecosystem-level processes. However, the magnitude and direction of these responses vary considerably depending on plant species, FQ compound, exposure concentration, and duration, as well as the experimental system used (e.g., hydroponic versus soil-based conditions), which can further influence contaminant availability and uptake (Martins et al., 2012; Zhao et al., 2024).

### 8.1. *Lactuca sativa*

Lettuce (*Lactuca sativa*) is a widely consumed leafy vegetable commonly used in phytotoxicity studies due to its sensitivity to contaminants and its relevance to food safety. Exposure of *L. sativa* to seven ciprofloxacin concentrations (0-500 $\mu g \cdot L^{-1}$) over 35 days under hydroponic conditions revealed progressive phytotoxic effects. By the end of the exposure period, plant mortality increased in a concentration-dependent manner, accompanied by visible changes in root morphology, including shortened, discolored roots, and a significant decrease in root length. (Mravcová et al., 2024).

The obtained evidence highlights the risks associated with irrigating lettuce with ciprofloxacin-contaminated water or growing it in FQ-contaminated soils, which are realistic scenarios in regions where reclaimed wastewater is used for agricultural irrigation. Lettuce is particularly vulnerable due to its leafy structure and high water content, which favor uptake and accumulation of soluble contaminants (Mravcová et al., 2024; Carée et al., 2021).

### 8.2. *Brassica parachinensis*

Exposure of plants to ciprofloxacin can cause markedly different effects depending on each variety's capacity to accumulate and tolerate the antibiotic. Studies on Chinese flowering cabbage (*Brassica parachinensis*) compared varieties with different levels of ciprofloxacin accumulation. Varieties with lower ciprofloxacin accumulation paradoxically experienced greater inhibition of growth and photosynthesis than those accumulating higher amounts, indicating that tolerance is not simply a function of exclusion, but also of the plant's ability to compartmentalise and detoxify accumulated compounds (Zhao et al., 2024).

Further transcriptomic and proteomic analyses revealed that, in sensitive varieties, genes and proteins involved in essential metabolic processes, including protein synthesis, carbon metabolism, and energy production, were more strongly downregulated than in tolerant varieties. The results explained in **Table 2** suggest that ciprofloxacin phytotoxicity depends not only on its concentration but also on the plant's capacity to regulate molecular networks involved in stress responses and secondary metabolism (Zhao et al., 2024).

**Table 2.** Summary of phytotoxic effects of fluoroquinolones (FQs) on selected plant species

| FQs | Tested species | Observed effects |
|---|---|---|
| Norfloxacin, enrofloxacin, lomefloxacin | *Gracilariopsis heteroclada* | Hormetic response: growth stimulation at low doses, inhibition at high doses; FQ sorption by algal biomass (Lin et al.,2025) |
| Ciprofloxacin | *Hydrilla verticillata* | Growth inhibition, decreased Fv/Fm, increased oxidative stress; elevated anthocyanins at low concentrations (Lu et al., 2025) |
| Ciprofloxacin | *Lemna minor* and *Lemna gibba* | Increased CAT activity, decreased lipid peroxidation; no change in chlorophylls a and b; reduced carotenoids (Nunes et al., 2019) |
| Ciprofloxacin | *Lactuca sativa* | Increased plant mortality, altered root morphology, decreased root length, concentration-dependent over 35 days (Mravcová et al., 2024) |
| Ciprofloxacin | *Brassica parachinensis* | Growth and photosynthesis inhibition; variety-dependent tolerance; downregulation of metabolic pathways in sensitive varieties (Zhao et al., 2024) |

## 9. FQs and the Spread of Antibiotic Resistance

Beyond their direct phytotoxic effects, FQs exert a profound indirect impact on ecosystems by promoting antibiotic resistance. Even at sub-inhibitory concentrations, FQs can exert selective pressure on soil and aquatic microbial communities, favouring the proliferation of resistant bacterial strains and the horizontal transfer of resistance genes via mobile genetic elements such as plasmids, transposons, and integrons (Martinez, 2019; Zhu et al., 2013).

Studies have consistently demonstrated positive correlations between FQ concentrations in soil and the abundance of FQ-resistance genes, such as qnr (plasmid-mediated quinolone resistance), and mutations in *gyrA* and *parC* (encoding the target enzymes DNA gyrase and topoisomerase IV). The concentration of FQs in agricultural soils, estimated at ng to $\mu g \cdot kg^{-1}$, is sufficient to select for resistance (Martinez, 2019; Zhu et al., 2013; Liu et al., 2022).

Resistant bacteria in environmental reservoirs can colonise the gastrointestinal tracts of animals and humans through the food chain, creating a difficult-to-break cycle of resistance dissemination. The World Health Organization (WHO) has listed FQ-resistant bacteria, including fluoroquinolone-resistant *Enterobacteriaceae* and *Pseudomonas aeruginosa*, among the critical priority pathogens requiring urgent development of new antibiotics (World Health Organization, 2017).

## 10. Phytoremediation as a Strategy to Reduce FQs Pollution

Phytoremediation is an environmentally friendly, cost-effective, and publicly acceptable technology that uses living plants and their associated rhizosphere microorganisms to remediate contaminated soil, sediment, and water. The mechanisms involved include phytoextraction (uptake and accumulation of contaminants in plant tissues), phytodegradation (enzymatic transformation by plant enzymes), rhizodegradation (microbial degradation in the rhizosphere), and phytostabilization (immobilization of contaminants in the root zone) (Chen et al., 2023; MacDonald et al., 2019).

Numerous studies have demonstrated that plants can effectively reduce FQ concentrations in contaminated environments. The general pattern of FQ distribution in plant tissues (root > stem > leaf) is advantageous for phytoremediation, since harvesting root biomass can remove accumulated FQs from the site (Chen et al., 2024; Chen et al., 2023).

### 10.1. *Vallisneria spiralis*

Studies on the submerged macrophyte *Vallisneria spiralis* have demonstrated its potential for phytoremediation of FQ-contaminated aquaculture sediments. Under controlled conditions, the presence of *V. spiralis* in enrofloxacin-contaminated sediment significantly accelerated the elimination of enrofloxacin and its metabolite ciprofloxacin. This effect was attributed to two complementary mechanisms: direct uptake of antibiotics by plant roots and modification of the sediment microbial community by the plant, which favoured the activity of antibiotic-degrading microorganisms (Zhang et al., 2019).

*Vallisneria spiralis* demonstrated considerable tolerance to enrofloxacin. At low and moderate concentrations, leaf growth and total biomass were maintained or even stimulated, and nutrient uptake was not significantly hindered. At high concentrations, slight negative effects on root development were observed, but the plant continued to survive, demonstrating a high capacity for chemical stress resistance (Zhang et al., 2019).

### 10.2. Wetland Plants and Multi-Species Systems

Beyond individual species, constructed wetland systems employing multiple plant species have shown promise for reducing antibiotic concentrations in contaminated effluents. Macrophytes such as *Phragmites australis*, *Typha latifolia*, and *Iris pseudacorus* have been investigated for their capacity to take up and transform FQs. The combination of direct plant uptake, rhizosphere microbial degradation, and photolysis in surface-flow wetland systems can achieve substantial FQ removal rates, with studies reporting removal efficiencies of over 90% for ciprofloxacin under optimised conditions (Chen et al., 2023; MacDonald et al., 2019).

Dynamic speciation techniques, such as the diffusive gradients in thin films (DGT) method, have been applied to phytoremediation systems to distinguish plant-available FQ fractions from those bound to sediment, providing a more accurate picture of the phytoremediation process and the flux of antibiotics to root surfaces (Chen et al., 2023).

## 11. Knowledge Gaps and Future Research Directions

Despite the growing body of literature on fluoroquinolone (FQ) phytotoxicity and phytoremediation, several important knowledge gaps remain. The majority of existing studies have been conducted under controlled laboratory conditions, often using relatively high, experimentally applied concentrations of single FQ compounds and single plant species. In contrast, environmentally relevant concentrations are typically lower and occur within complex mixtures of antibiotics and other co-contaminants. The interactive effects of such mixtures on plant systems remain poorly understood, and synergistic or antagonistic interactions may substantially modify phytotoxic outcomes compared with single-compound exposures (Martins et al., 2012; Calouro et al., 2020).

Long-term ecological studies addressing the cumulative effects of chronic, low-level FQ exposure on plant communities, soil microbial diversity, and overall ecosystem functioning are still scarce. Most available studies are short-term (ranging from days to weeks), which

limits understanding of multigenerational effects under environmentally realistic exposure scenarios. This is particularly relevant given the continuous environmental input of FQs from agricultural, veterinary, and pharmaceutical sources (Bhatt and Chatterjee, 2022; Chen et al., 2024).

In addition, the molecular mechanisms underlying FQ uptake, translocation, and tolerance in plants remain insufficiently characterised. Identifying specific transporter proteins, detoxification pathways, and stress-response signalling networks would provide a basis for developing more efficient phytoremediation systems through plant selection or molecular improvement strategies (Zhao et al., 2024; MacDonald et al., 2019).

Finally, the lack of standardised experimental protocols for assessing FQ phytotoxicity across different plant species and exposure systems limits comparability between studies. The development of harmonised guidelines, similar to OECD or ISO ecotoxicological testing frameworks, would improve reproducibility and strengthen the scientific basis for environmental risk assessment and regulatory decision-making (Calouro et al., 2020; OECD, 2006).

## Conclusions

FQs are globally significant antibiotics whose environmental persistence poses considerable ecological risks. Because of their low biodegradability, high excretion rates, and strong adsorption properties, FQs accumulate in soils, sediments, water bodies, and plants. Numerous studies demonstrate that both aquatic and terrestrial plants absorb FQs, with effects depending on concentration. While low doses may enhance growth or activate antioxidant defences through hormetic mechanisms, higher concentrations cause phytotoxic effects, including growth inhibition, reduced photosynthetic efficiency, oxidative stress, altered secondary metabolism, and structural damage to plant tissues.

Beyond plant-level toxicity, FQs contribute to the dissemination of antibiotic resistance in soil and aquatic microbial communities through selective pressure and horizontal gene transfer, with broader implications for ecosystem stability and public health. At the same time, the reviewed evidence highlights the potential of phytoremediation-based approaches, particularly submerged macrophytes and constructed wetland systems, as promising tools for mitigating FQ contamination in wastewater and aquatic environments.

Overall, this review consolidates current knowledge on the environmental fate, phytotoxic effects, and remediation potential of FQs, providing an integrated perspective on their ecological impact. The findings emphasize the need for improved environmental monitoring of antibiotic residues, stricter regulation of antibiotic use in agriculture and aquaculture, and enhanced wastewater treatment strategies to limit environmental dissemination. From a practical standpoint, integrating phytoremediation into wastewater management systems and environmental monitoring frameworks represents a promising and scalable approach for reducing FQ pollution. Continued research is required to better understand long-term ecological risks and to optimize remediation technologies for real-world applications.

## Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

## Author Contributions

Conceptualization: C.T.; Data curation: M.G.; Formal analysis: M.G. and C.T.; Investigation: C.T. and M.G.; Methodology: M.G., A.R., C.T.; Project administration: C.T.; Resources: C.T.; Supervision: C.T.; Validation: A.R. and C.T.; Visualization: C.T.; Writing – original draft: M.G., Preparation: M.G.; Writing – review & editing: A.R. and C.T.

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## Generative AI Statement:

During the preparation of this work, the author(s) used Grammarly *to improve language and grammar.* After using Grammarly, the author(s) reviewed and edited the content as needed and are fully responsible for the originality and integrity of the content of the manuscript.

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# EFFECT OF NaCl SALINITY ON BIOACTIVE COMPOUND ACCUMULATION AND ANTIOXIDANT POTENTIAL IN BASIL (*OCIMUM BASILICUM* L.): IMPLICATIONS FOR NUTRITIONAL VALUE

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**Abstract:** Salt stress is one of the most prevalent abiotic stresses limiting plant productivity worldwide, yet moderate salinity can act as an elicitor of secondary metabolism. This study investigated the effect of NaCl concentrations on germination, vegetative growth, photosynthetic pigment content, total polyphenol content (TPC), and ABTS (3-ethylbenzothiazoline-6-sulfonic acid) radical-scavenging capacity in basil (*Ocimum basilicum* L.). Following assessment of data normality and homogeneity of variance, the nonparametric Kruskal-Wallis test was applied to leaf number, while one-way ANOVA with Tukey HSD post hoc testing was applied to TPC and ABTS data. Low salinity (V1, 17.1mM NaCl) stimulated fresh biomass. Leaf number was significantly reduced in V2 and V3 relative to the control. Chlorophyll a and total chlorophyll were highest in V3. TPC decreased progressively with salinity, with V0 and V1 significantly higher than V3. ABTS antioxidant capacity showed a contrasting non-linear pattern, being significantly higher in V3 than in V0, V1, and V2. Pearson correlation across all triplicates (n = 12) between TPC and ABTS was significantly negative, indicating that non-phenolic compounds likely sustain antioxidant capacity in salt-stressed basil. The results provide a basis for optimizing controlled cultivation of basil to enhance its bioactive profile for functional food applications.

**Keywords:** *Ocimum basilicum*, salt stress, NaCl, photosynthetic pigments, polyphenols, ABTS, antioxidant capacity, functional food

## 1. Introduction

Basil (*Ocimum basilicum* L., Lamiaceae) is one of the most widely cultivated culinary and medicinal herbs globally, prized for its essential oil profile, aromatic properties, and rich content of bioactive phytochemicals, including phenolic acids, flavonoids, and terpenoids (Zheljazkov et al., 2008; Ekren et al., 2012). Beyond its culinary use, basil is increasingly studied for its antioxidant, anti-inflammatory, antimicrobial, and hepatoprotective activities, positioning it as a candidate crop for functional food development (Kwee and Niemeyer, 2011).

The phenolic profile of basil varies considerably among cultivars and accessions, reflecting differences in the relative content of these major phenolic acids and in the resulting antioxidant activity (Kwee and Niemeyer, 2011). Beyond their role as natural antioxidants that scavenge free radicals and limit oxidative stress, basil phenolics and other phytonutrients have been associated with a broad range of beneficial health effects, including antimicrobial, anti-inflammatory, antidiabetic, and cardioprotective properties (Filip 2017). These characteristics make basil an important model species for investigating how phenolic content and antioxidant capacity are influenced by growing and cultivation conditions, as addressed in the present study.

Soil salinity is one of the most damaging abiotic stresses limiting agricultural production, affecting over 800 million hectares of arable land worldwide (FAO, 2021). Sodium chloride (NaCl) induces osmotic stress, ionic toxicity, and oxidative damage in plants, leading to impaired germination, reduced growth, and altered metabolism (Munns and Tester, 2008). However, an emerging body of evidence suggests that mild-to-moderate salt stress can function as an abiotic elicitor of secondary metabolite biosynthesis in certain plant species, potentially increasing the nutraceutical value of crops grown under controlled conditions (Rouphael et al., 2018; Chrysargyris et al., 2019).

In basil, the relationship between salinity and secondary metabolism is complex and concentration-dependent. Low NaCl concentrations have been reported to stimulate phenolic compound accumulation, while high concentrations generally impair photosynthetic efficiency and biomass accumulation (Tarchoune et al., 2010; Attia et al., 2021). The modulation of photosynthetic pigments — chlorophylls and carotenoids — under salt stress reflects the interplay between oxidative damage and antioxidant protective responses (Ashraf and Harris, 2013). Despite growing interest in salinity-driven enhancement of bioactive compounds, the dose-response relationship and its nutritional implications remain incompletely characterized, particularly under controlled greenhouse conditions that simulate realistic urban or indoor farming scenarios.

The present study evaluated the effect of four NaCl concentrations on germination parameters, vegetative growth, photosynthetic pigments, total phenolic content, and ABTS radical-scavenging antioxidant capacity in basil seedlings grown in a controlled greenhouse environment. The aim was to identify the salinity level that optimizes bioactive compound accumulation without unacceptably compromising plant growth, with implications for controlled-environment cultivation strategies aimed at producing high-value functional ingredients.

Building on existing evidence that abiotic stress can act as an elicitor of secondary metabolism in medicinal and aromatic plants, we hypothesized that moderate NaCl exposure would stimulate the synthesis of phenolic compounds and enhance the antioxidant capacity of basil without significantly compromising vegetative growth, whereas prolonged exposure to high NaCl concentrations would reduce photosynthetic pigment content and biomass accumulation as a consequence of osmotic stress. This working hypothesis was addressed through two specific research questions: (i) Does low-to-moderate salinity enhance phenolic content and antioxidant capacity relative to non-saline control conditions without compromising plant growth and biomass accumulation? (ii) Does high salinity reduce photosynthetic pigment content and biomass yield as a consequence of osmotic and oxidative stress?

## 2. Materials and Methods

### 2.1. Plant Material and Experimental Design

The experiment was conducted at the greenhouse facilities of the Botanical Garden of the University of Medicine, Pharmacy, Sciences and Technology "George Emil Palade" of Târgu Mureș, Romania. Seeds of *Ocimum basilicum* L., from the collection of the Botanical Garden, were sown in plastic pots (diameter 10–12 cm) filled with commercial peat substrate. Three seeds were planted per pot, and 120 pots were used, distributed equally among four treatment groups (30 pots per treatment). Growth conditions were maintained at 25 ± 2 °C, with a relative humidity of 50–60% and a photoperiod of 12 h light/12 h dark, supplemented with artificial lighting when natural irradiance was insufficient. The chemical composition and electrical conductivity of the commercial peat substrate were not characterized prior to the experiment.

### 2.2. Salinity Treatments

Four salinity levels were applied via irrigation with NaCl solutions prepared in distilled water (**Table 1**). Each pot received 30 mL of the corresponding treatment solution at planting, at 7 days post-planting, and at 10 days post-planting, followed by regular watering with the treatment solution combined with plain water to maintain substrate moisture.

Salt tolerance varies widely across plant species; for many non-halophytic species, salinity stress is generally classified as low at around 50 mM NaCl, moderate at around 100 mM NaCl, and severe at 150–200 mM NaCl (Munns and Tester, 2008). However, basil (*Ocimum basilicum* L.) is documented as a moderately salt-tolerant species owing to its capacity to exclude $Na^+$ ions at the root level (Ciriello et al., 2024), and basil-specific dose-response studies have consequently established somewhat higher numerical thresholds for these categories. Lazarević et al. (2021) classified 100 mM NaCl as a moderate and 200 mM NaCl as a severe salinity stress in basil, while Khaliq et al. (2017) reported that basil tolerates salinity up to approximately 150 mM NaCl before photosynthetic performance becomes severely compromised. At the lower end of the range, Mousa et al. (2020) showed that ~1,000 ppm NaCl (~17 mM, numerically close to our V1 treatment) stimulated growth parameters in basil cv. 'Genovese' relative to untreated controls, whereas 2,000–4,000 ppm NaCl (~34–68 mM) progressively reduced growth — supporting the classification of our V1 concentration (17.1 mM) as a low, sub-inhibitory salinity level. Our V2 treatment (51.3 mM) falls within the range where basil studies report measurable but moderate growth without growth arrest (Menezes et al., 2017; Mousa et al., 2020), while our V3 treatment (171.1 mM) lies within the high/severe stress range established specifically for basil (Lazarević et al., 2021; Khaliq et al., 2017).

**Table 1.** NaCl salinity treatments applied to *Ocimum basilicum* L.

| Treatment | NaCl concentration (mM) | NaCl per 500 mL (g) | Salinity level |
|---|---|---|---|
| V0 | 0 | 0 | Control |
| V1 | 17,1 | 0.5 | Low |
| V2 | 51,3 | 1.5 | Moderate |
| V3 | 171,1 | 5.0 | High |

### 2.3. Germination Assessment

Germination capacity (GC) was recorded at 35 days post-sowing as the final cumulative germination count. This index was expressed as a percentage of the total number of seeds sown (3 seeds per pot).

### 2.4. Growth Parameters

At harvest, leaves were separated from stems and weighed individually using a KERN ACJ300-4M analytical balance (KERN & Sohn GmbH, Balingen, Germany). Leaf fresh weight (FW) and stem FW were recorded per plant. Leaf number per plant was counted.

### 2.5. Preparation of Plant Extracts

Ultrasound-assisted extraction was performed using a Hielscher UP200St processor (Hielscher Ultrasonics GmbH, Teltow, Germany) at 55% amplitude for 15 min with 40% ethanol at a solid-to-solvent ratio of 1:20 (1 g in 20 mL). Extracts were centrifuged at 1790 × g (4000 rpm; Nahita Blue 2615/1 digital centrifuge, Auxilab S.L., Beriain, Spain) for 10 minutes. For antioxidant and phenolic compound analyses, plant material was lyophilized using a Biobase BK-FD125S freeze dryer (Biobase Co. Ltd., Jinan, China).

### 2.6. Determination of Photosynthetic Pigments

For dry weight determination, a separate portion of the leaf samples was subjected to natural dehydration. Fresh leaves were spread in a single layer on paper and maintained at room temperature in a dark, well-ventilated environment protected from direct light exposure.

For photosynthetic pigment determination, 0.05 g dry leaf material was homogenized in 10 mL of 80% acetone - a mass selected to remain within the linear absorbance range of the spectrophotometer for this matrix and solvent system; the filtered extract was kept in aluminum-foil-covered tubes at −20°C until spectrophotometric analysis. Chlorophyll a (Chl a), chlorophyll b (Chl b), and total carotenoids were determined spectrophotometrically on dry leaves using an Analytik Jena SPECORD 200 PLUS spectrophotometer (Analytik Jena AG, Jena, Germany). Absorbances were recorded at 663, 646, and 470 nm. Pigment concentrations were calculated according to the equations of Lichtenthaler and Wellburn (1983): Chl $a = 12.21 \times A_{663} - 2.81 \times A_{646}$; Chl $b = 20.13 \times A_{646} - 5.03 \times A_{663}$; Carotenoids $= (1000 \times A_{470} - 3.27 \times \text{Chl a} - 104 \times \text{Chl b})/229$ (all expressed as mg $g^{-1}$ DW after dividing by sample mass and adjusting for extraction volume). Measurements were performed in triplicate.

