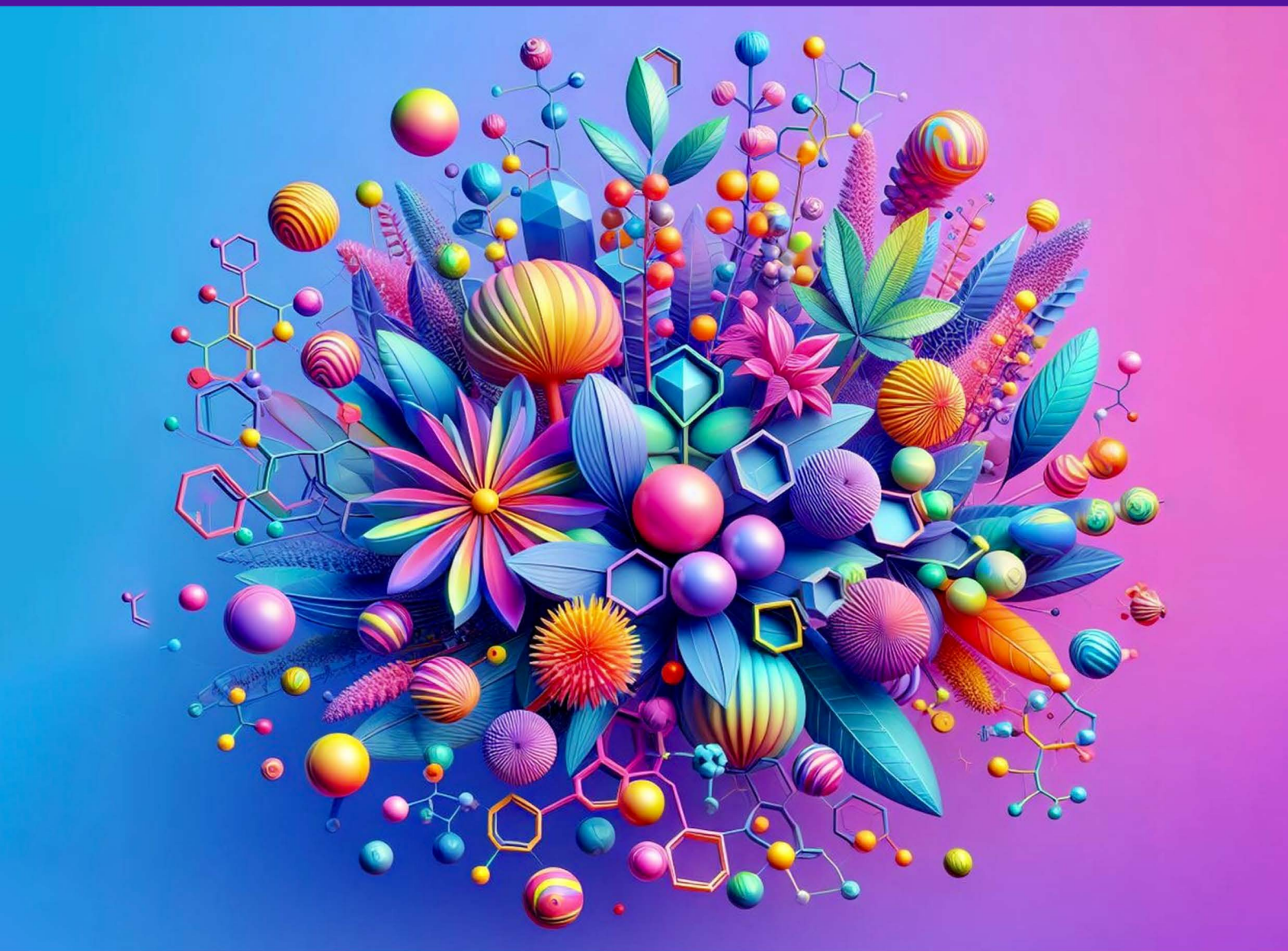
https://www.sciltp.com/journals/jmnp
Online ISSN: 2982-1975
Volume 2 , Issue 1, 2025

# Journal of Medicinal Natural Products



## Phytotherapy Targeting Rheumatoid Arthritis: A Clinically Based Approach

Scilight

# Contents

Vol. 2 No. 1 March 2025

*Journal of Medicinal Natural Products*

*Article*

# Bioactive Potential of *Premna esculanta*: A Study on Antioxidant, Antimicrobial and Antidiarrheal Efficacy

**Hafijul Yousuf, Al Mamun Shohag, Md Shawon Miah, Md Foyzur Rahman and As-Sazzad Mahmud ***

Department of Pharmacy, Dhaka International University, Dhaka 1212, Bangladesh
* Correspondence: sazzad.phar@diu.ac; Tel.: +880-1521305611
Received: 20 October 2024; Revised: 9 November 2024; Accepted: 14 November 2024; Published: 10 January 2025

**Abstract:** Objective: The discovery of the genus *Premna* and its traditional uses are the result of extensive information gained by living in forest or semi-forest areas and closely observing indigenous populations regarding the therapeutic qualities of plants. This investigation centered on examining the DPPH scavenging, antimicrobial and anti-diarrheal properties of ethanolic leaf extracts. This study aimed to investigate the bioactive components and antibacterial properties of ethanolic leaf extracts and their fractions. Methods: In vitro antioxidant was evaluated by DPPH scavenging assay and the disc diffusion method evaluated antimicrobial efficacy. In vivo screening for antidiarrheal was conducted, the latent period of defecation in castor oil-induced diarrhea in mice assessed antidiarrheal effects. Results: The extract showed an ability to scavenge DPPH with $IC_{50}$ values of 0.931 µg/mL for *Premna esculanta* extract and $IC_{50}$ 0.902 µg/mL for ascorbic acid standard. The extract demonstrated significant antimicrobial activity, with inhibition zones ranging from 12–19 mm against various microbial strains, notably *Shigella boydii*, *Shigella dysenteriae*, *Pseudomonas aeruginosa*, *Escherichia coli*, *Sarcina lutea*, *Bacillus subtilis*, and *Candida albicans*, at concentrations of 250 and 500 µg/disc. In anti-diarrheal test, Loperamide (3 mg/kg) reduced total feces and defecation by 56.62% with concentrations of 500 mg/kg. Significant changes were observed in anti-diarrheal studies. Conclusion: *Premna esculanta* leaf extract demonstrated significant antimicrobial, and antidiarrheal activities.

**Keywords:** DPPH; antimicrobial; anti-diarrheal

## 1. Introduction

The use of plants for medicinal purposes in both humans and animals dates back centuries. Many synthetic treatments, particularly those that are tropical, tend to have a range of side effects and are often too expensive for most people to afford to address this issue, people have turned to the plants available in their surroundings, despite the lack of scientific proof regarding their efficacy [1]. The health benefits of these plants come from phytochemicals, which are non-nutritive compounds that protect humans from various illnesses. The primary components include alkaloids, flavonoids, saponins, phenolic compounds, phytosterols, proteins, amino acids, gums, mucilage, and lignin. These phytochemical elements are fundamental to the development of various pharmaceutical industries and are crucial for identifying crude drugs [2]. Both epidemiological and in vitro research on medicinal plants and vegetables have strongly indicated that plant compounds with antioxidant properties may offer protective benefits against oxidative stress in biological systems [3]. One of the significant challenges in global healthcare is the urgent demand for new, effective, and affordable treatments for microbial infections, particularly in developing nations where infectious diseases account for nearly half of all deaths [4].

The World Health Organization (WHO) has initiated a control program focused on diarrheal diseases, incorporating traditional medicine alongside health education and preventive measures (Syder medicine). This program primarily relies on herbal remedies [5]. Antipyretic medications typically work by preventing or reducing the expression of COX-2, which helps lower elevated body temperatures by inhibiting the production of prostaglandin E2 (PGE2) [6]. Today, people around the globe increasingly prefer medicines derived from plants due to the undesirable side effects associated with synthetic drugs, which are often seen as more suitable for long-term treatment. Traditional plants may offer new compounds that can help mitigate the high costs and toxic effects of current medications for many rural communities in developing countries [7].

*Premna esculanta* (Roxb), a small, branching shrub from the Lamiaceae family, thrives in the moist, shaded areas of Bangladesh and India's primary rainforests. Tribal communities in the Chittagong Hill Tracts of Bangladesh have long used this plant to treat conditions such as gout, jaundice, lipomas (tumors), and edema. The leaves of *Premna esculanta* are applied to address arthritis and infections caused by bacteria and fungi [8].

Furthermore, this plant has been noted for its analgesic and thrombolytic properties [9], as well as its anti-inflammatory and anti-nociceptive effects [10], and its antioxidant and hepatoprotective activities [11].

This study aims to evaluate the therapeutic potential of *Premna esculanta* leaf extract by assessing its antibacterial, anti-diarrheal, antioxidant, neuropharmacological, and anti-inflammatory properties, with the goal of exploring its potential contributions to healthcare.

## 2. Materials and Methods

### *2.1. Chemical*

Analytical grade chemicals, including 2,2-diphenyl-1-picryl hydrazyl (DPPH) from Sigma, Burlington, MA, USA, $AlCl_3$ (aluminum chloride), NaOH (sodium hydroxide), $Na_2HPO_4 \cdot 2H_2O$ (disodium hydrogen phosphate dihydrate) from Loba, Mumbai, India, as well as $H_2O_2$ (hydrogen peroxide) from Merck, Darmstadt, Germany, and $C_{12}H_{12}N_2O_2S$ (phenazine methosulfate PMS) from Sigma, USA, were utilized. All standard medications employed for in vivo pharmacological evaluations were acquired from Square Pharmaceuticals Ltd. (Dhaka, Bangladesh) and Incepta Pharmaceuticals Ltd., Dhaka, Bangladesh.

### *2.2. Plant Collection and Extraction*

Plant leaves from *Premna esculanta* were gathered in Bandarban, Chittagong, Bangladesh, in July 2023. The identification of the plant specimen was conducted by Dr. Mohammad Sayedur Rahman, a senior scientific officer at the Bangladesh National Herbarium in Mirpur, Dhaka, Bangladesh, and the authentication number assigned was DACB-94772. The collected leaves were dried in the shade, crushed into a coarse powder, and then extracted using maceration, involving 250 g of the powdered leaves mixed with 1000 mL of 96% ethanol for 14 days. The extraction process yielded 6.3%.

### *2.3. Animals*

Young Swiss albino mice, aged between four to six weeks and weighing approximately twenty to twenty-five grams, were obtained from Jahangirnagar University in Bangladesh. After their acquisition, they were placed in the animal facility of the pharmacology lab within the pharmacy department for a period of two to three weeks to help them adjust to their new environment. This accommodation took place at Dhaka International University in Bangladesh. All experiments were performed in a calm, secluded, and controlled setting. The animal studies for this research adhered to the ethical standards set by the Committee of Clinical Pharmacy & Pharmacology at the Department of Pharmacy, Dhaka International University, located in Satarkul, Badda, Dhaka-1212. [Ref: CPP/DIU/EC/006].

### *2.4. DPPH Scavenging Activity*

DPPH scavenging assay, as described by Khan 2013 [12]. The experiment was carried out with ten test tubes, each containing specific amounts of plant extract and ascorbic acid at concentrations of: 500, 250, 125, 62.5, 31.25, 15.625, 7.813, 3.906, 1.953, and 0.977 μg/mL. To prepare these concentrations, the plant extract and ascorbic acid were measured three times and then dissolved in ethanol. Ascorbic acid acted as positive control. A precise amount of DPPH was weighed and dissolved in ethanol to prepare a 0.004% (*w*/*v*) solution, which was then blended using a sonicator. Each test tube received 1 mL of the various concentrations of ascorbic acid and plant extract. Subsequently, 3 mL of the 0.004% DPPH solution was pipetted into each test tube. The test tubes were then stored in a dark environment at room temperature for 30 min to allow the reaction to occur fully. In addition, blank test tubes containing only ethanol with DPPH were prepared. After this incubation period, the absorbance of each test tube was recorded at 517 nm using a UV spectrophotometer. The percentage of inhibition was determined with the following formula: % inhibition = [(Blank absorbance − Sample absorbance)/Blank absorbance] × 100.

### *2.5. Antimicrobial Activity*

To evaluate the extract's antimicrobial activity disc diffusion method was used which was outlined by Fakruddin [13]. Tests were performed on the extract against a range of eight gram-negative microorganisms, which

included Shigella boydii, Shigella dysenteriae, Pseudomonas aeruginosa, Salmonella paratyphi, Salmonella typhi, Vibrio parahaemolyticus, Vibrio mimicus, and Escherichia coli. Additionally, there were eight gram-positive bacteria tested as well, comprising Sarcina lutea, Bacillus cereus, Bacillus megaterium, Bacillus subtilis, Saccharomyces cerevisiae, Candida albicans, Aspergillus niger, and Staphylococcus aureus. Mueller-Hinton agar medium was used to prepare test plates. 25 and 50 micrograms per of crude leaf extract per microliter were placed onto 5-mm filter paper discs (Whatman No. 1). After that the discs were totally dry. Following the imbuing process, the discs were covered with 100 µL of the culture and cultured for 24 h at 37 °C on sterilized agar plates. Discs containing 5 µg of ciprofloxacin were used as a positive control. The inhibitory zone's diameter was measured in millimeters following the incubation period. The control reference was a sterile blank disc.