### 2.7. Determination of Total Phenolic Content (TPC)

TPC was determined using the Folin-Ciocalteu colorimetric method (Delgado-Alvarado et al., 2022) adapted for microplate format using a Thermo Scientific Multiskan FC microplate reader (Thermo Fisher Scientific, Singapore). In a 96-well microplate, 20 µL of each lyophilised extract (measured in triplicate) was mixed with 100 µL of 10-fold diluted Folin-Ciocalteu reagent (Merck KGaA, Darmstadt, Germany) and incubated in the dark for 3 min. Subsequently, 80 µL of 7.5% $Na_2CO_3$ solution was added, and the mixture was incubated in the dark for 30 min. Absorbance was read at 765 nm. Results were expressed as milligrams of gallic acid equivalent per milliliter of extract (mg GAE $mL^{-1}$) using a gallic acid calibration curve: $y = 3.0236x + 0.055$, $R^2 = 0.9979$.

### 2.8. Determination of Antioxidant Capacity (ABTS Assay)

The radical-scavenging activity of 2,2′-azinobis (3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) was assessed using the $ABTS^+$

decolorization method (Tanase et al. 2022). The radical cation was generated by reacting 25 mg ABTS dissolved in 25 mL distilled water with 9.375 mg $K_2S_2O_8$ (≥99%, Carl Roth GmbH, Karlsruhe, Germany) dissolved in 25 mL distilled water (1:1 ratio), followed by 14 h incubation in the dark at room temperature. The working $ABTS^+$ solution was diluted to an absorbance of ~0.70 at 734 nm. Lyophilized extracts (1.5 mg in 1.5 mL distilled water) were measured in triplicate. In a 96-well microplate, 20 µL of extract (or distilled water as the blank) was mixed with 200 µL of $ABTS^+$ solution and incubated for 6 min in the dark at room temperature. Absorbance was recorded at 734 nm. Results were expressed as mg Trolox equivalents per mL extract (mg TE $mL^{-1}$) using a Trolox calibration curve (Sigma-Aldrich, St. Louis, MO, USA): $y = 0.297x + 0.8459$, $R^2 = 0.9992$.

### 2.9. Statistical Analysis

All data are expressed as mean ± standard deviation (SD) from three replicates, except leaf number, which was recorded for each individual plant and is additionally reported as the median and interquartile range (IQR). Normality of data distribution was assessed using the Shapiro-Wilk test, and homogeneity of variances was assessed using Levene's test, prior to selecting parametric or non-parametric procedures. For TPC and ABTS data, both assumptions were satisfied (Shapiro-Wilk, $p > 0.05$ for all groups; Levene's test, $p = 0.518$ and $p = 0.690$, respectively), justifying the use of one-way analysis of variance (ANOVA) followed by Tukey HSD post-hoc test ($\alpha = 0.05$). For leaf number, the data significantly deviated from a normal distribution (Shapiro-Wilk test on pooled residuals, $p = 0.0003$), despite homogeneous variances across groups (Levene's test, $p = 0.954$); therefore, the non-parametric Kruskal-Wallis test was applied, followed by pairwise Mann-Whitney U tests with Bonferroni correction for multiple comparisons. Pearson correlation analysis was performed between TPC and ABTS values across all triplicates ($n=12$). For photosynthetic pigment data, only mean absorbance values from analytical triplicates were available at the treatment level; descriptive statistics and trends are reported without formal hypothesis testing. Statistical analyses were conducted in Python (SciPy library v.1.13). Significance thresholds: *$p<0.05$; **$p<0.01$; ***$p<0.001$.

## 3. Results

### 3.1. Germination Parameters

Despite The effect of NaCl on seed germination is illustrated in **Figure 1.** At this early 7-day checkpoint — well before the 35-day final assessment — V3 (171.1 mM NaCl) had no visible germinated seedlings, whereas V0 showed germination in 6 of 30 pots monitored; this early observation reflects only the proportion of seeds that had emerged within the first week and does not indicate poor overall germination capacity, which reached 70.0% in V0 by the final 35-day assessment. Germination capacity, recorded at 35 days post-sowing, was highest in V0 (70.0%) and V2 (73.3%), with V1 (60.0%) and V3 (63.3%) showing somewhat lower values (**Figure 1**).

### 3.2. Vegetative Growth Parameters

Fresh biomass data at harvest are illustrated in **Figure 2**. Plants treated with low salinity (V1, 17.1 mM NaCl) showed the highest mean leaf fresh weight (8.73 g $plant^{-1}$) and stem fresh weight (3.81 g $plant^{-1}$), exceeding control values (6.77 and 2.61 g $plant^{-1}$, respectively) by approximately 29% and 46%, respectively.

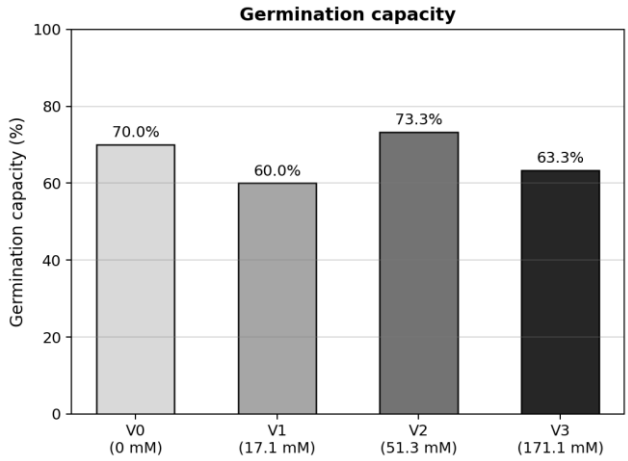


**Fig. 1.** Germination capacity (%) of *Ocimum basilicum* L. seeds under NaCl treatments (mean of 30 seeds per treatment). V0 = 0 mM; V1 = 17.1 mM; V2 = 51.3 mM; V3 = 171.1 mM NaCl.

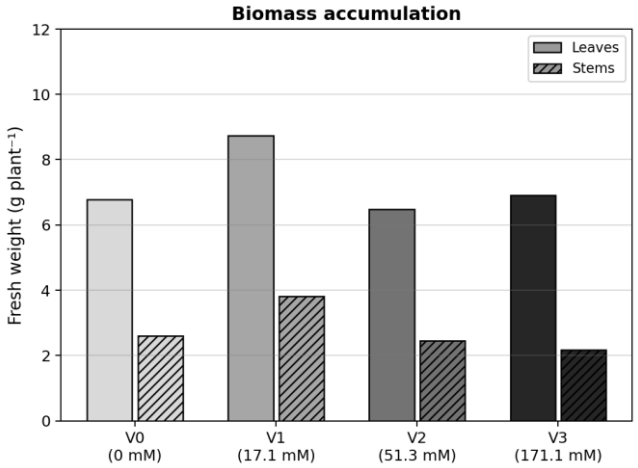


**Fig. 2.** Biomass accumulation (leaf and stem fresh weight, g plant$^{-1}$) *in Ocimum basilicum* L. under NaCl treatments V0 = 0 mM; V1 = 17.1 mM; V2 = 51.3 mM; V3 = 171.1 mM NaCl.

Moderate salinity (V2, 51.3 mM) produced leaf and stem fresh weights of 6.48 and 2.46 g plant$^{-1}$, while high salinity (V3, 171.1 mM) produced 6.91 and 2.18 g plant$^{-1}$, respectively — both yielding total shoot fresh weights (8.94 and 9.09 g plant$^{-1}$) comparable to the control (9.38 g plant$^{-1}$). Fresh weight parameters were evaluated descriptively; no formal statistical hypothesis testing was applied to biomass data, in contrast to leaf number (below), for which individual-plant replication permitted formal testing.

As leaf number data significantly deviated from a normal distribution, the non-parametric Kruskal-Wallis test was applied to individual-plant leaf counts (V0: median 7.0, IQR 6.0–7.2, n = 20; V1: median 6.5, IQR 6.0–7.0, n = 16; V2: median 5.5, IQR 5.0–6.0, n = 24; V3: median 6.0, IQR 4.8–6.0, n = 20), revealing a statistically significant treatment effect ($H = 15.041$, $p = 0.0018$). Pairwise Mann-Whitney U tests with Bonferroni correction showed that V2 and V3 had significantly fewer leaves than V0 ($p_{adj} = 0.013$ and $p_{adj} = 0.026$, respectively), while V1 did not differ significantly from V0 ($p_{adj} = 1.000$); V2 and V3 were not significantly different from each other ($p_{adj} = 1.000$), and V1 did not differ significantly from V2 or V3 after correction ($p_{adj} = 0.077$ and $p_{adj} = 0.137$, respectively) (**Figure 3**).

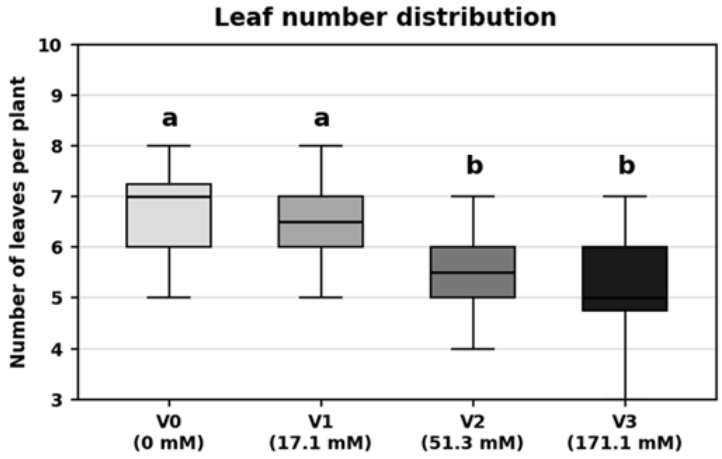


**Fig. 3.** Leaf number per plant in *Ocimum basilicum* L. under NaCl treatments (boxplots; different letters denote significant differences, Mann-Whitney U with Bonferroni correction, p_adj < 0.05). V0 = 0 mM; V1 = 17.1 mM; V2 = 51.3 mM; V3 = 171.1 mM NaCl.

### 3.3. Photosynthetic Pigments

Pigment concentrations from dry leaves are illustrated in **Figure 4**. Chlorophyll a ranged from 7.20 mg $g^{-1}$ DW (V2) to 8.58 mg $g^{-1}$ DW (V3), with V3 exceeding the control by ~17.5%. Total chlorophyll (Chl a + b) was also highest in V3 (13.15 mg $g^{-1}$ DW). The Chl a/b ratio was highest in V3 (1.88) and lowest in V1 (1.69). Carotenoid content was slightly reduced in V1 (1.39 mg $g^{-1}$ DW) relative to the control (1.66 mg $g^{-1}$ DW) and marginally elevated in V3 (1.70 mg $g^{-1}$ DW).

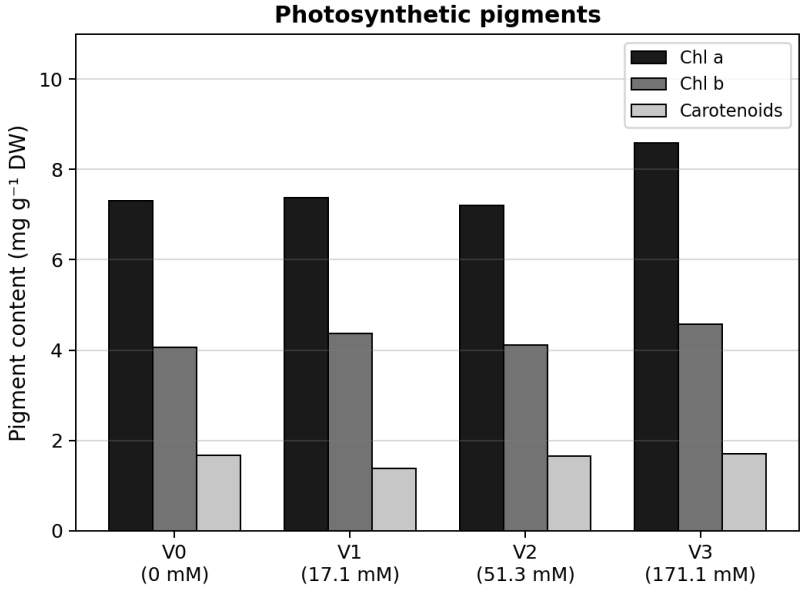


**Fig. 4.** Photosynthetic pigment content (mg $g^{-1}$ DW) in *Ocimum basilicum* L. under NaCl treatments (means of analytical triplicates). V0 = 0 mM; V1 = 17.1 mM; V2 = 51.3 mM; V3 = 171.1 mM NaCl.

### 3.4. Total Polyphenol Content (TPC)

TPC values determined from three independent biological replicates per treatment are shown in **Figure 5**. A statistically significant treatment effect was found (one-way ANOVA: F = 7.936, p = 0.0088). TPC decreased progressively with increasing NaCl concentration: V0 (0.123 ± 0.019 mg GAE $mL^{-1}$), V1 (0.110 ± 0.015), V2 (0.104 ± 0.012), and V3 (0.072 ± 0.003). Tukey HSD post-hoc analysis revealed that V0 was significantly higher than V3 (p = 0.0069), and V1 was significantly higher than V3 (p = 0.0325). No significant differences were detected between V0, V1, and V2, nor between V2 and V3 (p > 0.05). This indicates that high salinity (171.1 mM) substantially suppressed phenolic compound biosynthesis or stability, reducing TPC by approximately 41.5% relative to the control.

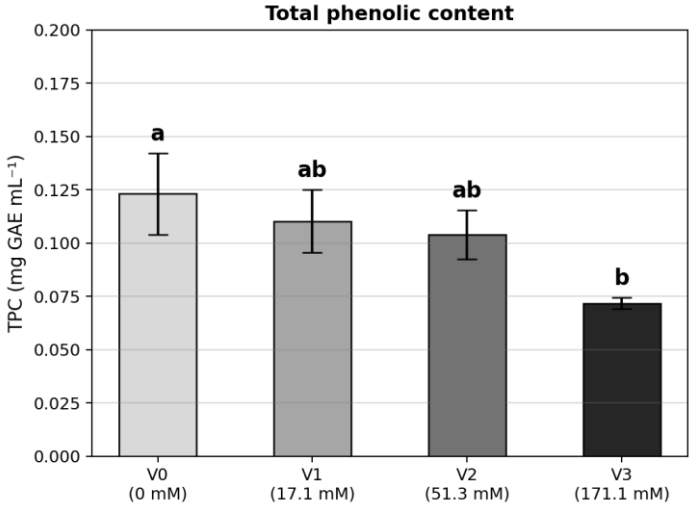


**Fig. 5.** Total phenolic content (TPC, mg GAE $mL^{-1}$ ± SD) of *Ocimum basilicum* L. extracts under NaCl treatments (different letters denote significant differences, Tukey HSD, p < 0.05). V0 = 0 mM; V1 = 17.1 mM; V2 = 51.3 mM; V3 = 171.1 mM NaCl.

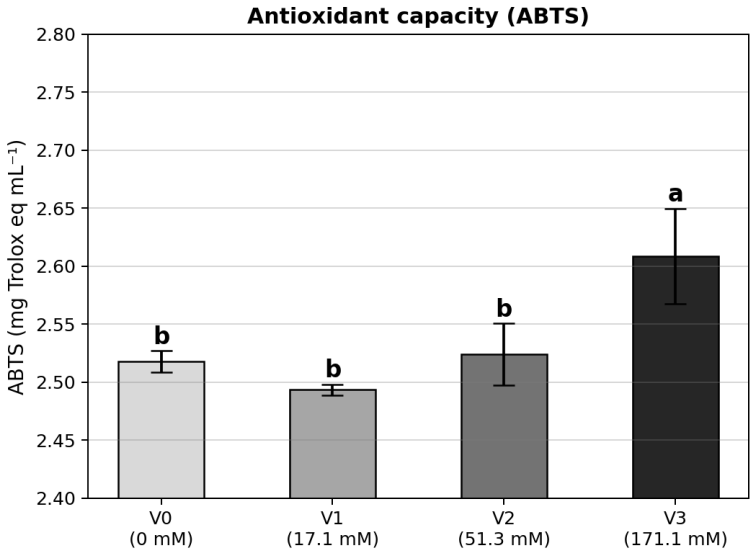


**Fig. 6.** ABTS antioxidant capacity (mg Trolox eq $mL^{-1}$ ± SD) of *Ocimum basilicum* L. extracts under NaCl treatments (different letters denote significant differences, Tukey HSD, p < 0.05). V0 = 0 mM; V1 = 17.1 mM; V2 = 51.3 mM; V3 = 171.1 mM NaCl.

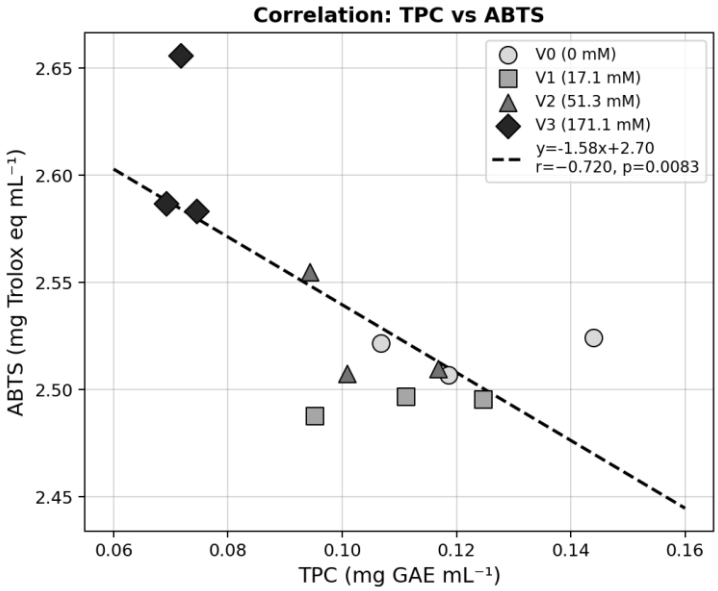


**Fig. 7.** Pearson correlation between total phenolic content (TPC, mg GAE $mL^{-1}$) and ABTS radical-scavenging capacity (mg Trolox eq $mL^{-1}$) across all biological replicates (n = 12). r = −0.720, p = 0.0083

### 3.5. Antioxidant Capacity (ABTS)

ABTS radical-scavenging capacity (**Figure 6**) showed a significant treatment effect (one-way ANOVA: F = 12.065, p = 0.0024). Values were: V0 (2.518 ± 0.009), V1 (2.493 ± 0.005), V2 (2.524 ± 0.027), and V3 (2.609 ± 0.041 mg TE $mL^{-1}$). Tukey HSD analysis revealed that V3 was significantly higher than V0 (p = 0.0092), V1 (p = 0.0022), and V2 (p = 0.0138), while V0, V1, and V2 did not differ significantly from each other (p > 0.05). The ~4.6% increase in ABTS at V3 relative to the control, despite the lowest TPC, suggests activation of non-phenolic antioxidant pathways under high-salt stress.

### 3.6. Correlation between TPC and Antioxidant Capacity

Pearson correlation analysis between TPC and ABTS across all 12 biological replicates (3 per treatment × 4 treatments) yielded r = −0.720 (p = 0.0083) (**Figure 7**). This statistically significant negative correlation indicates that treatments and replicates with higher phenolic content were systematically associated with lower ABTS radical-scavenging activity, and vice versa. When computed using only treatment means (n = 4), the trend was similar (r = −0.899, p = 0.101), though non-significant due to the low statistical power at that level. The significant result at the replicate level (n = 12) provides stronger evidence for a genuine decoupling between phenolic compound accumulation and ABTS-measured antioxidant capacity in basil under NaCl stress.

## 4. Discussions

The present study examined the dose-dependent effects of NaCl salinity on germination, vegetative growth, photosynthetic pigments, phenolic content, and antioxidant capacity in basil under controlled greenhouse conditions. Statistical analysis with biological replicates (n = 3 per treatment) for TPC and ABTS, complemented by individual-plant data for growth parameters, enabled rigorous hypothesis testing and several important biological insights.

No clear monotonic inhibitory trend was observed in germination parameters. The moderate-salinity treatment V2 (51.3 mM)

showed the highest germination capacity (73.3%), surpassing the control. This is consistent with reports that mild osmotic priming can stimulate germination in some species by accelerating water uptake and activating germination-associated enzymes (Farhoudi et al., 2011). The delayed germination observed at the highest salt concentration (V3) during the first week — with no visible seedlings — followed by recovery to 63.3% final germination capacity, suggests initial osmotic inhibition overcome by adaptive mechanisms in subsequent weeks.

The stimulatory effect of low salinity (V1, 17.1 mM NaCl) on biomass — with leaf and stem FW both exceeding control values by ~29% and ~46%, respectively — is consistent with a hormetic response, whereby a subinhibitory stressor stimulates growth. This phenomenon has been described in basil and other Lamiaceae under mild NaCl stress (Bernstein et al., 2010; Tarchoune et al., 2012). The significant reduction in leaf number observed at V2 and V3 reflects a concentration-dependent suppression of leaf initiation or accelerated senescence above 51.3 mM NaCl.

The paradoxical increase in total chlorophyll at V3 relative to control — despite the highest ionic stress — may reflect osmotic adjustment through chloroplast reorganization, or a leaf-age effect if V3 plants retained fewer but more mature, pigment-dense leaves at harvest. Similar non-linear chlorophyll responses under salt stress have been reported in basil (Attia et al., 2021) and other glycophytic herbs (Ashraf and Harris, 2013). The slight decrease in carotenoids at V1 relative to control is less straightforward and may reflect developmental variation in this small sample, given that carotenoids typically accumulate under stress as photoprotective pigments.