### *2.6. Evaluation of Antidiarrheal Activity*

The antidiarrheal activity was assessed using a model where mice induced with diarrhea by administration of castor oil were tested, as described by Golder [14]. Castor oil decreases the absorption of fluid content and promotes intestinal motility. While the positive control group was given the standard drug loperamide at a dose of 3 mg/kg 0.5 mL of castor oil orally to induce diarrhea 30 min later, the test groups were given the sample extract at 250 and 500 mg/kg body weight doses. After four hours, the latent period's duration and amount of feces were recorded for each mouse in its cage on blotting paper.

### *2.7. Statistical Analysis*

The means ± standard errors of means were used to express all experimental results. One-way analysis of variance was utilized to evaluate statistical significance using Dunnett's test.

With Prism 6.0 (Graph Pad Software Inc., San Diego, CA, USA), and Excel statistical analysis was carried out. When $p < 0.05$, the study's results were deemed statistically significant.

The means ± standard errors of the means were utilized to present all experimental findings. To assess statistical significance, a one-way analysis of variance was performed, employing Dunnett's test. Statistical analyses were conducted using Prism 6.0 (Graph Pad Software Inc., San Diego, CA, USA) and Excel. Results were considered statistically significant when the *p* value was less than 0.05.

## 3. Results and Discussion

### *3.1. DPPH Scavenging Activity*

The DPPH free radical scavenging assay was conducted to assess the quantitative antioxidant activity, revealing that the $IC_{50}$ values for the ethanol crude extract of *Premna esculanta* was measured at 0.931 µg/mL, while ascorbic acid, the standard reference, had a value of 0.902 µg/mL (Figure 1). *Premna esculanta*, a plant belonging to the Lamiaceae family found in Bangladesh, was evaluated for its capacity to neutralize free radicals by reacting with DPPH free radicals. The extract's effectiveness in scavenging DPPH was compared to that of ascorbic acid, which is recognized as a potent antioxidant. The ability to act as an antioxidant is a crucial pharmacological characteristic of plants.

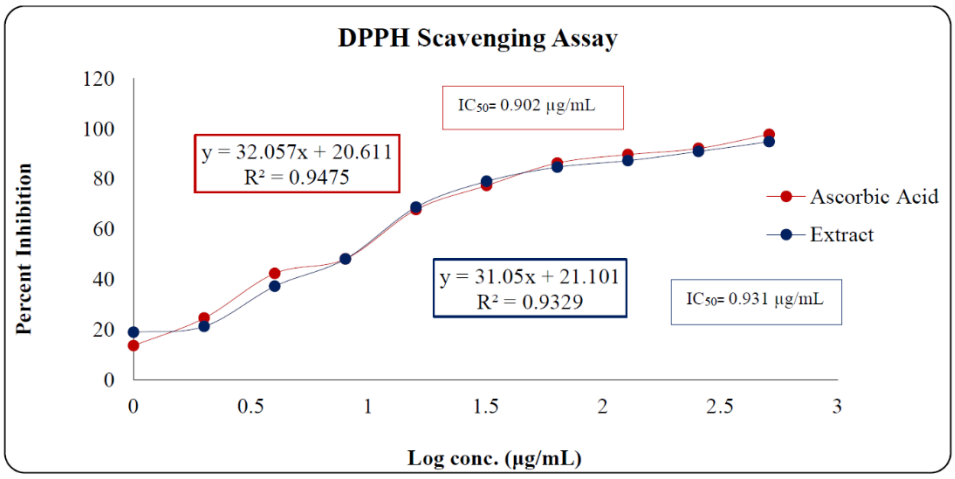



**Figure 1.** Comparison of absorbance vs. log concentration graph for ascorbic acid vs. *Premna esculanta.*

DPPH is commonly used to evaluate the free radical scavenging or antioxidant potential of plant extracts since it is easily neutralized by antioxidants [15]. The ability of the extract to scavenge was found to be dependent on its concentration, represented by $IC_{50}$ (the concentration of the sample needed to reduce the initial concentration of DPPH by 50%). A lower $IC_{50}$ value signifies greater antioxidant activity. In this study, the plant extract exhibited an $IC_{50}$ of 0.931 µg/mL, while standard ascorbic acid had a value of 0.902 µg/mL (Figure 1).

The ethanolic extract of *Premna esculanta* demonstrates significant antioxidant activity, indicated by its DPPH scavenging ability with an $IC_{50}$ close to that of ascorbic acid. This activity suggests the presence of bioactive phytochemicals, primarily phenolics, and flavonoids, which are known to neutralize free radicals by donating hydrogen atoms or electrons. Flavonoids and phenolics have been widely studied for their antioxidant capacities, correlating with the extract's effectiveness in reducing oxidative stress [12]. The high antioxidant potency of these compounds implies a protective role against cellular damage and potential therapeutic application in managing oxidative stress-related disorders.

### *3.2. Antimicrobial Activity*

The antimicrobial and antifungal properties of the extract were assessed at concentrations of 250 µg and 500 µg against various bacterial and fungal strains via the zone of inhibition technique. Ciprofloxacin at 5 µg served as the positive control, while a blank sample acted as the negative control. The extract demonstrated different levels of inhibition against both Gram-positive and Gram-negative bacteria. At the 250-µg concentration, notable activity was observed against *Shigella boydii* (17 mm), *Shigella dysenteriae* (12 mm), and *Pseudomonas aeruginosa* (14 mm).

At 500 µg, the inhibition zones significantly increased for *Shigella dysenteriae* (16 mm), *Pseudomonas aeruginosa* (18 mm), and *Escherichia coli* (19 mm). For the Gram-positive strains, the extract at 500 µg showed relevant activity against *Sarcina lutea* (17 mm), *Bacillus subtilis* (17 mm), and *Candida albicans* (17 mm) (Figure 2).

The findings suggest that the extract possesses broad-spectrum antimicrobial activity, with higher concentrations yielding larger zones of inhibition. Generally, the Gram-negative bacteria displayed greater inhibition zones than the Gram-positive strains, indicating a possible difference in susceptibility [16]. Gram-negative bacteria have an outer membrane that may be more vulnerable to the extract's ingredients, which could be explained by the differences in their cell wall architectures [17].

The antifungal activity, particularly against *Candida albicans*, highlights the extract's potential as an antifungal agent [18]. The extract's efficacy at higher concentrations suggests that its active compounds could be isolated and potentially used in higher doses to combat microbial infections effectively.

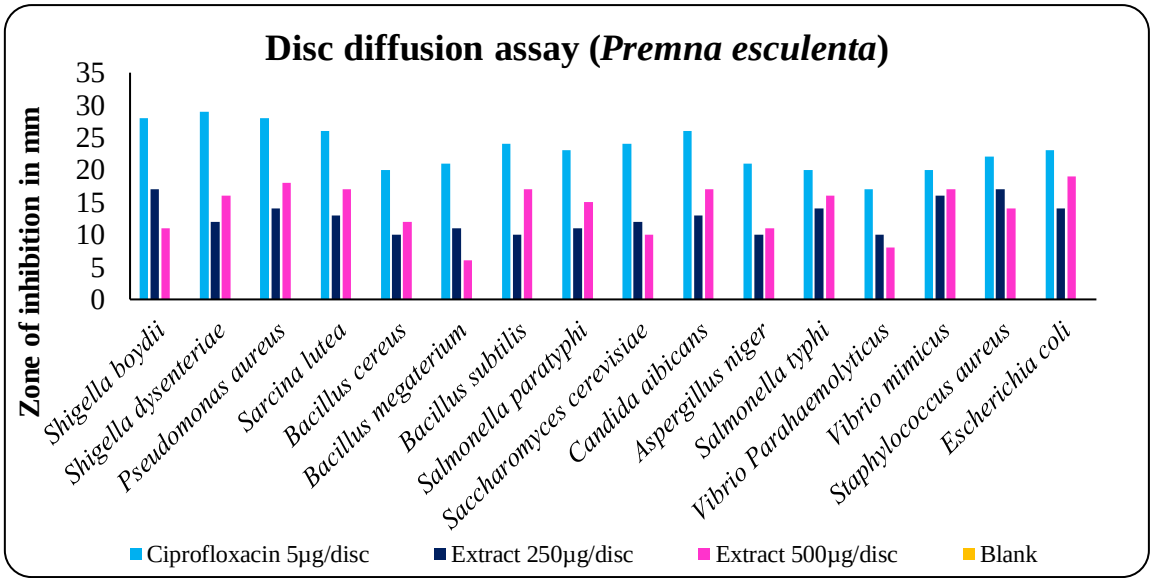



**Figure 2.** In vitro antimicrobial activity of ethanolic extract of *Premna esculanta.*

The antimicrobial effects of *Premna esculanta* are notable, particularly against a range of Gram-positive (+) and Gram-negative (−) bacteria, with inhibition zones of up to 19 mm for certain strains. Alkaloids, terpenoids, and saponins in the extract may be responsible for its antibacterial efficacy all of these compounds we got after the phytochemical screening. These compounds disrupt bacterial cell membranes and interface with intracellular functions, particularly in Gram-negative (−) bacteria, where their outer membrane may increase susceptibility [17].

The broad-spectrum antibacterial activity observed aligns with findings from other plant-based studies, which similarly attribute antimicrobial properties to alkaloids and terpenoids.

### *3.3. Antidiarrheal Activity*

*Premna esculanta* extract at doses of 250 and 500 mg/kg body weight significantly reduced the overall number of feces and delayed the beginning of diarrhea in the castor oil-induced diarrheal mice in a dose-dependent manner when the statistical significance level was established at 0.05%. At dosages of 250 and 500 mg/kg body weight, *Premna esculanta* inhibited defecation by 32.35% and 56.62%, respectively.

In the antidiarrheal test, the predominant symptoms of diarrhea caused by oral castor oil administration were altered intestinal motility and increased bowel movements. *Premna esculanta* extract increased the latent period of defecation in mice with castor oil-induced diarrhea by 127.6 and 158.4 min at doses of 250 mg/kg and 500 mg/kg body weight, respectively. In contrast, the latent period of defecation in the standard (loperamide at 3 mg/kg dose) and control groups was 30.8 and 184 min, respectively. At doses of 250 mg/kg and 500 mg/kg, the extract significantly reduced defecation by 32.35% and 56.62%, respectively (Figure 3). A prior small-scale study on the plant's antidiarrheal properties likewise verified our findings [3].

The antidiarrheal effect of *Premna esculanta* extract, demonstrated through reduced fecal output in castor oil-induced diarrhea models, suggests bioactivity potentially linked to flavonoids, tannins, and saponins we got all of these compounds in our phytochemical screening study. Flavonoids and tannins stabilize the intestinal membrane by inhibiting fluid secretion, while saponins reduce gut motility. This plant's efficacy in increasing the latent period of defecation aligns with studies highlighting the roles of these metabolites in antidiarrheal activities [14]. The presence of such compounds supports traditional uses of *Premna esculanta* for gastrointestinal alignments.

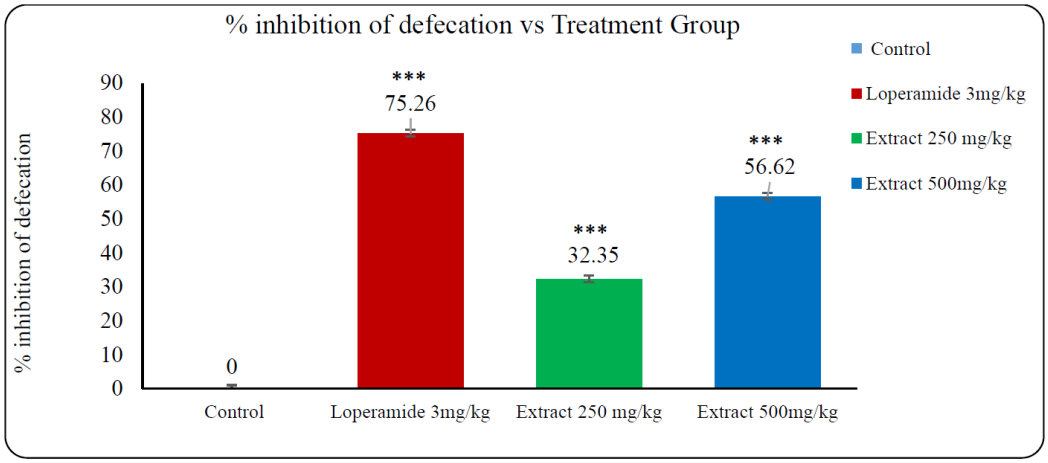



**Figure 3.** Percentage of inhibition defecation in case of castor-oil-induced diarrhea. *** $p < 0.001$

## 4. Conclusion

In this study, we evaluated the medicinal value of the *Premna esculanta* plant frequently used in traditional medicine, using thorough pharmacological studies conducted both in vitro and in vivo. The plant demonstrates significant pharmacological potential based on its evaluated properties. It exhibits robust antimicrobial, and antidiarrheal activities, indicating its efficacy in treating infections and diarrhea. Additionally, its strong antioxidant capacity suggests a protective role against oxidative stress. These findings collectively highlight the plant's promising application in medicinal treatments, supporting its potential development as a natural remedy for various health conditions. More research is necessary to clarify the underlying mechanisms and maximize their application in therapeutic settings.

**Author Contributions:** M.F.R., M.S.M., A.M.S., and H.Y. performed the literature review and experiment under the guidance of A.-S.M.; M.F.R. and M.S.M. performed the writing—original draft; A.-S.M. performed the review, evaluation, and supervision. All authors have read and agreed to the published version of the manuscript.

**Funding:** This research received no external funding.

**Institutional Review Board Statement:** The study was conducted in accordance with the Declaration of Helsinki and approved by the Institutional Review Board of [Dhaka International University] (Ref: CPP/DIU/EC/006).

**Informed Consent Statement:** Informed consent was obtained from all subjects involved in the study.

**Data Availability Statement:** Data are available upon reasonable request from the corresponding author.

**Conflicts of Interest:** The authors declare no conflict of interest.

## Abbreviation

| | |
|---|---|
| DPPH | 2,2-diphenyl-1-picrylhydrazyl |
| PGE2 | prostaglandin E2 |
| COX-2 | Cyclooxygenase-2 |
| $AlCl_3$ | aluminum chloride |
| NaOH | sodium hydroxide |
| $Na_2HPO_4 \cdot 2H_2O$ | disodium hydrogen phosphate dihydrate |
| $H_2O_2$ | hydrogen peroxide |
| $C_{12}H_{12}N_2O_2S$ | phenazine methosulfate PMS |
| UV spectrophotometer | Ultraviolet spectrophotometer |
| $IC_{50}$ | Inhibition concentration of DPPH by 50% |
| G-positive | Gram-positive |
| G-negative | Gram-negative |

## References

1. Aziz, M.A.; Khan, A.H.; Adnan, M.; et al. Traditional Uses of Medicinal Plants Used by Indigenous Communities for Veterinary Practices at Bajaur Agency, Pakistan. *J. Ethnobiol. Ethnomed.* **2018**, *14*, 1–18.
2. Kumar, A.; P, N.; Kumar, M.; Jose, A.; et al. Major Phytochemicals: Recent Advances in Health Benefits and Extraction Method. *Molecules* **2023**, *28*, 887.
3. Phaniendra, A.; Jestadi, D.B.; Periyasamy, L. Free Radicals: Properties, Sources, Targets, and Their Implication in Various Diseases. *Indian J. Clin. Biochem.* **2015**, *30*, 11–26.
4. Coque, T.M.; Cantón, R.; Pérez-Cobas, A.E.; et al. Antimicrobial Resistance in the Global Health Network: Known Unknowns and Challenges for Efficient Responses in the 21st Century. *Microorganisms* **2023**, *11*, 1050.
5. Wolfheim, C.; Fontaine, O.; Merson, M. Evolution of the World Health Organization's Programmatic Actions to Control Diarrheal Diseases. *J. Glob. Health* **2019**, *9*, 020802.
6. Timbadiya, M.J.; Nishteswar, K.; Acharya, R.; et al. Experimental Evaluation of Antipyretic and Analgesic Activities of Amalakyadi Gana: An Ayurvedic Formulation. *AYU (Int. Q. J. Res. Ayurveda)* **2015**, *36*, 220–224.
7. Kifayatullah, M.; Mustafa, M.S.; Sengupta, P.; et al. Evaluation of the Acute and Sub-Acute Toxicity of the Ethanolic Extract of *Pericampylus Glaucus* (Lam.) Merr. in BALB/c Mice. *J. Acute Dis.* **2015**, *4*, 309–315.
8. Mahmud, Z.A.; Bachar, S.C.; Qais, N. Antihyperlipidemic Activity of Leaf and Root Extracts of *Premna esculanta* (Roxb.) in Poloxamer-407 Induced Hyperlipidemic Mice and Rats. *Orient. Pharm. Exp. Med.* **2011**, *11*, 263–270.
9. Al Mahmud, Z.; Emran, T.B.; Qais, N.; et al. Evaluation of Analgesic, Anti-Inflammatory, Thrombolytic and Hepatoprotective Activities of Roots of *Premna esculanta* (Roxb). *J. Basic Clin. Physiol. Pharmacol.* **2016**, *27*, 63–70.
10. Qais, N.; Mahmud, Z.A.; Karim, M.R.; et al. Anti-Nociceptive, Anti-Inflammatory and Sedating Activities of Leaf Extracts of *Premna esculanta* (Roxb). *J. Pharm. Res* **2011**, *4*, 3463–3465.
11. Bachar, S.C.; Mahmud, Z.A.; Qais, N. Antioxidant and Hepatoprotective Activities of Ethanolic Extracts of Leaves of *Premna esculanta* Roxb. against Carbon Tetrachloride-Induced Liver Damage in Rats. *J. Young Pharm.* **2012**, *4*, 228–234.
12. Khan, M.A.; Rahman, A.A.; Islam, S.; et al. A Comparative Study on the Antioxidant Activity of Ethanolic Extracts from Different Parts of *Morus Alba* L. (Moraceae). *BMC Res. Notes* **2013**, *6*, 24.
13. Fakruddin, M.; Mannan, K.S.B.; Mazumdar, R.M.; et al. Antibacterial, Antifungal and Antioxidant Activities of the Ethanol Extract of the Stem Bark of *Clausena heptaphylla*. *BMC Complement. Altern. Med.* **2012**, *12*, 232.
14. Golder, M.; Sadhu, S.K.; Biswas, B.; et al. Comparative Pharmacologic Profiles of Leaves and Hypocotyls of a Mangrove Plant: *Bruguiera gymnorrhiza*. *Adv. Tradit. Med.* **2020**, *20*, 395–403.
15. Hossain, A.; Hossain, A.; Mannan, S.J. Evaluation of Antioxidant and Analgesic Activities of Three Medicinal Plants. *Pharmacogn. Res.* **2019**, *11*, 248–253.
16. Kaur, G.J.; Arora, D.S. Antibacterial and Phytochemical Screening of *Anethum graveolens*, *Foeniculum vulgare* and Trachyspermum ammi. *BMC Complement. Altern. Med.* **2009**, *9*, 30.
17. Nikaido, H. Molecular Basis of Bacterial Outer Membrane Permeability Revisited. *Microbiol. Mol. Biol. Rev.* **2003**, *67*, 593–656.
18. Pinto, E.; Pina-Vaz, C.; Salgueiro, L.; et al. Antifungal Activity of the Essential Oil of Thymus Pulegioides on Candida, Aspergillus and Dermatophyte Species. *J. Med. Microbiol.* **2006**, *55*, 1367–1373.

*Article*

# Evaluation of the Phytochemical Composition and Antibacterial Efficacy of *Momordica balsamina* and *Luffa aegyptiaca* Leaf Extracts

**Muhammad Shehu Giwa [1], Basira Ibrahim [1], Fatima Musa [1], and Emad M. Abdallah [2,3,*]**

[1] Department of biological sciences, Faculty of Life Sciences, College of Science, Computing and Engineering, Kaduna State University, Kaduna 800283, Nigeria
[2] Department of Biology, College of Science, Qassim University, Qassim 51452, Saudi Arabia
[3] Faculty of Health and Life Sciences, INTI International University, Nilai 71800, Negeri Sembilan, Malaysia
* Correspondence: emad100sdl@yahoo.com
Received: 21 September 2024; Revised: 7 October 2024; Accepted: 2 November 2024; Published: 17 January 2025

**Abstract:** The current study evaluated the antibacterial activities of methanol leaf extracts from *Momordica balsamina* and *Luffa aegyptica* against clinical isolates of *Staphylococcus aureus*, *Salmonella typhi*, and *Escherichia coli*. Phytochemical analysis revealed the presence of various bioactive compounds, including alkaloids, flavonoids, and tannins, while anthraquinones were absent. Both extracts demonstrated significant antibacterial activity, particularly against Gram-negative bacteria (*S. typhi*), with minimum inhibitory concentrations (MIC) as low as 12.5 mg/mL and minimum bactericidal concentrations (MBC) of 25 mg/mL for *M. balsamina*, and MIC of 12.5 mg/mL and MBC of 50 mg/mL for *L. aegyptica*. These findings suggest that these plants have potential as sources of antibacterial agents, warranting further pharmaceutical investigation.

**Keywords:** antibacterial activity; in vitro study; ethnomedicine; plant extract; bioactive compounds

## 1. Background

Herbal medicine is now regarded as a rapidly expanding health system globally. Approximately 80% of the global population depends on medicinal plants for primary healthcare, indicating a hopeful future for health maintenance via these natural resources [1]. The use of medicinal plants as remedies predates the emergence of Western medicine alongside the advancement of science and technology. It may serve as a viable alternative source for several medicines, especially after the recent significant failures of antibiotics against pathogens [2]. Recently, it is estimated that there are 14 million deaths due to infections, making them the second most common cause of death after heart diseases. Bacterial pathogens were responsible for 7.7 million of these fatalities, with antibiotic-resistant bacteria contributing to 1.3 million deaths, highlighting the urgent need for novel classes antibiotics [3,4].

Medicinal plants are plants in which one or more of their organs produce substances that can be used for medicinal purposes or as precursors for pharmaceutical synthesis. The practice of herbal medicine traces its origins to the earliest epochs of human history. Historical evidence indicates the utilization of herbs across various ancient civilizations, including Egyptian, Chinese, Greek, and Roman societies, for the treatment of diseases and the restoration of bodily vitality [5,6]. Hence, numerous plants have been acknowledged for their therapeutic and curative attributes and are commonly referred to as medicinal plants. Plants like picorhiza, garlic, cloves, slippery elm, neem fruit and leaves, Kushen, nutmeg, cinnamon, ginger, peppermint, sage, thyme, mustard and fenugreek were studied and reported to possess organic compounds that are vital for human medicine and provide a cure for infection by microbial agents [7].

Phytonutrients are among the chemicals detected to possess pharmacological value in medicinal plants. Phytochemicals exhibit properties that aid in ameliorating bacterial infections by limiting their growth in host cells. Certain phytochemicals, such as alkaloids, flavonoids, tannins, and glycosides, exert their medicinal purpose against microbial infections by limiting bacterial cell wall synthesis, cell division, and DNA repair mechanisms [8]. Saponins aid in the reduction of bacterial proliferation by creating free radicals that hinder bacterial cell growth [9].

Many plants have been screened and tested for their antimicrobial efficacy against certain bacterial strains by studying the phytonutrient contents of their extracts. A previous study [10] indicated the presence of various phytochemicals in methanolic Chaya leaves. This suggests that this plant is a good source of flavonoids, saponins,

and phenolic compounds. Similarly, research showed the presence of tannins, terpenes, glycosides and alkaloids in *Moringa* leaves [11]. While these studies indicate the potential of locally grown plants to contain phytochemicals that can limit the growth of microbes, there is a need to explore the chemical profile of other plants.

*Momordica balsamina* Linn is widely distributed across Nigeria and is known for its diverse medicinal and nutritional applications. However, its bioactivities remain largely underexplored [12,13]. *Momordica balsamina* (balsam apple) is an annual vine characterized by tendrils, and it originates from tropical regions in Africa. This plant, belonging to the Cucurbitaceae family, is a significant medicinal and nutritional resource [14]. It exhibits remarkable antimicrobial properties against bacteria, making it a promising source of antimicrobial agents for the treatment of various diseases. On the other hand, *Luffa aegyptica* is a member of the Cucurbitaceae family, which is also referred to as the sponge gourd plant [15]. The fruit of the plant contained a network of fibers that enveloped a substantial quantity of flat, blackish seeds and is mostly found in African countries such as Nigeria and parts of Asia such as India.

Previous studies have indicated the potential of its phytochemical compounds in antibacterial efficacy utilizing different parts of *Luffa aegyptica* and *Momordica balsamina*, such as seeds, fruits, and stem bark, using ethanol and fractions [16,17]. However, little is known about their antibacterial activity using leaf and methanolic extracts as solvents for extraction. The current study aimed to evaluate the phytochemical and antibacterial profile of methanolic leaf extracts of *Momordica balsamina* and *Luffa aegyptica* against clinical bacterial isolates.

## 2. Materials and Methods

### *2.1. Sample Collection, Identification and Processing*

The leaves of *Momordica balsamina* and *Luffa aegyptiaca* were collected from the Zaria Local Government Area of Kaduna State and authenticated by a taxonomist in the Herbarium Unit of the Department of Botany Ahmadu Bello University, Zaria. Fresh leaves were washed with distilled water, shade-dried at room temperature, and then ground into a fine powder using a mortar and pestle. The powder samples were stored in clean polythene nylon for further analysis.

### *2.2. Preparation of Extract*

The methanolic extract of the plant leaves was prepared following the protocol outlined by Yusuf et al. [10]. A total of 100 g of plant material was immersed in 500 mL of 100% methanol and subjected to vigorous agitation in conical flasks. The mixture was kept in a well-tighten bottle in dark for 72 h and subsequently filtered through muslin cloth and filter paper. The filtrate was concentrated using a water bath set at 40 °C for 48 h. The resulting crude extract was stored in a sterile container at 4 °C until further use. The extract yield was calculated using the following formula:

$$\text{Yield (\%)} = \frac{weight\ of\ dry\ extract}{weight\ of\ dry\ plant\ material} \times 100$$

The extract concentrations were prepared as follows: Briefly, 0.1 g of the methanolic leaf extract of the plant was weighed and added to 10 mL of 10% dimethyl sulfoxide (DMSO). Using a two-fold serial dilution, concentrations of 50 mg/mL, 25 mg/mL, and 12.5 mg/mL were prepared. The various concentrations were labelled and stored in bijou bottles until needed [18].

### *2.3. Qualitative Phytochemical Screening*

The methanolic extract of *M. balsamina* and *L. aegyptiaca* were tested for the presence of plant secondary metabolites using the following screening procedures described in literature [19], to check for the presence of saponins, flavonoids, tannins, terpenoids, steroids, alkaloids, anthraquinones and cardiac glycosides, as follows:

#### 2.3.1. Test for Saponins

2 mL of the plant extract was taken in a test tube, and exactly 10 mL of distilled water was added. The mixture was vigorously shaken for 30 s and then allowed to stand for 5 min. The presence of saponins was indicated by the formation of a 2 cm layer of foam, which persisted for 10 min.

#### 2.3.2. Test for Flavonoids

The presence of flavonoids in the plant extract was determined by adding 1 mL of NaOH to 3 mL of the extract. The formation of a yellow colouration indicated the presence of flavonoids.