The statistically significant, progressive decrease in TPC with increasing NaCl contrasts with several published reports suggesting that moderate salt stress stimulates phenolic biosynthesis in basil (Tarchoune et al., 2010; Chrysargyris et al., 2019). The NaCl concentrations used here (17.1–171.1 mM) may have exceeded the mild-stress optimum for phenolic elicitation in this cultivar, with V3 representing clearly inhibitory ionic levels. The 41.5% reduction in TPC at V3 is consistent with oxidative damage to phenolic biosynthetic enzymes and metabolic disruption expected under severe salt stress. Importantly, the use of three independent biological replicates in this study allows this conclusion to be made with statistical confidence, strengthening the validity of the finding compared with single-pool extraction approaches.

The most striking finding of this study is the significant increase in ABTS antioxidant capacity at V3, despite V3 having the lowest TPC. This decoupling is supported by the statistically significant negative Pearson correlation between TPC and ABTS across all replicates. This pattern strongly suggests that non-phenolic antioxidants are the primary contributors to radical-scavenging activity under high salinity. Candidates include ascorbate, tocopherols, and stress-induced terpenoids such as rosmarinic acid precursors or volatile essential oil components known to accumulate in basil under stress conditions (Munns and Tester, 2008; Rouphael et al., 2018). The ABTS assay measures total hydrogen-donating and electron-transfer capacity regardless of compound class, and is therefore sensitive to these alternative antioxidant pools. These findings caution against using TPC alone as a proxy for antioxidant capacity in stress physiology studies, particularly when comparing treatments with divergent metabolic profiles.

From an applied perspective, V1 (17.1 mM NaCl) emerges as the most favorable treatment for controlled basil cultivation, maximizing

biomass yield without reducing polyphenol content. For applications where enhanced ABTS antioxidant capacity is the target — at the cost of lower phenolic content and reduced leaf number — V3 (171.1 mM) may be relevant, though the practical stress imposed on plants and the reduced harvestable biomass limit its utility for most commercial scenarios.

## Conclusions

This study demonstrates that NaCl salinity exerts concentration-dependent effects on vegetative growth, and statistically significant effects on leaf number, total phenolic content and antioxidant capacity in *Ocimum basilicum*. Low salinity (17.1 mM) stimulated fresh biomass accumulation (hormetic response) without significantly reducing leaf number, while moderate and high salinity (51.3 – 171.1 mM) significantly suppressed leaf production. Chlorophyll content increased at 171.1 mM NaCl, consistent with a stress-adaptive pigment response. TPC decreased progressively with salinity, with V3 significantly lower than V0 and V1, whereas ABTS antioxidant capacity was significantly elevated in V3. The significant negative Pearson correlation between TPC and ABTS indicates that antioxidant activity under high salinity is driven by non-phenolic compounds. These findings have practical implications for optimizing basil cultivation under controlled conditions, with low salinity identified as the optimal strategy for maximizing both biomass and phenolic yields. Future work should include flavonoid profiling, DPPH and FRAP assays, essential oil quantification by GC-MS, and expanded biological replication for pigment determinations.

## Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

## Author Contributions

Conceptualization: Conceptualization: C.T.; Data curation: S.S.; Formal analysis: S.S., I.N. and C.T.; Investigation: C.T., I.N. and S.S.; Methodology: S.S., I.N., M.G. and C.T.; Project administration: C.T.; Resources: C.T.; Supervision: C.T.; Validation: I.N. and C.T.; Visualization: C.T.; Writing – original draft: S.S., Preparation: S.S.; Writing – review & editing: I.N. and C.T.

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## Generative AI Statement:

During the preparation of this manuscript, the authors used Claude to assist with language editing and the generation of data visualizations. The authors reviewed and edited all AI-assisted output and take full responsibility for the accuracy and integrity of the content presented in this publication.

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# PHYTOESTROGENS AS PLANT BIOACTIVES FOR SKIN AGING MANAGEMENT: A NARRATIVE REVIEW OF MECHANISMS, FORMULATION CHALLENGES, EFFICACY, AND SAFETY

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**Abstract:** The use of phytoestrogens in anti-aging cosmetics has generated growing interest, as they have the potential to mimic the effects of estrogens on aging skin without the risks associated with systemic hormone therapy. This narrative review aims to summarize the mechanisms of action of the main classes of phytoestrogens and to evaluate their efficacy for the intended purpose. The existing data, although limited in number, suggest moderate benefits, particularly in cases of photoaging or in postmenopausal women, where estrogen deficiency is a significant factor. However, efficacy is limited by the instability of cutaneous bioavailability, the stability of cosmetic formulations, and the lack of clinical studies conducted over a longer period of time. From a practical standpoint, the inclusion of phytoestrogens in cosmetic products may represent a complementary option for mature skin care; however, at this time, their use should be supplemented with specialized therapeutic interventions. Future research should focus on optimizing delivery systems, standardizing extracts, and conducting well-controlled clinical studies to accurately determine their efficacy and safety for short-, medium-, and long-term use.

**Keywords:** phytoestrogens, phytohormones, isoflavones, skin aging, topical cosmetics

## 1. Introduction

Skin aging is a complex process influenced by both external factors—such as exposure to pollution, UV rays, and dietary habits—and internal factors, including genetic and hormonal influences.

Skin aging is characterized by progressive structural and functional alterations affecting both the epidermis and dermis. At the molecular level, aging is associated with reduced fibroblast activity, fragmentation of collagen fibers, degradation of elastin, impaired extracellular matrix turnover, and increased oxidative stress. Furthermore, chronic exposure to ultraviolet radiation promotes the formation of reactive oxygen species (ROS), which activate matrix metalloproteinases (MMPs) responsible for collagen degradation and wrinkle formation. As a result, aged skin exhibits reduced elasticity, increased dryness,

thinning, and impaired wound-healing capacity (Verdier-Sévrain et al., 2006; Thornton, 2013).

Hormone levels decline with age, serving as a major endogenous contributor to skin deterioration, particularly in women during the menopausal transition. These age-related changes are primarily driven by the decline of testosterone and estrogen secretion, which directly impacts collagen synthesis, dermal thickness, hydration, and elasticity. Estrogen, in particular, exerts vital trophic effects on the skin by stimulating the secretion of collagen and hyaluronic acid, improving microcirculation, and reducing oxidative stress. The extensive regulatory role of estrogens in skin physiology is underscored by the expression of estrogen receptors (ERα and ERβ) across keratinocytes, fibroblasts, melanocytes, sebaceous glands, and hair follicles. Consequently, declining estrogen levels lead to decreased collagen content, impaired barrier function, reduced hydration, and diminished antioxidant defenses. Ultimately, clinical observations suggest that this post-menopausal loss of estrogen accelerates cutaneous aging far beyond the expectations of chronological aging alone (Lephart, 2018; Thornton, 2013; Rzepecki et al., 2019).

Phytoestrogens are of interest in anti-aging cosmetics due to their ability to interact with estrogen receptors that are predominant in the skin. Through mechanisms similar to those of estrogen (Lephart, 2018), these compounds can help maintain skin structure and function, with beneficial effects on elasticity, hydration, and the skin aging process. It is assumed that topical application allows for relevant local effects with a reduced potential for systemic reactions.

Medical evidence indicates that systemic and topical estrogen interventions effectively increase dermal collagen density while optimizing skin thickness, elasticity, and moisture retention (Sauerbronn et al., 2000; Son et al., 2005; Troxel et al., 2026). Although topical delivery minimizes localized adverse reactions, systemic absorption remains a documented effect (Troxel et al., 2026). Due to the established correlation between estrogen therapy and increased risks for endometrial and breast malignancies, phytoestrogens have emerged as a highly sought-after substitute in both dermatology and hormone replacement therapy (Patra et al., 2023; Intharuksa et al., 2025).

Despite growing commercial interest and an increasing number of cosmetic products containing phytoestrogen-rich extracts, the available evidence remains heterogeneous. Differences in plant sources, extraction methods, formulation strategies, concentrations, and study designs make it difficult to draw definitive conclusions regarding efficacy and safety. Furthermore, while several *in vitro* and small-scale clinical studies have reported favorable outcomes, larger controlled trials are still needed to establish the long-term benefits and risks associated with topical phytoestrogen use (Farkas, 2026; Lephart, 2021).

In this context, the present narrative review examines plant-derived phytoestrogens used in anti-aging cosmetics, focusing on their plant sources, cutaneous mechanisms of action and the strength of available experimental and clinical evidence. Additionally, the review seeks to highlight existing formulation-related limitations and potential safety concerns.

## 2. Review Methodology

To conduct this narrative review, experimental, clinical, and review studies were selected that examined the effects of phytoestrogens (particularly isoflavones) on the skin and the mechanisms through which they interact with biological processes relevant to skin aging.

Searches were conducted in PubMed, Scopus, Web of Science databases, using terms such as „phytoestrogen", „isoflavones", „skin aging", „topical phytoestrogens", „anti-aging", „estrogen receptors", as well as combinations thereof, with no time limit. Last search was performed on April 1, 2026.

Studies published in English with original data (*in vitro, ex vivo, in vivo*) and clinical studies or critical reviews on the efficacy of phytoestrogens on the skin were included. Studies in which dietary supplements or hormonal drug interventions were administered internally were excluded. We prioritized papers presenting clear mechanistic evidence to explain biological plausibility and controlled clinical trials to assess efficacy.

In accordance with the hypothesis presented above, scientific interest in cosmetology has focused primarily on phytoestrogens, particularly isoflavones, as evidenced by the results obtained from the keyword combinations used in searches. A search in the Web of Science database for the combination "topical phytoestrogens" and "anti-aging" yielded 10 results; searching for "phytoestrogens" and "anti-aging" yielded 58 results, while the terms "topical isoflavones" and "anti-aging" yielded 21 results (**Figure 1**).

At the same time, various areas of interest in which the studies were conducted are emerging. A review of recent literature indicates that phytoestrogens, particularly isoflavones, have the potential to significantly modulate skin aging processes associated with declining estrogen levels (Amini et al., 2025; Lephart, 2021; Lephart, 2025).