#### 2.3.3. Test for Tannins

The presence of tannins in the plant extract was confirmed by adding 3 drops of 0.1% ferric chloride to 2 mL of the extract. The formation of a brownish-green precipitate indicated the presence of tannins.

#### 2.3.4. Test of Terpenoids

The detection of terpenoids in the plant extract was determined by dissolving 2 mL of chloroform in 5 mL of the extract, followed by the careful addition of 3 mL of concentrated sulfuric acid. The formation of a reddish colouration at the interphase indicated a positive result for terpenoids.

#### 2.3.5. Test for Alkaloids

The presence of alkaloids in the methanolic extract of the plant was determined by adding a few drops of Wagner's reagent (a solution of potassium iodide and iodine) to 2 mL of the extract in a test tube. The formation of an orange-brown precipitate indicated the presence of alkaloids.

#### 2.3.6. Anthraquinone Test

A portion of the plant sample was dissolved in 5 mL of chloroform, shaken, and filtered. An equal amount of 10% ammonia solution was added to the filtrate with continuous shaking. The presence or absence of anthraquinones was indicated by the formation or non-formation of a bright pink colour in the upper aqueous layer.

#### 2.3.7. Cardiac Glycoside Test

A small portion of the plant extract was dissolved in 1 mL of glacial acetic acid (solution containing some traces of ferric chloride), and exactly 1 mL of $H_2SO_4$ was carefully added to the mixture. The presence or absence of cardiac glycosides was determined by observing the formation or non-formation of a brown ring at the interface.

### *2.4. Bacterial Isolation and Culturing*

Clinical bacterial isolates, specifically *Staphylococcus aureus* (*S. aureus*), *Salmonella typhi* (*S. typhi*), and *Escherichia coli* (*E. coil*) strains were clinical isolates obtained and identified from the Microbiology Laboratory unit at Barau Dikko Teaching Hospital (BDTH) in Kaduna. The samples were collected in nutrient agar slants, appropriately labelled, placed in a cold box, and then transferred to the Microbiology Laboratory at Kaduna State University, Nigeria. Subsequently, the samples were incubated at 37 °C for 24 h for further analysis. An inoculum from an overnight growth culture of the clinical isolates was streaked on freshly prepared plates of Manitol Salt agar, Salmonella-Shigella agar and Eiosin Methylene Blue agar, respectively. All plates were incubated for 24 h at 37 °C to obtain pure cultures of the test isolates.

### *2.5. Bacterial Identification*

A complete loop of the stock cultures of *S. aureus*, *E. coli*, and *S. typhi* were streaked on both blood agar and nutrient agar plates and then incubated for 18–24 h at 37 °C. The morphology of colonies was recorded, and single colonies were selected for basic confirmation tests. These included Gram stain and cultivation onto various differential agars (MacConkey agar, xylose lysine deoxycholate agar, Salmonella-Shigella agar, mannitol salt agar, and eosin methylene blue (EMB) agar). Additionally, biochemical identification tests such as the indole test, oxidase test, coagulase test, catalase test, motility test, Simmon citrate test, MRVP (methyl red, Voges-Proskauer), and TSI (triple sugar iron) were conducted following the procedures documented previously [20]. The identified bacteria were then used in the experiment.

### *2.6. Antibacterial Susceptibility Test*

Antibacterial susceptibility of the plant extract was determined using the agar well diffusion method, following the procedures previously reported [21]. Approximately 0.1 mL of a standardized inoculum from a bacterial suspension was inoculated onto Mueller Hilton agar plates in triplicate measurements. The inoculum was

evenly spread over the surface of the plates using a clean cotton swab stick. After allowing the plates to stand for 10 min, wells with a diameter of 6 mm were created in the agar using a sterile cork borer. A volume of 0.1 mL from each of the various concentrations of the extract (100 mg/mL, 50 mg/mL, 25 mg/mL, and 12.5 mg/mL) was carefully filled into the respective wells. Additional wells were filled with dimethyl sulfoxide (DMSO) to serve as the negative controls. After allowing the plates to stand for 10 min at room temperature to facilitate the diffusion of extracts into the agar, they were then incubated at 37 °C for 24 h. Following incubation, the zone of inhibition for the growth of each tested bacteria was observed, and the diameter of each zone was measured in millimeters using a ruler. The means and standard deviation were statistically calculated.

### *2.7. MIC and MBC Tests*

The minimum inhibitory concentration (MIC) and the minimum bactericidal concentration (MBC) of the plant extracts against tested bacteria were carried out following the methodology previously published [22], with some modifications. Briefly, Mueller-Hinton broth (1 mL) was dispensed into five test tubes and autoclaved for sterilization. Afterward, 1 mL of the plant extract was added to the first test tube to create a 50% concentration. A series of twofold dilutions was carried out by transferring 1 mL from one tube to the next, producing concentrations of 50%, 25%, 12.5%, 6.25%, 3.12%. Then, 100 μL of the standardized bacterial culture was introduced into each test tube. The lowest concentration that showed no turbidity, indicating no bacterial growth, was identified as the MIC and recorded. For the MBC test, 50 μL was taken from each MIC tube and placed onto nutrient agar plates, which were incubated overnight at 30 °C to 35 °C. The lowest MIC concentration that showed no visible bacterial growth was considered the MBC.

## 3. Results and Discussion

The phytochemical Constituents of Methanlic Leaf Extract of *M. balsamina* and *L. aegyptiaca* are shown in (Table 1). Both tested plants showed the presence of alkaloids, flavonoids, saponins, tannins, steroids, terpenes, cardiac glycosides upon qualitative screening, but no anthraquinones detected in both plants. The results of phytochemicals of *M. balsamina* leaf extract align with the result reported in the previous investigation on apple cannel methanolic leaf extract [23]. Also, Adeyeni et al. [16] have documented the presence of saponin, alkaloids, and tannins upon the phytochemical of *L. aegyptiaca* leaf extract collected in Ilorin, Nigeria. Alkaloids manifest their antibacterial properties by interfering with the peptidoglycan constituents within bacterial cells, impeding the synthesis of the cell wall layer and resulting in cellular demise. Also, they serve as DNA intercalators, impeding the activity of bacterial cell topoisomerase enzymes [24]. In addition to these phytonutrients, other studies on *L. aegyptiaca* leaf extract detected the presence of anthraquinones which the present study was not able to detect. Maamoun et al. [11], detected the presence of anthraquinones on etthanolic leaf extract of *L. aegyptiaca* sample analyzed. The disparity between our study with these studies may likely attributed to the types of solvents used during Soxhlet extraction. Indicating that type of solvent yields different results upon preliminary phytochemical screening. The absence of anthraquinones in the plants under study may be notable or expected depending on their phytochemical pathways and evolutionary traits. Anthraquinones are typically synthesized via the polyketide pathway and are common in certain plant families like Polygonaceae and Rubiaceae [25]. Also, their absence could be expected as they produce other protective compounds like flavonoids and alkaloids instead. This absence may also reflect species-specific chemistry or ecological adaptations where the plants prioritize different metabolites for defense or survival.

**Table 1.** Phytochemical Constituents of Methanolic Leaf Extract of *M. balsamina* and *L. aegyptiaca.*

| Bioactive Compounds | *M. balsamina* | *L. aegyptica* |
|---|---|---|
| Alkaloids | + | + |
| Flavonoids | + | + |
| Saponins | + | + |
| Tannins | + | + |
| Steroids | + | + |
| Terpenes | + | + |
| Cardiac glycosides | + | + |
| Anthraquinones | − | − |

Key: +: indicates presence; −: indicates absent.

In line with our results, Valizadeh et al. [26], and Rahmi and Sari [27] reported the presence of saponin during phytochemical screening of *Momordica charantia* leaf extract. Even though different solvents were used between the present study and these previous studies saponin was detected. This suggests that the types of solvents used during extraction do not influence saponin detection. Saponins have been acknowledged for their anti-inflammatory properties, hemolytic activity, and ability to bind cholesterol [15]. In line with the efficacy of saponin on microbial growth, flavonoids also possessed an antibacterial effect against strains of microbes, particularly bacteria. Flavonoids retards bacterial growth by mitigating cellular oxidative stress in bacterial cells making them favourable agents for retarding bacterial growth [15]. The present study has detected the presence of flavonoids in both *M. balsamina* and *L. aegyptiaca* leaf extract which align with what was reported by Yusuf et al. [10] who also reported the presence of flavonoids upon qualitative phytochemical screening. In general, both plants under study belong to the same family (Cucurbitaceae), which explains the presence of similar phytochemical classes. Therefore, we recommend conducting a GC-MS analysis to identify their bioactive compounds in future research.

The antibacterial activity of *M. balsamina* leaf extract shown in Table 2, indicated the extract demonstrated high antibacterial efficacy against *S. typhi* compared with other bacterial isolates at all levels of extract concentrations. *E. coli* has the second inhibition zone after *S. typhi* indicating that the antibacterial activity of the extract was significant ($p < 0.05$) in gram-negative compared to gram-negative bacterial isolates. As such *M. balsamina* leaf extract has more antibacterial efficacy with gram-negative bacteria. However, we observed that the inhibitory activity of the *M. balsamina* extract is concentration-dependent with a varying concentration of 12.5, 25, 50, and 100 mg/mL of the extract. Additionally, the result shows that the extract possessed antibacterial activity against S. aureus at a considerably 50 mg/mL concentration. The observed reduced inhibition zone in the *S. aureus* strain may be attributed to its gram-positive nature, characterized by the presence of an extracellular envelope that confers resistance to specific chemicals, including antibiotics. Hence, the variability in susceptibility may stem from distinctions in the permeability barriers of the outer membrane, characterized by lipopolysaccharides in gram-negative bacteria. Furthermore, *E. coli* exhibited greater adaptability in comparison to *S. typhi*, attributed to its inherent resistance to a broad spectrum of antibiotics and its capacity to form biofilms on surfaces. This biofilm formation renders the cells less susceptible to medicinal antimicrobial agents at specific concentrations [28,29]. The variation in antibacterial activity of plant extracts against Gram-positive and Gram-negative bacteria is largely influenced by the structural defenses of the bacteria, the chemical properties of the bioactive compounds, and the specific interactions between these compounds and bacterial cells. Understanding these factors helps explain why some plant extracts are moree effective against one group of bacteria over the other.

**Table 2.** Antibacterial activity of Methanolic Leaf Extract of *M. balsamina* against Bacterial Isolates.

| Bacteria | Zones of Inhibition (mm) at Different Concentration (mg/mL) | | | | |
|---|---|---|---|---|---|
| | **100** | **50** | **25** | **12.5** | **Ciprofloxacin** |
| *Staphylococcus aureus* | 12.23 ± 0.31 $^{c}$ | 10.07 ± 0.15 $^{c}$ | 4.73 ± 0.25 $^{b}$ | 2.70 ± 0.44 $^{b}$ | 35.43 ± 0.31 $^{c}$ |
| *Salmonella typhi* | 20.90 ± 0.10 $^{a}$ | 17.07 ± 0.25 $^{a}$ | 10.73 ± 0.50 $^{a}$ | 6.77 ± 0.21 $^{a}$ | 39.13 ± 0.47 $^{b}$ |
| *Escherichia coli* | 17.67 ± 0.15 $^{b}$ | 15.27 ± 0.25 $^{b}$ | 10.03 ± 0.32 $^{a}$ | 6.10 ± 0.36 $^{a}$ | 42.47 ± 0.15 $^{a}$ |

Values are mean ± standard deviation, means with same letter(s) in a column are not significantly different according to Duncan Multiple Range Test (DMRT) at ($p < 0.05$).

Regarding *L. aegyptiaca*, the results show that it has more antibacterial against all the clinical bacterial isolates tested (Table 3). Even at a lower concentration of 12.5 mg/mL *L. aegyptiaca* extract has good antibacterial activity against *S. typhi* strain compared with other strains of organism. However, at 25 mg/mL and 12.5 mg/mL concentrations of *L. aegyptiaca* extract the zone of inhibition between *S. aureus* and *E. coli* strains is not statistically significant ($p < 0.05$). Indicating that twice the concentration of the extract is needed to exert the antibacterial efficacy of *S. aureus* by the plant extract. In the control group *E. coli* has the highest zone of inhibition compared with other strains. This indicated that the organism has more sensitivity to ciprofloxacin than *S. aureus* and *S. typhi*. A similar study on antibacterial activity has also recorded similar trends in antibacterial activity against *E. coli* [30]. The level of inhibition in *S. typhi* is probably due to the presence of alkaloids and flavonoids which have been previously summarized to possess antimicrobial activity through inhibition of cellular membrane synthesis and as well acting as bacterial cytotoxins [31].

**Table 3.** Antibacterial activity of Methanolic Leaf Extract of *L. aegyptiaca* against Bacterial Isolates.

| Bacteria | Zones of Inhibition (mm) at Different Concentration (mg/mL) | | | | |
|---|---|---|---|---|---|
| | **100** | **50** | **25** | **12.5** | **Ciprofloxacin** |
| *S. aureus* | 6.77 ± 0.25 [c] | 5.17 ± 0.12 [c] | 3.90 ± 0.10 [b] | 2.50 ± 0.00 [b] | 35.43 ± 0.31 [c] |
| *S. typhi* | 11.63 ± 0.35 [a] | 9.80 ± 0.30 [a] | 6.73 ± 0.25 [a] | 3.67 ± 0.40 [a] | 39.13 ± 0.47 [b] |
| *E. coli* | 9.87 ± 0.64 [b] | 6.17 ± 0.15 [b] | 4.13 ± 0.23 [b] | 2.70 ± 0.26 [b] | 42.47 ± 0.15 [a] |

Values are mean ± standard deviation, means with same letter(s) in a column are not significantly different according to Duncan Multiple Range Test (DMRT) at ($p < 0.05$).