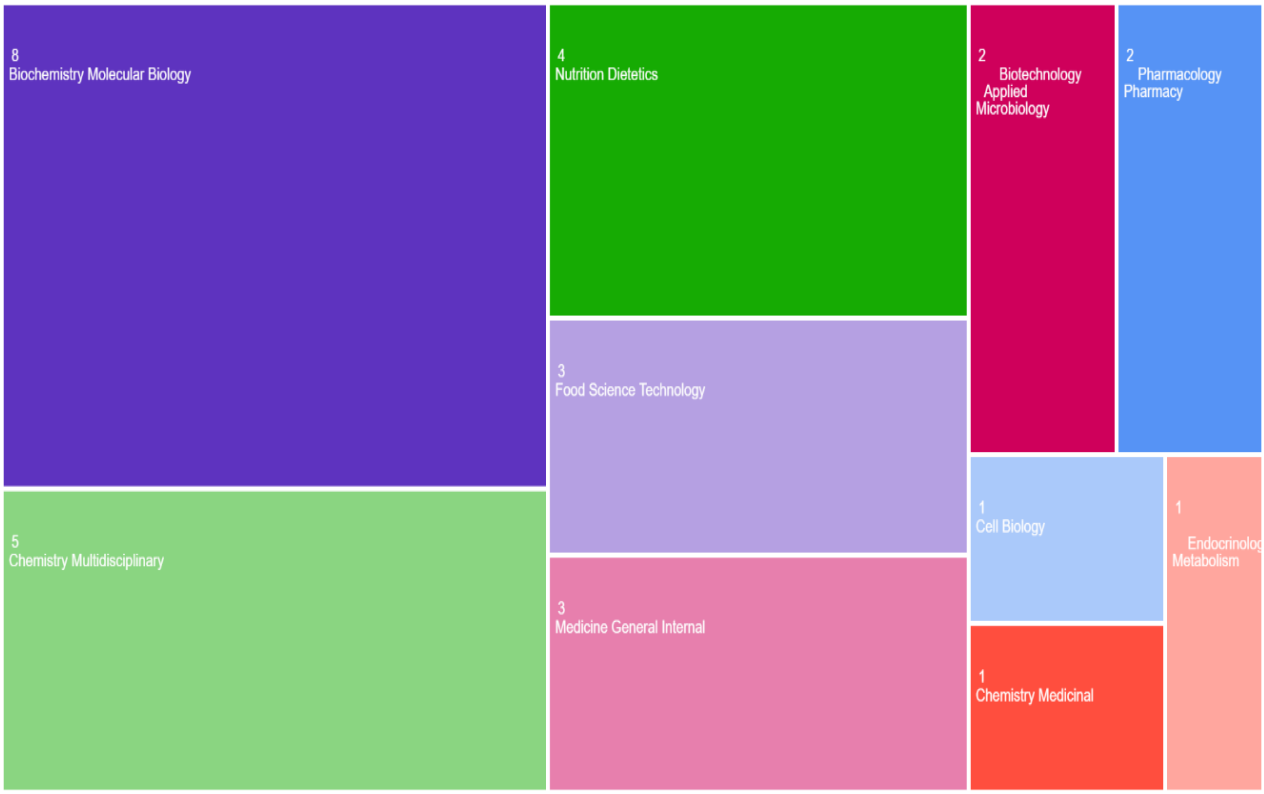


**Fig. 1**. Number of publications found on Web of Science using the terms "topical isoflavones" and "anti-aging," grouped by field (n=21) (image generated by Web of Science)

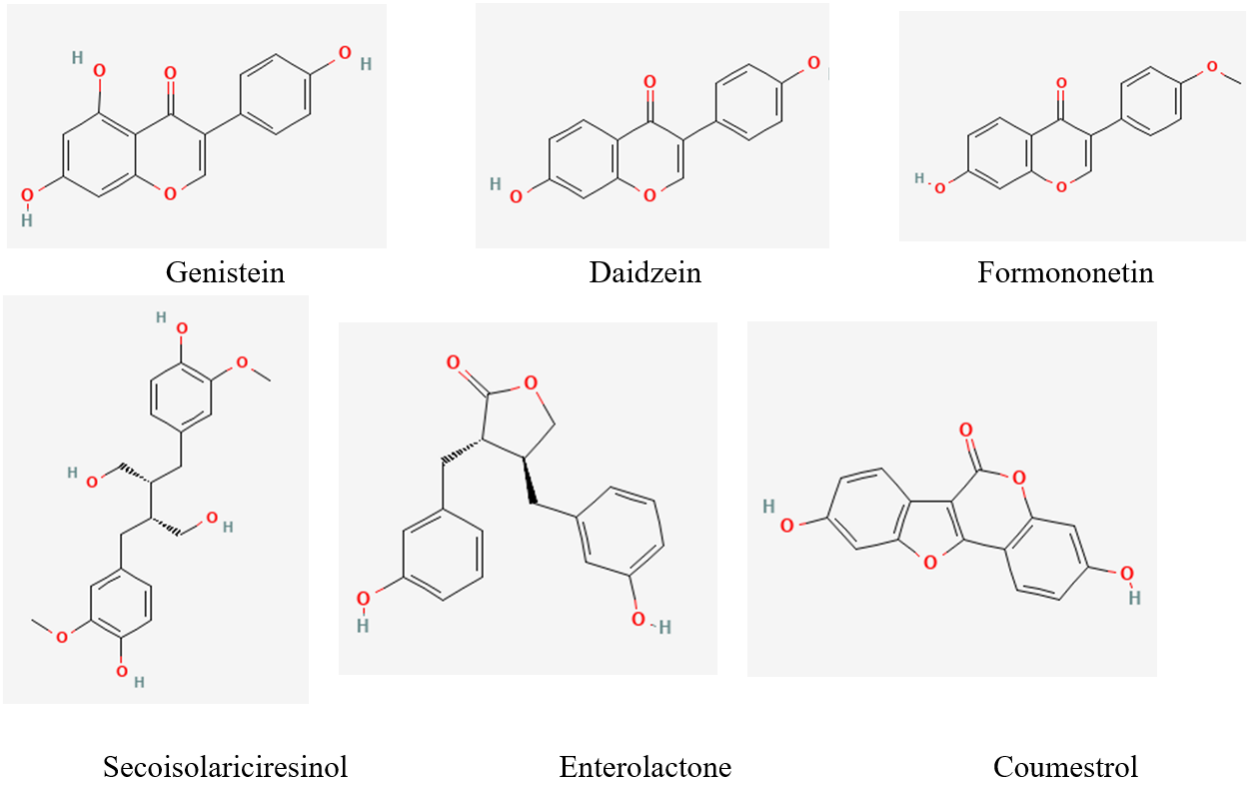


**Fig. 2.** Chemical structures of the most relevant isoflavones, lignans and coumestans used in cosmetics (structures generated by PubChem)

## 3. Definition and Classification of Phytoestrogens Relevant to Skin Aging

While phytohormones and phytoestrogens belong to the same botanical semantic sphere, they are not equivalent and serve entirely different functions. Phytohormones represent the totality of hormones produced by plants – such as auxins, gibberellins, and cytokinins – and their role is strictly linked to regulating plant growth, development and physiology. In contrast, phytoestrogens do not play a hormonal role within the plant itself; instead, they are plant-derived compounds that share structural similarities with mammalian estrogens, allowing them to exert estrogen-like effects specifically within animal or human organisms. Thus, phytohormones act exclusively within plant systems, whereas the external biological action of phytoestrogens makes them the relevant focus for the study of this topic.

Phytoestrogens can be grouped by chemical structure into isoflavones (genistein, daidzein), lignans (secoisolariciresinol, enterolactone), and other phytoestrogens with weak estrogenic activity—cumestans (coumestrol), and stilbenes (resveratrol) (Lephart, 2021). Isoflavones, due to their ability to bind to estrogen receptors and influence intracellular processes of collagen synthesis and other anti-aging mechanisms (Amini et al., 2025), continue to dominate research (**Figure 2**).

## 4. Major Plant Sources and Phytochemical Profiles

The Fabaceae family is the primary source of phytoestrogens studied for their anti-aging effects. The primary focus is on isoflavones from soy (*Glycine max*), but other species are also being investigated for their efficacy in stimulating collagen synthesis and protecting the extracellular matrix, such as Chinese

liquorice (*Glycyrrhiza uralensis*). Recent studies note the presence of phytoestrogens primarily in seeds and underground parts, as extracts obtained from these parts tend to be the most effective in cosmetic applications (Amini et al., 2025).

### 4.1. Soybean - *Glycine max* (L.) Merr.

A recent study shows that an isoflavone-rich extract from soybean leaves can counteract the loss of collagen fibers in the skin of an animal model exposed to estrogen deficiency, suggesting antioxidant and collagen-modulating potential. The study focused on soybean leaves enriched with isoflavones, a product obtained by treating soybean plants with ethylene, with the aim of achieving a final isoflavone content of 11 mg/g. Thus, the enriched leaves reached an isoflavone content approximately fifty times higher than that of typical leaves. The identified isoflavones were daidzein and genistein (Yoo et al., 2024).

### 4.2. Red Clover - *Trifolium pratense* L

In the cosmetics industry, red clover is used as a complementary ingredient in formulations designed for mature or sensitive skin. Recent studies suggest that the isoflavones in red clover help maintain skin homeostasis, interacting primarily with β-type estrogen receptors (ER-β) in the skin (Amini et al., 2025). Red clover has long been an ingredient of interest due to its phytoestrogen content, as demonstrated by a study from 2006. This study examines the potential of a standardized red clover extract containing 11% isoflavones. From a biological standpoint, red clover extracts have demonstrated the ability to reduce oxidative stress and inhibit collagen degradation (Circosta et al., 2006), mechanisms relevant to combating skin aging.

### 4.3. Chinese Liquorice - *Glycyrrhiza uralensis* Fisch. ex DC.

Recent research highlights the presence of compounds such as glycyrrhizin, isoliquiritigenin, liquiritigenin, liquiritin, and glabrol in the roots of Chinese liquorice (Amini et al., 2025; Jiang et al., 2021). The main compounds quantified in extract evaluated on healthy volunteers were liquiritin-apioside, liquiritin, and liquiritigenin. Thus, the potential of Chinese liquorice extracts, rich in flavonoids, to inhibit the production of matrix metalloproteinase 1 (MMP-1) - an enzyme responsible for collagen degradation - is emerging, both in *in vitro* and *in vivo* models (Amini et al., 2025), suggesting significant benefits in anti-aging cosmetic products.

## 5. Mechanisms of Action at the Skin Level

Recent studies show that phytoestrogens can influence certain skin characteristics through various actions. Among these, the following stand out: collagen production via β-estrogen receptor-dependent signaling, skin hydration through the regulation of hyaluronic acid synthesis, and the reduction of extracellular matrix degradation through the inhibition of MMPs (Amini et al., 2025). These mechanisms are significant in managing skin aging.

### 5.1. Interaction with Cutaneous Estrogen Receptors

Compared to endogenous estrogens, phytoestrogens have a lower affinity for estrogen receptors (Amini et al., 2025), resulting in milder, dose-dependent effects, but also a superior safety profile.

Soy isoflavones are considered compounds with increased selectivity for β-estrogen receptors. This explains their beneficial effects on skin homeostasis without excessive systemic estrogenic stimulation. The reviewed studies highlight that the activation of these

receptors in dermal fibroblasts is associated with the stimulation of the synthesis of structural skin components and with the improvement of changes induced by estrogen deficiency (Yoo et al., 2024).

Similarly, the isoflavones in red clover, particularly biochanin A and formononetin, act through estrogen-like mechanisms, exhibiting a higher affinity for β-estrogen receptors. This interaction reduces the impact of estrogen deficiency on dermal structure and fibroblast metabolism (Circosta et al., 2006).

Licorice extracts contain phytoestrogenic compounds such as liquiritigenin, which is known for its selective activity on β-estrogen receptors. The study in question demonstrated that the activation of these signaling pathways is associated with increased expression of genes involved in collagen synthesis and with improved dermal density (Amini et al., 2025).

### 5.2. Action on the Extracellular Matrix

The influence on the extracellular matrix is one of the most important anti-aging mechanisms attributed to phytoestrogens.

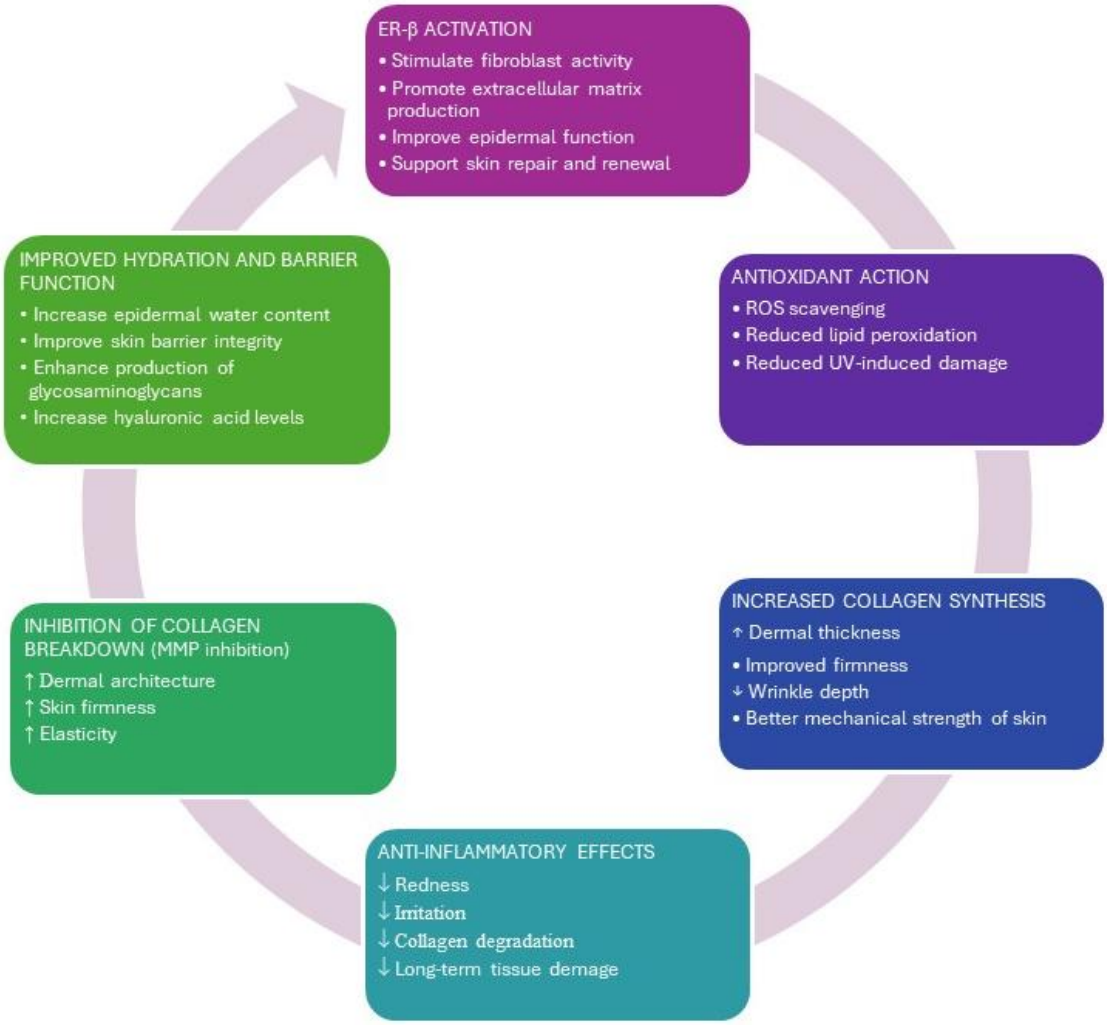


**Fig. 3.** Potential mechanisms through which phytoestrogens influence skin aging and their consequences

At the dermal level, fibroblasts are the primary cellular target of these compounds (Amini et al., 2025), with the activation of estrogen receptors leading to increased synthesis of type I and III collagen and the maintenance of skin elasticity.

According to the studies reviewed, administration of isoflavone-enriched soybean leaf extract led to the restoration of dermal collagen fibers in ovariectomized rats, suggesting a direct effect on extracellular matrix remodeling and fibroblast activity (Yoo et al., 2024).

A recent study shows that isoflavonoids from soy increased collagen and hyaluronic acid, but were less effective than the control, estradiol (Farkas E et al., 2026).

In the case of red clover, administration of isoflavones reduced skin changes induced by ovariectomy, preventing a decrease in dermal thickness and a reduction in collagen content. These results suggest a protective effect on the extracellular matrix through the stimulation of fibroblasts and the limitation of structural degradation. Chinese liquorice extract has been shown to affect the COL1A1 and COL3A1 genes—which are essential for collagen production—in human dermal fibroblasts, thereby increasing procollagen I production. In addition, the study highlighted the inhibition of MPP-1 induced by UVB rays and pollution (Circosta et al., 2006), which contributes to reducing collagen degradation and preserving dermal structure (**Figure 3**).

### 5.3. Protection Against Photoaging

Photoaging results from chronic exposure to ultraviolet radiation and the associated oxidative stress. These processes lead to collagen degradation, impairment of the skin barrier, and hyperpigmentation. Phytoestrogens possess significant antioxidant properties, helping to limit the oxidative degradation of proteins and membrane lipids. Isoflavones and flavonoids from soy, red clover, and Chinese liquorice have demonstrated the ability to reduce oxidative stress caused by UV rays and to diminish the inflammatory processes associated with photoaging. Liquorice extract prevents dermal collagen breakdown by blocking the MMP-1 production triggered by UVB rays and pollutant particles. Furthermore, active compounds in licorice, such as glabridin and liquiritigenin, possess antioxidant and photoprotective activity (Amini et al., 2025), reducing the oxidative effects associated with UV radiation exposure.

Numerous studies have suggested that phytoestrogens could reduce UV-induced erythema by decreasing inflammation and lipid peroxidation (Lin, 2008; Widyarini, 2001). At the same time, the flavonoid compounds in licorice produce depigmenting effects by inhibiting tyrosinase, thereby limiting inflammatory hyperpigmentation and skin pigmentation irregularities (Amini et al., 2025). These mechanisms support the use of phytoestrogens in the prevention and management of the clinical signs of photoaging.

### 5.4. Anti-inflammatory Effect and Other Relevant Mechanisms

Inflammation drives skin aging by progressively degrading the extracellular matrix and impairing skin barrier function. Phytoestrogens can reduce inflammation-induced tissue degradation through their antioxidant and estrogen-like activity (Amini et al., 2025).

Isoflavones from soy and red clover help maintain dermal homeostasis by supporting fibroblast activity and reducing changes associated with estrogen deficiency (Yoo et al., 2024; Circosta et al., 2006), including loss of elasticity and thinning of the dermis.

In the case of Chinese liquorice extract, the anti-inflammatory effects are associated with

both a reduction in oxidative stress and a limitation of extracellular matrix degradation through the inhibition of MMP-1. At the same time, the improvement in dermal density observed in the clinical study (Amini et al., 2025) suggests beneficial effects on the microstructure of the dermis and on the maintenance of vascular and skin tissue support (**Table 1**).

**Table 1**. Preclinical and clinical evidence for topical phytoestrogens in skin aging

| **Plant source, active compounds** | **Study outcome and mechanisms of action in the skin** | **Study design** | **Bibliographic reference** |
|---|---|---|---|
| Human studies | | | |
| Genistein | Protection against peroxidation by modulating oxidant/antioxidant system and mitochondria membrane potential; mechanisms related to ERs and GPER30 and kinases activation | *In vitro*, human fibroblasts/keratinocytes | Savoia et al., 2018 |
| *Glycyrrhiza uralensis* extract, with liquiritigenin as a quantitative chemical marker | Induces Collagen I and III Gene Expression<br>Induces Pro Collagen I Protein Expression<br>Inhibits UVB Induced MMP1<br>Inhibits Pollution Induced MMP1 | *In vitro*, human epidermal keratinocytes and dermal fibroblasts | Amini et al., 2025 |
| Gel with isoflavones (genistein 4%) | Estrogens have a stronger effect on histomorphometrical parameters than isoflavones | DB-RCT, postmenopausal women (n=46); 24 weeks; control group: gel with 17-beta-estradiol 0.01% | Moraes et al., 2009 |
| Active formulation containing 1% *Glycyrrhiza uralensis* extract, with liquiritigenin as a quantitative chemical marker | Improvements in dermal density for sub-group above 50 years old | *In vivo*, healthy female volunteers (n=46, mean age=58 years); 8 weeks, twice daily | Amini et al., 2025 |
| Genistein (99% purity), vitamin E, vitamin B3, and ceramide | Skin wrinkling parameters showed a significant reduction | DB-RCT, postmenopausal women (n=50, mean age=55,8 years), 6 weeks | Na Takuathung et. al., 2023 |
| Resveratrol, 2% emulsion | Reduces the appearance of skin wrinkles; increases skin firmness; decreases skin redness | *In vivo*, observation study female subjects (n=20, average age=44.6 years); 8 weeks | Brinke et al., 2021 |
| Trans-resveratrol: one capsule of 75 mg/day and apply 1 g of cream (1.5% trans-resveratrol) twice daily | When taken orally and applied topically, trans-resveratrol was effective at wrinkle reduction, and when applied topically, it increased sebum levels. | DB-RCT, Females aged 40 years and above (n=122); 8 weeks | Rao et al., 2025 |
| Animal studies | | | |
| Genistein, solution 0.5%<br>Daidzein, solution 0.5% | Provide effective photoprotection (measured by sunburn cell formation and/or erythema) | White Yorkshire pigs, 4 days | Lin et al, 2008 |

| | | | |
|---|---|---|---|
| Biochanin A, solution 0.5% | | | |
| Isoflavones from *Trifolium pratense,* red clover: genistein, daidzein, biochanin A, formononetin; the metabolites equol and isoequol and the derivative dehydroequol. | Protect from inflammation and immune suppression induced by UV radiation | Hairless albino mice | Widyarini et al., 2001 |

## 6. The Efficacy of Phytoestrogens in Anti-Aging Products and Evaluation Methods

### 6.1. Parameters Used in Evaluating the Efficacy of Anti-Aging Products

The efficacy of anti-aging cosmetic products is determined through a series of clinical tests that monitor structural and functional changes in the skin. Among the most important parameters analyzed are the degree of hydration, skin elasticity and firmness, wrinkle reduction, transepidermal water loss (TEWL), pigmentation levels, and skin tone uniformity (Fluhr et al., 2006; Lodén and Maibach, 2000).

Hydration of the stratum corneum is one of the main indicators of the skin's barrier function. It is measured by corneometry, using a device called a corneometer, which determines moisture levels by measuring the skin's electrical conductivity (Rogiers, 2001). Thus, the drier the skin, the lower its electrical conductivity, and increased hydration and an increased dielectric constant are associated with the reduction of fine wrinkles and improved skin elasticity.