The Minimum Inhibitory Concentration (MIC) and the Minimum Bactericidal Concentration (MBC) of the methanol leaf extracts of *M. balsamina* and *L. aegyptiaca* against bacterial isolates are presented in Table 4. The lowest MIC was 12.5 mg/mL and the highest was 50 mg/mL, while lowest MBC was 25 mg/mL and highest was 50 mg/mL respectively. In both plant extracts *S. typhi* has the highest response with strong antibacterial activity at a lower concentration of 12.5 mg/mL extract. *E. coli* and *S. aureus* responded to the antibacterial of the two plant extracts at 25 mg/mL for *M. balsamina*. Previous studies have indicated the potentiality of *M. balsamina* to inhibit the growth of microbes even at a considerably low concentration of 25 mg/mL [32,33]. The antibacterial efficacy recorded in both plant extracts may likely be due to the presence of bioactive phytochemical compounds such as cardiac glycosides and tannins which disrupt bacterial growth. Cardiac glycosides exert antibacterial activity by inhibiting bacterial ATPase, disrupting cellular ion gradients critical for bacterial viability [34]. Tannins, on the other hand, display antibacterial effects by precipitating proteins in bacterial cell walls, leading to structural alterations and membrane destabilization [7]. Generally, Gram-positive bacteria have a thick, exposed peptidoglycan layer that is easily targeted by antibiotics, making them more susceptible. In contrast, Gram-negative bacteria possess an additional outer membrane containing lipopolysaccharides and porins, which limit antibiotic entry. This outer membrane, along with efficient efflux pumps and periplasmic beta-lactamases, provides Gram-negative bacteria with greater resistance. Their complex cell wall structure and defense mechanisms make Gram-negative bacteria generally more resistant to many antibiotics [35].

**Table 4.** Minimum Inhibitory Concentration (MIC) and Minimum Bactericidal Concentration (MBC) of Plant Extracts.

| Bacteria | MIC (mg/mL) | | MBC (mg/mL) | |
|---|---|---|---|---|
| | ***M. balsamina*** | ***L. aegyptica*** | ***M. balsamina*** | ***L. aegyptica*** |
| *S. aureus* | 25 | 50 | 50 | 50 |
| *S. typhi* | 12.5 | 12.5 | 25 | 50 |
| *E. coli* | 25 | 25 | 25 | 50 |

The MBC of the test organism indicated bacterial growth retards at approximately 25 to 50 mg/mL concentrations of *M. balsamina* extract indicating the plant has more inhibitory compared with *L. aegyptiaca*. *S. typhi* has the lowest MBC (Table 4) compared with other bacterial isolates against *M. balsamina* leaf extract. The lower MBC observed in *S. typhi* is presumably attributable to the characteristics inherent in its bacterial cell membrane. Also, the variations in antibacterial efficacy between *Luffa aegyptica* and *Momordica balsamina* are attributed to differences in their chemical compositions and the concentrations of individual compounds. While our qualitative analysis has identified the major phytochemical classes, further in-depth investigation using GC-MS is essential to elucidate the specific compounds, as each phytochemical class comprises thousands of bioactive constituents.

Concurrently, in both bacterial isolates, we have found that *L. aegyptiaca* exerts its antibacterial efficacy at a considerably at 25 to 50 mg/mL concentration. This indicates that *M. balsamina* has more antibacterial effects compared with *L. aegyptiaca*. The efficacy of medicinal plant in exerting its antibacterial property depends on the concentration of the extract. Scientific investigations shown that these natural phytochemical substances exert both direct and indirect impacts on human, animal, and microbial physiology. Some phytochemical substances possess the capability to inhibit or eliminate harmful microorganisms via distinct mechanisms of action [36]. Finally, the results of our current study reveal the main active ingredients in the leaves of these two plants. The study recommends conducting further research to identify these active compounds and determine their mechanisms of action on bacteria using advanced techniques and approaches, such as Metabolomics and Proteomics, Transcriptomics (RNA-Sequencing), Molecular Docking and in Silico Modeling, Fluorescence Microscopy and Confocal Laser Scanning Microscopy, Atomic Force Microscopy (AFM), Flow Cytometry, and Mass Spectrometry-Based Metabolic Profiling. These methods will help us understand how plant-derived compounds

combat bacteria, guiding the development of new, effective antimicrobial agents from these plants. Metabolomics can provide a detailed profile of the metabolites present in the plant extracts, helping to pinpoint which specific compounds contribute to their antibacterial effects. Molecular docking, on the other hand, allows researchers to simulate the interaction between these bioactive compounds and bacterial targets, giving insights into their mode of action. By combining these approaches, the study would gain a deeper understanding of how the extracts work at a molecular level, enhancing the scientific validity of the findings.

## 4. Conclusion

This research successfully identified a broad spectrum of phytochemicals, including alkaloids, flavonoids, saponins, steroids, tannins, cardiac glycosides, terpenoids, and carbohydrates in the methanolic extracts of *Momordica balsamina* and *Luffa aegyptiaca* leaves, although anthraquinones were notably absent. The study highlighted the significant antibacterial potential of these extracts, particularly against the Gram-negative bacterium *Salmonella typhi*, demonstrating substantial inhibition zones even at a low concentration of 25 mg/mL. The extracts exhibited high phytonutrient content and showed remarkable efficacy against both Gram-positive (*Staphylococcus aureus*) and Gram-negative bacteria (*S. typhi* and *Escherichia coli*), with a pronounced effectiveness against Gram-negative strains. These findings suggest that the methanolic extracts of *M. balsamina* and *L. aegyptiaca* could be promising candidates for the development of novel antibacterial agents to combat infectious diseases, particularly those caused by Gram-negative pathogens. Future studies are recommended to focus on the isolation and characterization of individual bioactive compounds within these extracts.

**Author Contributions:** M.S.G., B.I., and F.M.: methodology; M.S.G.: experiments; M.S.G., B.I., and F.M.: conceptualization and writing—original draft; B.I., and F.M.: visualization, E.M.A.: writing—review, editing, evaluation, correspondence, revision, and consultations. The draft manuscript was read and approved by all authors. All authors have read and agreed to the published version of the manuscript.

**Funding:** This particular research did not receive any funding from any government or non-government organizations.

**Institutional Review Board Statement:** The study was conducted according to the guidelines of the Declaration of Helsinki.

**Informed Consent Statement:** Not applicable.

**Data Availability Statement:** Data available upon request from the 1st authors (M.G., email: kasumscbio180016@kasu.edu.ng).

**Acknowledgments:** The authors would like to thank the Department of Biological Sciences, Faculty of Life Sciences, College of Science, Computing, and Engineering, Kaduna State University, 800283, Kaduna State, Nigeria, for providing research and laboratory facilities.

**Conflicts of Interest:** The authors declare no conflict of interest.

## References

1. Sulieman, A.M.E.; Abdallah, E.M.; Alanazi, N.A.; et al. Bioactive profiling of *Rumex vesicarius* L. from the Hail region of Saudi Arabia: A study on its phytochemical and elemental analysis, antibiofilm, antibacterial, antioxidant properties, and molecular docking analysis. *Front. Microbiol.* **2024**, *15*, 1421143.
2. Abdallah, E.M. Plants: An alternative source for antimicrobials. *J. Appl. Pharm. Sci.* **2011**, *1*, 16–20.
3. Brüssow, H. The antibiotic resistance crisis and the development of new antibiotics. *Microb. Biotechnol.* **2024**, *17*, e14510.
4. Mendelson, M.; Lewnard, J.A.; Sharland, M.; et al. Ensuring progress on sustainable access to effective antibiotics at the 2024 UN General Assembly: A target-based approach. *Lancet* **2024**, 403, 2551–2564.
5. Aumeeruddy, M.Z.; Mahomoodally, M.F. Traditional herbal therapies for hypertension: A systematic review of global ethnobotanical field studies. *S. Afr. J. Bot.* **2020**, *135*, 451–464.
6. Yousofvand, N.; Moloodi, B. An overview of the effect of medicinal herbs on pain. *Phytother. Res.* **2023**, *37*, 1057–1081.
7. Parham, S.; Kharazi, A.Z.; Bakhsheshi-Rad, H.R.; et al. Antioxidant, antimicrobial and antiviral properties of herbal materials. *Antioxidants* **2020**, *9*, 1309.
8. Bashir, M.; Ibrahim, A.; Bilyaminu, M.; et al. Phytochemical screening and antibacterial activity of leaf and stem bark extracts of Adansonia digitata on E. coli, S. aureus and S. typhi. *Microbes Infect. Dis.* **2022**, *3*, 217–223.
9. Li, Z.; Xia, A.; Li, S.; et al. The pharmacological properties and therapeutic use of bitter melon (*Momordica charantia* L.). *Curr. Pharmacol. Rep.* **2020**, *6*, 103–109.
10. Yusuf, A.; Abubakar, J.; Lawal, A. Phytochemicals Screening and Nutritional Profile of Cnidosculus aconitflius Leaves collected in Birnin Kebbi, Nigeria. *Sch. Int. J. Biochem.* **2022**, *5*, 85–89.
11. Maamoun, A.A.; El-Akkad, R.H.; Farag, M.A. Mapping metabolome changes in Luffa aegyptiaca Mill fruits at different maturation stages via MS-based metabolomics and chemometrics. *J. Adv. Res.* **2021**, *29*, 179–189.

12. Okusanya, O.; Ola-Adams, B.; Bamidele, J. Variations in size, leaf morphology, and fruit characters among 25 populations of *Luffa aegyptiaca*. *Can. J. Bot.* **1981**, *59*, 2618–2627.
13. Kadiri, O.J.; Okafor, S.I.; Ogaji, J.I. Safety and health benefits profile studies of leaf extracts of *Momordica balsamina* Linn (Cucurbitaceae) found in North Central Nigeria. *GSC Biol. Pharm. Sci.* **2020**, *11*, 278–286.
14. Coleman, M.I.; Khan, M.; Gbodossou, E.; et al. Identification of a novel anti-HIV-1 protein from Momordica balsamina leaf extract. *Int. J. Environ. Res. Public. Health* **2022**, *19*, 15227.
15. Harfiani, E.; Pradana, D.C.; Yusmaini, H. A Review on the Phytochemical and Pharmacological Activitities of *Luffa acutangula* (L.) Roxb. *Pharm. J. Farm. Indones.* **2020**, *17*, 396–406.
16. Adeyeni, E.; Ayodele, E.; Adedosu, T. Phytochemical Constituents and Antimicrobial Activities of Ethanolic Extract of Luffa Cylindrica Seed. *Ilorin J. Sci.* **2020**, *7*, 127–143.
17. Mashiane, P.; Manhivi, V.E.; Shoko, T.; et al. Cooking African pumpkin leaves (*Momordica balsamina* L.) by stir-frying improved bioactivity and bioaccessibility of metabolites—Metabolomic and chemometric approaches. *Foods* **2021**, *10*, 2890.
18. Hassan Cheong, N.D.; Zakaria, L.A.; Yusof, H. Qualitative Phytochemical Screening and Antibacterial Properties of *Momordica charantia* Methanolic Extract Against Selected Bacterial Strains. *Malays. J. Med. Health Sci.* **2022**, *18*, 154–161.
19. Mishra, B.B.; Tiwari, V.K. Natural products: An evolving role in future drug discovery. *Eur. J. Med. Chem.* **2011**, *46*, 4769–4807.
20. Muribeca, A.d.J.; Gomes, P.W.P.; Paes, S.S.; et al. Antibacterial Activity from *Momordica charantia* L. Leaves and Flavones Enriched Phase. *Pharmaceutics* **2022**, *14*, 1796.
21. Doğaroğlu, Z.G.; Uysal, Y.; Çaylalı, Z.; et al. Antibacterial and phytotoxicological properties assessment of *Momordica charantia* extract-based ZnO nanoparticles. *J. Sci. Food Agric.* **2024**, *104*, 2851–2861.
22. Abdallah, E.M. Antibacterial activity of Hibiscus sabdariffa L. calyces against hospital isolates of multidrug resistant Acinetobacter baumannii. *J. Acute Dis.* **2016**, *5*, 512–516.
23. Abdulhamid, A.; Jega, S.; Sani, I.; Bagudo, A.; Abubakar, R. Phytochemical and antibacterial activity of Mormodica balsamina leaves crude extract and fractions. *Drug Discov.* **2023**, *17*, e11dd1012.
24. Egamberdieva, D.; Jabborova, D.; Babich, S.; et al. Antimicrobial activities of herbal plants from Uzbekistan against human pathogenic microbes. *Environ. Sustain.* **2021**, *4*, 87–94.
25. Nallakaruppan, N.; Thiagarajan, K. In vitro elicitation of anthraquinones—A review. *Plant Cell Tissue Organ Cult.* **2024**, *156*, 70.
26. Valizadeh, M.; Beigomi, M.; Fazeli-Nasab, B. Antibacterial and Anti biofilm effects of ethanol and aceton leaf extract of *Momordica charantia* and *Tecomella undulata* against Acinetobacter baumannii. *Int. J. Adv. Biol. Biomed. Res.* **2020**, *8*, 403–418.
27. Rahmi, M.; Sari, T. Antibacterial activity of ethanol extract, n-hexan, ethyl acetate and butanol fraction of *Momordica charantia* L. seed against Staphylococcus epidermidis. *J. Phys. Conf. Ser.* **2021**, *1918*, 052013.
28. Sherekar, P. Antimicrobial agent from Plants and Herbs: A systemic Review. *Res. J. Pharmacogn. Phytochem.* **2021**, *13*, 179–181.
29. Samadov, B.S. The use of the medicinal plant *Momordica charantia* L in folk medicine. *Asian J. Pharm. Biol. Res.* **2022**, *11*.
30. Dalkılıç, S.; Dalkılıç, L.K.; Küçüktüfekçi, M.C.; et al. Determination of cytotoxic, apoptotic, necrotic, antimicrobial and antioxidant activities of Aloe vera and Abies cilicia subsp. cilicica. *Int. J. Plant Based Pharm.* **2023**, *3*, 165–175.
31. Oyelere, S.F.; Ajayi, O.H.; Ayoade, T.E.; et al. A detailed review on the phytochemical profiles and anti-diabetic mechanisms of *Momordica charantia*. *Heliyon* **2022**, *8*, e09253.
32. Choudhary, B.; Berwal, M.; Ram, H.; et al. *Momordica balsamina* L.: An unexploited vegetable crop rich in medicinal and nutritional properties. *J. Agric. Ecol.* **2022**, *14*, 84–92.
33. Dien, T.N.; Thi, H.N. Antibacterial activity of silver nanoparticle LED-synthesized using Citrus maxima peels. *Int. J. Plant Based Pharm.* **2023**, *3*, 141–147.
34. Shahrajabian, M.H.; Cheng, Q.; Sun, W. The most important medicinal herbs and plants in traditional Chinese and Iranian medicinal sciences with antioxidant activities. *Lett. Drug Des. Discov.* **2023**, *20*, 1171–1184.
35. Alegun, O.; Pandeya, A.; Cui, J.; et al. Donnan potential across the outer membrane of gram-negative bacteria and its effect on the permeability of antibiotics. *Antibiotics* **2021**, *10*, 701.
36. Sadeek, A.; Abdallah, E.M. Phytochemical compounds as antibacterial agents a mini review. *Saudi Arab. Glob. J. Pharmaceu Sci.* **2019**, *53*, 555720.

*Journal of Medicinal Natural Products*

*Article*

# In Vitro Activity of Isolated Bioactive Metabolites from Endophytic Fungus Associated with *Aegiceras corniculatum*

**Sharika Noshin [1], Rahul Dev Bairagi [1], Sadia Airin [1], Dipa Debnath [1], As-Sazzad Mahmud [1,2], Md. Sohanur Rahaman [1,3], Amit Kumar Acharzo [1], Raiyan Rahman Reon [1], and Md. Amirul Islam [1,3,*]**

[1] Pharmacy Discipline, School of Life Sciences, Khulna University, Khulna 9208, Bangladesh
[2] Department of Pharmacy, Dhaka International University, Dhaka 1212, Bangladesh
[3] Department of Pharmacy, East West University, Dhaka 1212, Bangladesh
***** Correspondence: ma.islam@pharm.ku.ac.bd or amirul.islam@ewubd.edu
Received: 18 November 2024; Revised: 3 January 2025; Accepted: 6 January 2025; Published: 7 February 2025

**Abstract:** A vast and unexplored source of diverse and unique compounds and biological properties is provided by the mangrove fungi. The primary goal is to investigate the biological effects of secondary bioactive compounds produced by endophytic fungi that reside in *Aegiceras corniculatum* bark, focusing on their antioxidant, alpha-glucosidase inhibitory, and antimicrobial properties. Seven distinct strains of endophytic fungi were isolated, of which three particular strains (ACSF-1, ACSF-3, and ACSF-5) were selected for further examination. These strains were cultivated in potato dextrose broth (PDB) and underwent extraction using dichloromethane (DCM) and ethyl acetate (EtOAc). In the DPPH assay, the fraction ACSF-3 of the DCM showed a good $IC_{50}$ value of 239.88 µg/mL, whereas the ascorbic acid $IC_{50}$ was 15.985 µg/mL. Additionally, the crude extract ACSF-3 exhibited the highest levels of total phenolic content (89.89 mg GAE/g), total flavonoid content (288.52 mg QE/g), and total tannin content (53.85 GAE/g). To evaluate antihyperglycemic activity, the ACSF-3 n-Hexane fraction, which showcased the highest efficacy with a value of 0.91 µg/mL. The extracts of ACSF-1 and ACSF-3 demonstrated significant zones of inhibition against Escherichia coli, with sizes reaching up to 16 and 12 mm, respectively, and ACSF-5 displayed the highest zone of inhibition against *Staphylococcus aureus*.

**Keywords:** *Aegiceras corniculatum*; endophytic fungus; antioxidant; antimicrobial; antihyperglycemic

## 1. Introduction

Endophytes are microbes that present inside the plant tissues and do not produce any harm to the plants for a significant portion of their life cycle [1]. Endophytic fungi are inherent elements of the plant micro-ecosystem that exert a beneficial influence on the physiological functions of the host plant through various mechanisms such as synthesis of hormones, biosynthesis, and acquisition of nutrients to support plant growth and development. In addition, these secret metabolites aid in the plant's defense against herbivores and pathogens, and the enhancement of the plant's ability to adapt to abiotic stressors [2]. In reciprocation, plants provide both a habitat and nourishment for these endophytic fungi [3]. Microorganisms that are endophytic a reservoir of unique secondary metabolites that can serve as a potential source of drug discovery across diverse therapeutic areas, including antiarthritic, antimicrobial, anticancer, antidiabetic, anti-insect, and immunosuppressant activities [4,5].

*Aegiceras corniculatum*, a member of the Myrsinaceae family, is primarily a diminutive shrub that thrives in the wetlands of Asia and Australia. It possesses the ability to withstand an extensive array of environmental conditions, thereby enabling its presence in a diverse range of tidal habitats. The species exhibits resilience in the face of varying degrees of salinity and exposure to sunlight, allowing it to prosper in an assortment of soil types. Frequently, it can be observed forming a dense sub-canopy along the periphery of tidal creeks and river margins. However, in coastal mangrove ecosystems, it predominantly flourishes along the inland boundaries [6].

The members of the family Myrsinaceae characteristically show presence of secondary metabolite. The identification and characterization of bioactive metabolites from endophytic fungi inhabiting the tissues of *Aegiceras corniculatum* have drawn substantial attention in recent times because of their potential applications across diverse domains such as medicine and agriculture. From several researches, it is obvious that *A.*

*corniculatum* possesses anti-tuberculosis, antibacterial, antioxidant, and anti-inflammatory activities [7,8]. In addition, anti-diabetic, effectivity for chronic granulomatous inflammation and arthritis has been proved in animal models [9,10]. However, bioactive metabolites of endophytic fungus associated with *Aegiceras corniculatum* is still create ambiguity. Therefore, we have endeavored to identify the secondary metabolites and their corresponding pharmacological activities, which could potentially aid in future drug discovery.

## 2. Materials and Methods

### *2.1. Sample Collection and Isolation of Endophytic Fungi*

Fresh barks of *Aegiceras corniculatum* were collected in September, from Mangrove Sundarbans, Bangladesh. The samples were kept in disinfected individual plastic bags and transferred to the laboratory within 24–48 h. The gathered plant specimens underwent a thorough cleansing process involving rinsing under flowing tap water, followed by immersion in distilled water. The plants were mounted on paper, and the sample was identified by the experts of the Bangladesh National Herbarium (voucher specimen no. 44815). After cutting into small pieces about 0.5 $cm^2$, the samples underwent a sterilization process. This involved sequential treatments with 75% ethanol (1 min), 5% sodium hypochlorite (3 min), and another round of 75% ethanol (30 s), followed by meticulous rinsing with sterile distilled water [11]. Further, Sterilized specimens of tissues were spread out over Potato Dextrose Agar medium in Petri dishes horizontally along with Chloramphenicol (150 µg/mL). After that, the plates were placed in incubation at room temperature until fungal growth appeared (1–3 week). From the first culture plate, a minute amount of the fungus was carefully extracted from the distinct colony and subsequently introduced into a Petri dish infused with potato dextrose culture media. In order to prevent bacterial contamination, Lactic Acid or Chloramphenicol may be incorporated into the medium. Once a pure culture is achieved, the fungus is cultivated for 5 to 10 days. The storage procedure may then commence.

### *2.2. Identification and Morphological Characterization of Endophytic Fungi*

The identification of the endophytic fungi was carried out through the analysis of their morphological characteristics. This involved a comprehensive analysis the colony's characteristic, including the morphology, coloration of the aerial hyphae, growth rate, margin features, surface texture, and the presence of spores or reproductive structures. To induce sporulation, the mycelia of the endophytic fungi were placed on sterilized segments of host plants. Following this, the spores and mycelia were extracted from the refined colonies, deposited onto glass slides, and subjected to staining with lactophenol cotton blue (1% *v*/*v*). The prepared slides were then scrutinized under a light microscope for further examination (Olympus, Bartlett, TN, USA). The taxonomic classification was determined utilizing a handbook dedicated to the identification of fungi [12]. Fungi are primarily recognized through meticulous scrutiny of their morphology and distinctive traits. In the technique, fungi are grown on a slide directly which was prepared by a layer of agar. This approach eliminates the necessity of transferring fungus from a culture plate to a specific slide, reducing the risk of damage to vital identifying features, particularly the spore-bearing structures. The isolates were subjected to a thorough identification process involving a meticulous review conducted 2–3 times, in conjunction with proper reference. The identification of the isolates yielded the following results: ACSF-1, ACSF-3, and ACSF-5, and the yields were 1.7 g, 2.1 g, and 2 g, respectively.

### *2.3. Extraction of Fungus and Preparation of Crude Extract*

Potato dextrose broth media was prepared through the addition of water (24 mg/mL) to a dehydrated product. Sterilized Potato dextrose broth media, Scott bottle, Erlenmeyer flask as well as its cotton cap, and Cork borer (maintained temperature of 121 °C and a pressure of 15-lbs/sq inch for 15 min) placed in a laminar hood and UV light 30 min before working. The endophytic fungi were subsequently cultivated in potato dextrose broth (PDB) utilizing a cubic shaker operating at 150 rpm, with an incubation period of 21 days at a temperature of 28 ± 2 °C. During the phase of separation, the mycelium was meticulously separated from the broth by means of cotton filtration to ensure aseptic conditions. In a separatory funnel where n-hexane was added in a 2:1 ratio to facilitate defatting, the broth had been subsequently relocated [13]. The upper layer of n-hexane which contained the fatty material was then separated from the separatory funnel. Following this, the secondary metabolite was extracted from the defatted broth using dichloromethane (DCM) or ethyl acetate. Lastly, with the help of a rotary evaporator at a temperature of 37 °C the extracted liquid was evaporated [14].

### *2.4. Screening of Antioxidant Activity*

2.4.1. Screening of DPPH Activity

The DPPH scavenging assay was conducted in accordance with an established procedure, with specific modifications to suit the experimental requirements [15]. The endophytic fungal extracts which was dissolved in methanol at 1 mg/mL, were used to prepare the stock solutions. It was then serially diluted into a 96-well plate along with methanol so that concentrations lie from 1000 to 3.90 µg/mL range can be achieved. Then, we added 100 µL of 0. 2 mM DPPH solution (Sigma-Aldrich, St. Louis, MO, USA) to each well and incubated the plates in the dark at 25 °C for 30 min, covered with a lid and aluminum foil. We measure absorbance at 517 nm using a microplate reader and some standards like ascorbic acid (Merck, Darmstadt, Germany) and butylated hydroxytoluene (BHT) (Merck, Indianapolis, India) were used. Each sample was monitored thrice.

2.4.2. Quantification of Total Phenol Content (TPC)

The quantification of total polyphenol content in the endophytic fungal extract was carried out following a standardized protocol employing Folin-Ciocalteu's reagent [16]. In this procedure, 200 µL of each extract solution (1 mg/mL) was used along with 200 µL of Folin-Ciocalteu's reagent from Sigma-Aldrich in the United States and 2 mL of distilled water. The mixing was continued and 1.6 mL of a 7.5% $Na_2CO_3$ solution was added meanwhile. After that the mixture was placed in the darkness at room temperature for 90 min for incubation. The measurement of absorbance was taken at 760 nm and distilled water was used as a blank in a spectrophotometer. Finally, we calculated total polyphenol content by using a standard calibration curve which was generated with six gallic acid concentrations from 6.25 to 200 µg/mL). The results were demonstrated as gallic acid equivalent (GAE) in µg $mg^{-1}$ of dried fungal extract. Each sample was observed thrice.

2.4.3. Determination of Total Flavonoid Content (TFC)

We have followed colorimetric method where aluminum chloride was used to ascertain the flavonoid content of the given sample [17] with some possible modifications. In short, to assess the flavonoid amount in fungal extract (1 mg/mL), we took 300 µL of the extract which was added to 3.4 mL of 30% methanol, and 150 µL sodium nitrite ($NaNO_2$) solution 5% concentration. Further, we combined Aluminum chloride ($AlCl_3$) 150 µL solution of 10% concentration, and 1 mL of 1 M sodium hydroxide (NaOH) solution. After thorough mixing, the absorbance had been measured at 510 nm weave length using a spectrophotometer where distilled water was taken as the blank. For calculating the final data, we used a standard calibration curve in which six concentrations (6.25–200 µg/mL) of quercetin was added, and the flavonoid concentration was measured and expressed as quercetin equivalents (QE) in µg/mg of fungal extract that had been dried. Each sample was tested three times.