TEWL (Transepidermal Water Loss) reflects the integrity of the skin barrier and is measured using a TEWL meter. Elevated TEWL values are associated with skin aging (Pinnagoda et al., 1990). When testing cosmetic products, a decrease in TEWL following product use suggests an improvement in the skin's barrier function attributable to the product.

Skin elasticity is assessed via cutometry using a cutometer. This measures the skin's ability to return to its original shape after the application of controlled negative pressure.

The R2 and R7 parameters are considered relevant indicators of biological elasticity and skin firmness (Dobrev, 2000). Reduced skin elasticity and firmness are associated with the degradation of collagen and elastin fibers, which are among the main causes of skin aging.

Wrinkle evaluation involves analyzing parameters such as depth, surface area, roughness, and skin texture. Assessments are conducted both clinically and instrumentally, utilizing standardized photography, 3D imaging analysis (Luebberding et al., 2013), or specialized systems such as Visioscan, PRIMOS, and Antera 3D.

Hyperpigmentation and skin color unevenness are assessed via colorimetry or mexametry. These methods allow for the measurement of melanin levels and erythema using the mexameter, a narrow-band reflectance spectrophotometer (Fullerton et al., 1996).

Reducing pigmentation spots and evening out skin tone are considered key factors in determining the effectiveness of anti-aging products.

Beyond traditional approaches, we must consider how phytoestrogens can influence the body. For cosmetic products containing phytoestrogens, a detailed analysis should also be conducted on how they act throughout the entire body. Thus, we should determine the extent to which they are absorbed—both at the skin level and whether they are taken up into the bloodstream—how these substances are eliminated from the body, and how these parameters vary depending on concentration and frequency of use.

### 6.2. Clinical Evidence on the Efficacy of Phytoestrogens in Anti-Aging

Genistein is one of the most studied phytoestrogens used in anti-aging products due to its ability to bind to β-estrogen receptors present in the skin. Activation of these receptors can stimulate collagen synthesis, increase hyaluronic acid secretion, and reduce oxidative stress (Patra et al., 2023; Intharuksa et al., 2025) involved in skin aging.

A randomized, double-blind, placebo-controlled clinical trial evaluated the efficacy of a topical product containing genistein, combined with vitamin E, vitamin B3, and ceramides, in postmenopausal women. The study included 50 participants who applied the product over a 6-week period. The authors reported an increase in skin hydration, a reduction in fine pores, and an improvement in certain wrinkle parameters, particularly in participants over the age of 56. These results must, however, be interpreted with caution, as the tested product contained several ingredients with proven anti-aging effects (vitamin E, ceramides, and niacinamide) (Na et al., 2023), making it difficult to attribute the results exclusively to genistein. Additionally, the study duration was short, and the sample size was small.

## 7. The Cosmetic Formulation of Anti-Aging Products Containing Phytoestrogens

The phytoestrogens from the targeted plant sources are isoflavones and polyphenolic flavonoids with fairly weak estrogenic activity. Structurally, most isoflavones have relatively low molecular weights, which promotes diffusion through the *stratum corneum* and skin permeability. However, the chemical form strongly influences absorption. Thus, aglycones are more easily absorbed through membranes due to their increased lipophilicity, unlike glycosides, which are more hydrophilic (Setchell et al., 2001).

In the case of red clover, biochanin A and formononetin are more lipophilic than genistein and daidzein, which may limit their dispersion in aqueous vehicles. Thus, solubilization systems are often necessary for efficient absorption (Del Rio et al., 2013).

Similarly, glabridin from licorice is lipophilic and highly sensitive to oxidation and light, which makes it difficult to formulate into stable aqueous systems (Ao et al., 2010).

In general, isoflavones are relatively stable in a slightly acidic to neutral range (approximately pH 4–7), a range comparable to the natural pH of the skin. However, under alkaline conditions, accelerated oxidation, isomerization, and phenolic degradation may occur, and sensitivity to light and oxygen increases (Hu, 2007). In the case of glabridin, instability is accentuated both in an alkaline environment and under conditions of light exposure, where oxidative degradation occurs more rapidly (Ao et al., 2010).

Polyphenols are more susceptible to photooxidative processes because they absorb UV radiation and can lead to changes in chemical structure and photoinduced oxidation. These processes can lead to reduced biological activity, color changes in the formulation, precipitation, and diminished efficacy of the cosmetic product (Fernandes et al., 2023; Savic

et al., 2026). To promote stability, specific packaging (opaque and airless), antioxidants such as vitamin E, chelating agents, and lipid encapsulation systems are used.

The compatibility of phytoestrogens with excipients is a key consideration in the development of dermo-cosmetic formulations. Common issues include complexation with metal ions, precipitation in the presence of electrolytes, incompatibility with ionic surfactants, and oxidative degradation in insufficiently stabilized emulsions (Savic et al., 2026). Thus, isoflavones exhibit superior stability in anhydrous systems, W/O (water-in-oil) emulsions, phospholipid carriers, and nanoencapsulated systems.

Finally, phytoestrogens are also compatible with other active ingredients: antioxidants (vitamins C and E), peptides, or other moisturizing ingredients, which results in a synergistic anti-aging effect without significantly increasing the risk of skin irritation (Tomas et al., 2025).

To improve solubility, stability, and skin permeability, modern delivery systems for phytoestrogens, such as liposomes and nanoemulsions, have been developed. Liposomes and nanoemulsions are advanced, small-sized colloidal systems designed to facilitate dispersion. Liposomes are phospholipid vesicles frequently used in cosmetic formulations to protect active compounds from oxidation and to improve skin penetration. These systems allow for the controlled release of active substances and reduce the irritation potential of formulations (Akombaetwa et al., 2023).

Nanoemulsions increase the contact surface area and facilitate the solubilization of lipophilic compounds, improving distribution and skin permeability. Among their advantages are superior stability compared to other types of delivery systems, as they increase dermal penetrability and facilitate the development of an improved cosmetic formulation (Woo et al., 2025).

Solid lipid nanoparticles and nanostructured lipid carriers reduce oxidative degradation and facilitate the incorporation of highly lipophilic compounds into stable lipid bases (Eroğlu et al., 2023). These systems prolong contact time with the skin surface and improve the permeability of the stratum corneum. They are considered promising for active ingredients such as glabridin and biochanin A.

Self-emulsifying systems allow for the spontaneous formation of fine dispersions upon contact with the aqueous phase of the formulation, increasing the dispersion and solubilization of lipophilic compounds (Eid et al., 2023). In cosmetic products, these systems can contribute to the uniform distribution of phytoestrogens and increased skin penetration.

The use of standardized extracts encourages a potential synergistic effect between bioactive compounds and complex antioxidant and anti-inflammatory activity (Stallings and Lupo, 2009). However, these extracts have disadvantages such as lower stability and difficulty in standardization within cosmetic formulations.

Currently, modern trends in cosmetic formulation aim for the rigorous standardization of plant extracts and their combination with nano-encapsulated systems to optimize stability and skin bioavailability (Woo et al., 2025; Eroğlu et al., 2023).

Standardization of extracts (Stallings and Lupo, 2009) is essential because phytoestrogen content varies depending on species, cultivation methods, harvesting, processing, and extraction.

## 8. Safety and Controversies

The benefits of using phytoestrogens to combat the skin aging process have been mentioned previously, highlighting their

antioxidant and moisturizing actions and their influence on collagen synthesis; however, studies also outline other important benefits for the skin.

One benefit is the improvement of the skin's barrier function. The combination of phytoestrogens and phytosterols can stimulate the production of ceramides and hyaluronic acid in the epidermis, reducing transepidermal water loss (Carneiro et al., 2023), thus highlighting, from a distinct perspective, a previously noted benefit.

The cosmetics industry also relies heavily on product presentation. Thus, consumer acceptance will be much higher for products—even hormonal ones—derived from natural sources.

Despite their promising potential, the use of phytoestrogens in anti-aging cosmetics poses certain significant limitations.

First, their hormonal activity is weak and fluctuating. The efficacy of phytoestrogens depends on their concentration, chemical composition, and ability to bind to estrogen receptors. This may limit their anti-aging effect compared to other types of hormonal therapies (Baber, 2010).

Another drawback is their limited ability to penetrate the skin. Phytoestrogens vary in terms of lipophilicity, which can reduce their bioavailability in the dermis unless delivery systems such as nanovectors or advanced lipid formulations are used (Cheng et al., 2025).

Furthermore, from a regulatory standpoint, there is a lack of standardization for these extracts. Concentrations of active ingredients can vary between products, and some formulations may contain insufficient amounts to produce significant effects (Tomas et al., 2025), which highlights the discrepancy between marketing claims and scientific reality.

Clinical data are still limited, particularly regarding long-term topical treatments, and although the risk is considered low, there are potential safety precautions for individuals with a history of hormone-dependent diseases (Lephart, 2025), where systemic absorption could interfere, even minimally, with hormonal therapies.

Furthermore, some phytoestrogens can negatively affect skin health if not used in appropriate amounts or formulations. They can cause increased sensitivity or even skin irritation, particularly in formulations containing irritating ingredients (alcohol, fragrances).

## 9. Research Gaps and Future Directions

Phytoestrogens found in soy, red clover, and Chinese liquorice are promising compounds due to their estrogen-like, antioxidant, and anti-inflammatory properties. Among these, the isoflavones in soy and red clover have the strongest scientific support, particularly regarding their interaction with estrogen receptors and their effects on skin elasticity and hydration.

The best-supported mechanisms are antioxidant activity and the reduction of inflammation. Although some anti-aging or regenerative effects remain insufficiently demonstrated clinically, the available clinical evidence suggests favorable effects on skin parameters; however, these are limited by small sample sizes, the short duration of the studies, and the lack of standardization in formulations.

Major obstacles in interpreting the results include variations in the concentrations used, extraction methods, and the characteristics of the populations studied. Furthermore, the lack of uniform evaluation methods and data on long-term safety limits the credibility of current results.

At the same time, the report highlights gaps that can provide a clear and useful perspective for further exploration of the topic and the development of the industry. Although most recommendations for further development

are based on the assessment of safety and stability over time, it is also important to investigate how these phytoestrogens are assimilated by the body and how they are subsequently eliminated to avoid potential adverse reactions in the glands or other organs.

In this context, the future development of products containing phytoestrogens should aim for the standardization of plant extracts, clear reporting of concentrations, and the use of objective, reproducible testing methods. More extensive clinical studies, conducted on well-defined populations and with longer follow-up periods, are needed to clarify the efficacy and safety of these compounds.

## Conclusions

This paper has highlighted the significant potential of phytoestrogens, as plant-based active ingredients in anti-aging cosmetic formulations, by analyzing scientific studies that demonstrate their beneficial effects.

Overall, plant phytoestrogens show considerable potential as alternatives or adjuvants in skin care, but full validation of their efficacy and safety requires a much broader approach.

## Conflict of Interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

## Author Contributions

MA. P: Conceptualization, Investigation, Visualization, Writing – original draft. E. LZ: Conceptualization, Supervision, Writing – review & editing.

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The authors declare that the research was conducted in the absence of any Artificial Intelligence (AI) Tools.

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