2.4.4. Determination of Total Tannin Content (TTC)

The tannin content test procedure comprised several sequential steps [18]. Initially, separate test tubes were used to hold 0.1 mL of standard solutions at varying concentrations (0.5, 0.4, 0.3, 0.2, 0.1 mg/mL) and 0.1 mL of the extract sample. Subsequently, each test tube received distilled water in the amount of 7.5 mL. Additionally, we took FC reagent in the amount of 0.5 mL was introduced in test tube. Then we added 1 mL of 35% $Na_2CO_3$. Following the addition of 0.1 mL standard solution or extract sample to separate test tubes, 7.5 mL water specially distilled water was added to each tube. Subsequently, FC reagent had been taken 0.5 mL along with 1 mL $Na_2CO_3$ in which the concentration was 35%, and the solutions were diluted to with distilled water of 10 mL. After vertexing for 15 s, the test tubes were kept 30 min at the room temperature condition. The measurement of absorbance of the solutions had then measured at the wavelength of 725 nm. A blank was prepared following the same steps as above, excluding the addition of gallic acid and the sample. It's important to note that a blank was prepared by replicating the aforementioned steps, excluding the inclusion of gallic acid and the sample in the process.

*2.5. Alpha-Glucosidase Inhibitory Activity*

The experiment was conducted in accordance with a previously established procedure with certain alterations [19]. At first, we collected endophytic fungal extracts which was then dissolved in dimethyl sulfoxide (DMSO) and prepared stock solutions at the amount of 8 mg/mL. Subsequently, we took each stock solution of 50 µL which was aimed at serial 2 × dilution in a 96-well plate using potassium phosphate buffer solution (PBS) of 100 mM with a pH of 6.8. This process yielded a concentration range from 4 to 0.031 mg/mL. Subsequently, each well was enriched with α-glucosidase enzyme (Sigma-Aldrich, United States) solution 20 µL, measuring 0.4 unit/mL. The samples were then incubated at 37 °C for 10 min, after which 4-nitrophenyl α-D-glucopyranoside (pNPG) (Sigma-

Aldrich, United States) (5 mM) was introduced at the amount of 10 µL. The mixtures were further incubated at one significant temperature which was 37 °C for an additional 30 min. The conclusion of the reaction was achieved by the addition of 50 µL of a solution of sodium carbonate ($Na_2CO_3$) with a concentration of 0.2 M. To quantify the absorption, a microplate reader was employed at a wavelength of 405 nm. Negative control was established employing SDW, while the standard was executed with acarbose. Employing an established method [20] mixture was remained at the same temperature before like 37 °C for 20 min within a 96-well plate for each concentration. Next, to each well, we added 20 µL α-glucosidase enzyme solution with possessing of 0.4 µ/mL concentration. The plates were subsequently mixed thoroughly and subjected to re-incubation for a duration of 10 min at a temperature of 37 °C. Following the incubation process, the reaction was ceased by incorporating 50 µL of a solution consisting of 0.2 M sodium carbonate ($Na_2CO_3$) into each well. The measurement of optical density (OD) was conducted at a wavelength of 405 nm using a microplate reader. To ascertain the initial velocity of the reaction, the OD value was correlated with time for different concentrations of the substrate. The determination of Km and Vmax values was accomplished through the utilization of Lineweaver-Burk and Michaelis-Menten plots. The experiments were performed on three separate occasions.

### *2.6. Test for Antimicrobial Activity*

For determination of antimicrobial activity, we followed microdilution assay of broth, hence achieved minimum inhibitory concentrations (MICs) for each fungal extract, adapting established methodologies for endophytic fungi [21]. At the beginning, one fresh stock solution, total amount 6 mg/mL was prepared by dissolving the extracts (ACSF-1, ACSF-3, ACSF-5) in acetone. In addition, this stock solution went through serial dilution process with water which was sterilized as well as distilled. It allowed to achieve different concentrations stared from 3 mg/mL and end up with 0.005 mg/mL. Standardized bacterial strains, including *Staphylococcus aureus*, *Bacillus subtilis*, *Escherichia coli*, and *Salmonella enterica* suspensions were inoculated into wells containing the extract and incubated under specific conditions. After incubation, MICs were determined by introducing resazurin dye (Sigma-Aldrich, United States), where a blue color signified the absence of visible microbial growth. After incubation, we checked for the minimum bactericidal concentrations (MBC) and minimum fungicidal concentrations (MFC). These concentrations were determined as the lowest where we saw fewer than one visible colony. To compare and validate our results, we included positive controls like the standard antibiotic Ciprofloxacin. The presence of more than one colony indicated significant effect called bacteriostatic/fungistatic effect means it cannot kill the bacteria whereas suppress their growth. The whole test was repeated thrice for accuracy.

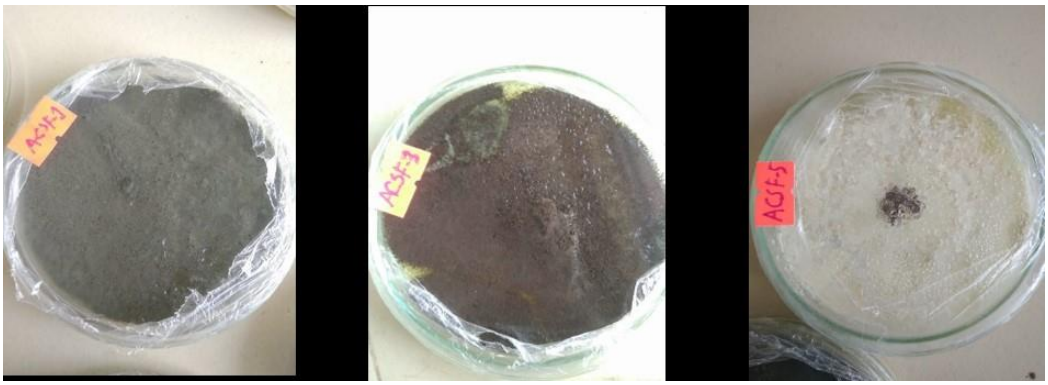
### *2.7. Statistical Analysis*

GraphPad Prism 8.0 (GraphPad Software by Dotmatics, Boston, USA) as used to perform statistical analysis for the antioxidant experiment. We calculated mean and SEM for data calculation in terms of each test parameter. $y = mx + c$ equation has been employed for $IC_{50}$ and p value 0.05 has been considered as statistical significance.

## 3. Results

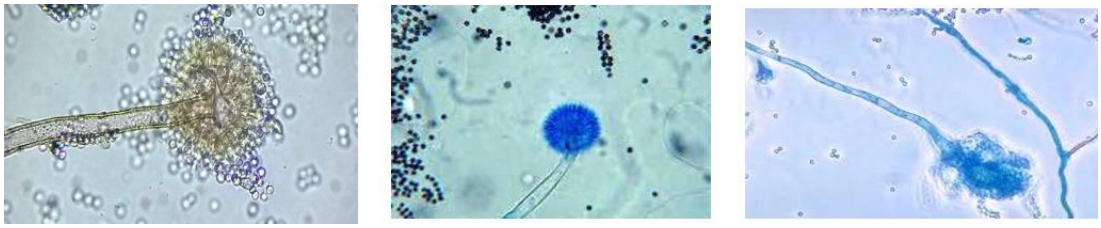
### *3.1. Isolation, Identification and Extraction*

Seven strains of endophytic fungi were procured from the bark of the mangrove plant, *Aegiceras corniculatum.* Among the seven strains the 3 were isolated and further extracted. The identity of the isolates is: ACSF-1, ACSF-3, ACSF-5 (Figures 1 and 2). After isolation, these three strains were extracted and so achieved the crude extract through the solvent partitioning system. They were fermented on potato dextrose broth (PDB) and extracted using dichloromethane (DCM) and ethyl acetate (EtOAc) after defatting by using n-hexane.



**a** **b** **c**

**Figure 1.** Identity of the isolates (ACSF-1, ACSF-3 and ACSF-5).



**a** **b** **c**

**Figure 2.** Microscopic Identification of ACSF-1, ACSF-3 and ACSF-5.

### *3.2. DPPH Free Radical Scavenging Activity*

The methanolic extract of bark of the *Aegiceras corniculatum* and extracted endophytic fungi demonstrated DPPH free radical scavenging activity which was really significant. The well-known standard antioxidant, ascorbic acid, exhibited an $IC_{50}$ value of approximately 15.985 µg/mL, confirming its efficacy as a reference compound for antioxidant activity. The crude extracts and fractions of ACSF-1, ACSF-3, and ACSF-5 exhibited varying degrees of DPPH free radical scavenging activity, with $IC_{50}$ values of 156.31 µg/mL, 87.48 µg/mL, and 202.25 µg/mL, respectively (Figure 3). Fractionation of the ACSF-3 extract yielded n-Hexane, DCM, and Ethyl acetate fractions, with $IC_{50}$ values of 213.79 µg/mL, 239.88 µg/mL, and 72.44 µg/mL, respectively (Figure 3). These results suggest that different fractions may possess distinct antioxidant potential.

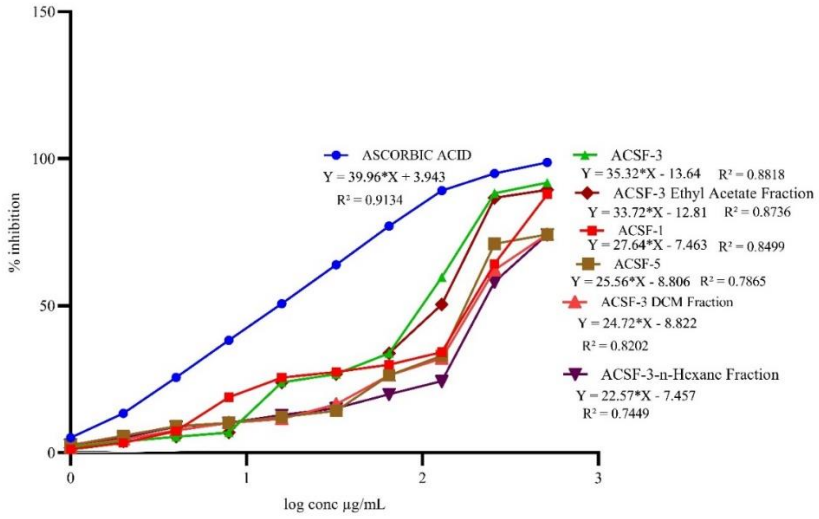



**Figure 3.** DPPH scavenging activity of endophytic fungal extracts.

### *3.3. Total Polyphenolic Content*

ACSF-1 Crude Extract (ACSF-1) possess Total Phenolic Content Approximately 68.77 mg GAE/g. In addition, n-Hexane Fraction, DCM (Dichloromethane) and Ethyl Acetate Fraction (ACSF-1) exert Total Phenolic Content approximately 5.29 mg GAE/g, 9.89 mg GAE/g and 49.67 mg GAE/g respectively (Figure 4). Moving on to ACSF-3, the crude extract shows a remarkably high Total Phenolic Content, measuring approximately 89.89 mg GAE/g. However, the n-Hexane Fraction of ACSF-3 exhibits a lower Total Phenolic Content, approximately 8.17 mg GAE/g. The DCM and Ethyl Acetate Fraction of ACSF-3 demonstrates an intermediate Total Phenolic Content, measuring about 19.19 mg GAE/g and 65.11 mg GAE/g (Figure 4). The crude extract from ACSF-5 was found to possess a Total Phenolic Content of approximately 27.45 mg GAE/g. In contrast, the n-Hexane Fraction of ACSF-5 exhibited a notably lower Total Phenolic Content, measuring approximately 6.12 mg GAE/g. Moving forward, the DCM (Dichloromethane) Fraction of ACSF-5 displayed an intermediate Total Phenolic Content, measuring about 11.54 mg GAE/g. Finally, the Ethyl Acetate Fraction of ACSF-5 demonstrated a relatively higher Total Phenolic Content, approximately 23.88 mg GAE/g (Figure 4).

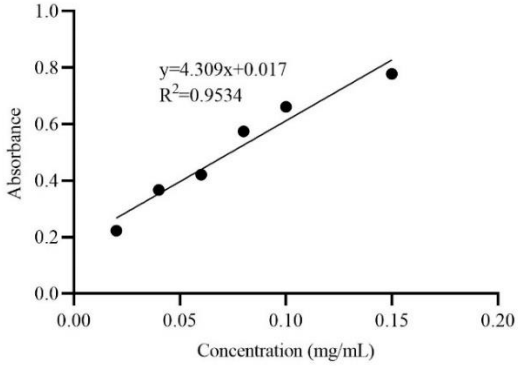



**Figure 4.** Total Phenolic Content of endophytic fungal extracts based on gallic acid standard calibration curve.

### *3.4. Total Flavonoid Content*

The analysis across ACSF-1, ACSF-3, and ACSF-5 fractions, along with their respective crude extracts, reveals distinct variations. Within the DCM (Dichloromethane) fractions, ACSF-3 exhibits the highest flavonoid content at approximately 57.6 mg QE/g, followed by ACSF-1 with about 29.31 mg QE/g, and ACSF-5 with approximately 22.1 mg QE/g (Figure 5). Conversely, the n-Hexane fractions show lower flavonoid content, with ACSF-3 having the highest at approximately 8.76 mg QE/g, followed by ACSF-1 with about 5.56 mg QE/g, and ACSF-5 at approximately 3.12 mg QE/g (Figure 5). Notably, the Ethyl Acetate fractions consistently demonstrate relatively higher flavonoid content across all three samples, with ACSF-3 having the highest at approximately 131.96 mg QE/g, followed by ACSF-1 at about 94.92 mg QE/g, and ACSF-5 with approximately 76.2 mg QE/g (Figure 5). Additionally, the crude extracts of ACSF-1, ACSF-3, and ACSF-5 exhibit substantial flavonoid content, measuring approximately 180.8 mg QE/g, 288.52 mg QE/g, and 116.43 mg QE/g, respectively. This comprehensive summary encompasses flavonoid content variations within the DCM, n-Hexane, and Ethyl Acetate fractions, along with the crude extracts of ACSF-1, ACSF-3, and ACSF-5.

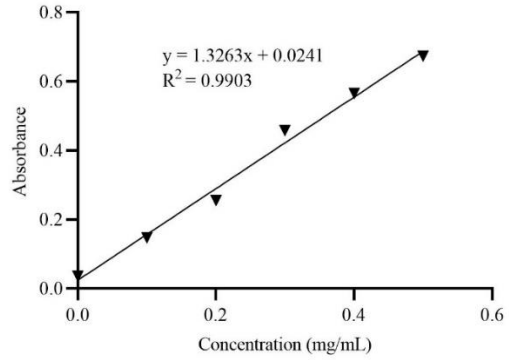



**Figure 5.** Total Flavonoid Content of endophytic fungal extracts based on quercetin standard calibration curve.

### *3.5. Total Tannin Content*

Across ACSF-1, ACSF-3, and ACSF-5, the tannin content varies within the different fractions. In ACSF-1, the crude extract exhibits a tannin content of approximately 23.63 mg GAE/g, while the DCM fraction measures about 7.78 mg GAE/g, and the Ethyl Acetate fraction shows around 15.23 mg GAE/g (Figure 6). For ACSF-3, the crude extract has a tannin content of approximately 53.85 mg GAE/g, with the DCM fraction measuring about 14.69 mg GAE/g, and the Ethyl Acetate fraction showing approximately 45.6 mg GAE/g (Figure 6). Finally, in ACSF-5, the crude extract demonstrates a tannin content of approximately 38.05 mg GAE/g, with the DCM fraction measuring about 9.24 mg GAE/g, and the Ethyl Acetate fraction showing approximately 31.23 mg GAE/g (Figure 6).

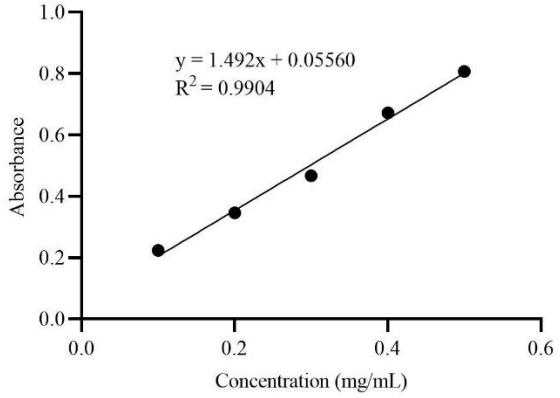



**Figure 6.** Total Tannin Content of endophytic fungal extracts based on standard curve.

### *3.6. Antimicrobial Activity*

In our antimicrobial susceptibility testing, we assessed the inhibitory effects of different extracts and fractions derived from endophytic fungi against various bacterial strains. The standard antibiotic, ciprofloxacin (30 µg/disc), exhibited robust inhibitory activity with zone diameters ranging from 25 to 30 mm against the tested bacterial strains (Figure 7). Among the fractions of ACSF-1, the crude extract at 250 µg/disc displayed modest inhibition, with zone diameters ranging from 6 to 9 mm against the bacterial strains (Figure 7). When the concentration was increased to 500 µg/disc, the inhibitory effects improved, with zone diameters ranging from 11 to 16 mm. The n-Hexane fraction showed limited inhibitory activity, while the DCM fraction and ethyl acetate fraction demonstrated no inhibitory effects against the tested bacteria at both concentrations. Similar trends were observed for ACSF-3 and ACSF-5 fractions. The crude extracts exhibited moderate to mild inhibitory effects. Notably, the ethyl acetate fractions of ACSF-3 and ACSF-5 displayed relatively better inhibitory activity at 500 µg/disc, with zone diameters reaching up to 14.5 mm against some bacterial strains (Figure 7).

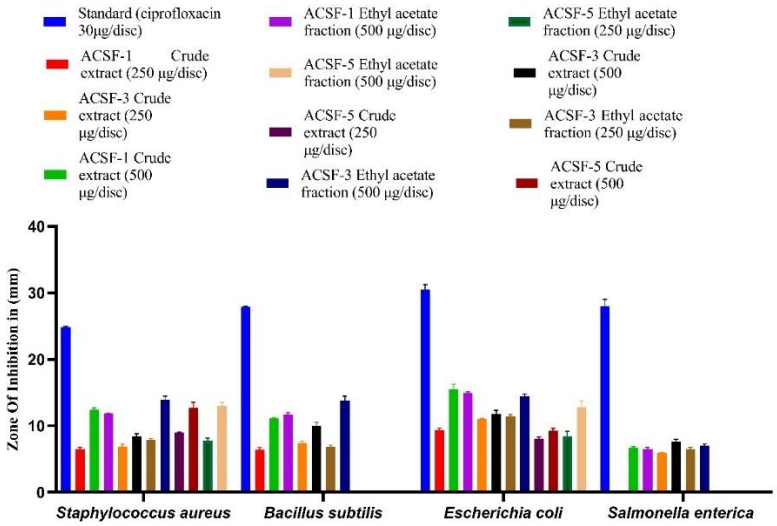



**Figure 7.** In-vitro antimicrobial activity of ACSF-1, ACSF-3 and ACSF-5 extracts by disc diffusion method.

### *3.7. α-Glucosidase Inhibitory Activity*

The $IC_{50}$ values means the concentration at which half of the total i.e., 50% inhibition of alpha-glucosidase activity occurs, were assessed for various extracts and fractions. Acarbose, a standard antidiabetic agent, exhibited an $IC_{50}$ value of 0.413 μg/mL, indicating its potent inhibitory effect on alpha-glucosidase (Figure 8). Among the crude extracts, ACSF-1 displayed an $IC_{50}$ value of 0.59 μg/mL, while ACSF-3 and ACSF-5 exhibited $IC_{50}$ values of 0.47 μg/mL and 0.67 μg/mL respectively (Figure 8), suggesting their potential as alpha-glucosidase inhibitors. Within the ACSF-3 fractions, the n-Hexane fraction showed 0.91 μg/mL, while the DCM fraction as well as ethyl acetate fraction exhibited values of 0.73 μg/mL and 0.5 μg/mL respectively (Figure 8). These findings indicate variable inhibitory activities of the tested extracts and fractions against alpha-glucosidase, highlighting their potential in managing diabetes.

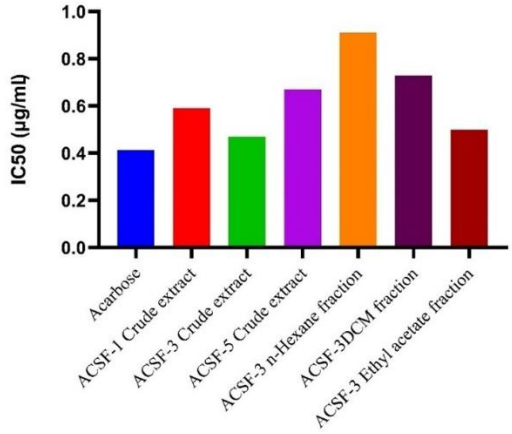



**Figure 8.** α-Glucosidase inhibitory activity of endophytic fungal extracts.

## 4. Discussion

The separation and characterization of bioactive adduct from endophytic fungi related to *Aegiceras corniculatum* have provided valuable insights into the potential of these fungal strains as sources of pharmacologically active compounds [22]. Parts of the *Aegiceras corniculatum* plant and the fungi that coexist with it contain new chemicals. These include 3,7-dihydroxy-2,5-diundecylnaphthoquinone, 2-methoxy-3-nonylresorcinol, 5-*O*-ethylembelin, 2,7-dihydroxy-8-methoxy-3,6-diundecyl-dibenzofuran-1,4-dione, and. Three known compounds were also found: 5-*O*-methylembelin, 3-undecylresorcinol, and 2-dehydroxy-5-*O*-methylembelin. 3,7-dihydroxy-2,5-diundecylnaphthoquinone is a derivative of naphthoquinone, and naphthoquinone derivatives show anticancer and antimicrobial activities [23]. In this investigation, we successfully obtained seven strains of endophytic fungi from the bark of *Aegiceras corniculatum*, thus encompassing a diverse array of fungal biodiversity. From this collection, ACSF-1, ACSF-3, and ACSF-5 were specifically chosen for subsequent extraction and assessment of their bioactive properties.

One of the key findings of this study was the remarkable DPPH free radical scavenging activity exhibited by the bark extract which was methanolic of *Aegiceras corniculatum* and extracted endophytic fungi. This indicates the presence of potent antioxidants in both the plant and the associated fungal strains, highlighting their potential in combating oxidative stress-related disorders. The graphical representation of the scavenging activity provides a visual representation of the dose-dependent response, further supporting the antioxidant capacity of these extracts (Figure 2).

The assessment of total polyphenolic content revealed substantial quantities in ACSF-1 crude extract, underscoring its role as a potential source of polyphenolic compounds. The graphical representation of the concentration-absorbance relationship allows for a clear visualization of the phenolic content and its variation across different concentrations.

Additionally, the evaluation of total flavonoid content further elucidated the bioactive potential of the isolated endophytic fungi. The graphical representation aids in understanding the concentration-dependent variation in flavonoid content, which holds significance in various pharmacological applications. The determination of total tannin content added another layer of understanding regarding the chemical composition of the extracts. Tannins, known for their astringent properties and potential health benefits, were found in varying quantities within the tested samples [24,25].

The assessment of antimicrobial activity demonstrated varying inhibitory effects among the extracts and fractions. The importance of antimicrobial assays was reviewed by the following works where researcher was synthesized seven complete lactam $Arg_{10}$-teixobactin analogues using solid-phase peptide synthesis. The entire lactam ring was obtained by replacing d-$Thr_8$ with (2R,3S)-diamino-propionic acid. Each analogue was evaluated with MIC against resistant bacteria to confirm their antimicrobial activity and efficacy. $Nle^{11}$-containing compounds showed noteworthy activity [26]. Notably, the standard antibiotic ciprofloxacin exhibited strong inhibitory activity, providing a benchmark for comparison (Figure 7). These results suggest the potential of certain extracts and fractions in combating bacterial infections, albeit with varying degrees of efficacy. Non-polar compound(s) may potentially contribute to the manifestation of antibacterial activity in disc diffusion assays, as they may demonstrate an inability to effectively disperse in agar media. In accordance with their investigation, the plant extracts showcased noteworthy minimum inhibitory concentrations (MIC) [8].

Finally, the evaluation of alpha-glucosidase inhibitory activity revealed promising results, indicating the potential of the tested extracts and fractions in managing diabetes. The graphical representation of this inhibitory activity provides valuable insights into the dose-dependent response.

Medicinal flora and their endophytes play a significant role as a valuable reservoir for biologically active substances and secondary metabolites, which constitute a majority of the herbal pharmaceuticals present in existing commercial sector [27].

## 5. Conclusion

The examination and characterization of biologically active substances from endophytic fungi connected with *Aegiceras corniculatum* present stimulating possibilities for pharmaceutical and medicinal applications. The wide array of biologically active compounds recognized like antioxidants, polyphenols, flavonoids, tannins, antimicrobial agents as well as alpha-glucosidase inhibitors which emphasize the pharmacological potential of these fungal strains. Further investigation is justified to separate and identify particular biologically active compounds and investigate their mechanisms of action, paving the way for the development of innovative therapeutic agents derived from these endophytic fungi. This examination highlights the potential of endophytic fungi linked to *Aegiceras corniculatum* as valuable origins of diverse bioactive compounds, providing a multitude of pharmacological advantages. It contributes notably to our comprehension of the natural substances present within the ecosystem of mangroves.

**Author Contributions:** Conceptualization: S.N., R.D.B., S.A. and M.A.I.; Data curation: S.N., R.D.B., S.A. and M.S.R.; Formal analysis: S.N., R.D.B., S.A. and A.K.A.; Investigation: S.N., R.D.B., S.A. and D.D.; Methodology: S.N., R.D.B., S.A. and M.A.I.; Project administration: M.A.I.; Resources: M.S.R., A.-S.M. and A.K.A.; Software: S.N., R.D.B., A.-S.M., D.D. and R.R.R.; Supervision: M.A.I.; Validation: M.A.I. and M.S.R.; Writing—original draft—Preparation: R.D.B., S.N., D.D. and R.R.R.; Writing—review & editing: M.A.I., A.-S.M. and A.K.A. All authors have read and agreed to the published version of the manuscript.

**Funding:** This research received no external funding.

**Institutional Review Board Statement:** Not applicable.

**Informed Consent Statement:** Not applicable.

**Data Availability Statement:** Not applicable.

**Acknowledgments:** We are very grateful to Pharmacy Discipline, Khulna University, Bangladesh for providing the laboratory facilities to carry out the research. The Research and Innovation Centre at Khulna University provided partial support for this study.

**Conflicts of Interest:** The authors declare no conflict of interest.

## References

1. Compant, S.; Cambon, M.C.; Vacher, C.; et al. The Plant Endosphere World—Bacterial Life within Plants. *Environ. Microbiol.* **2021**, *23*, 1812–1829.
2. Odelade, K.A.; Babalola, O.O. Bacteria, Fungi and Archaea Domains in Rhizospheric Soil and Their Effects in Enhancing Agricultural Productivity. *Int. J. Environ. Res. Public Health* **2019**, *16*, 3873.
3. Igiehon, N.O.; Babalola, O.O.; Cheseto, X.; et al. Effects of Rhizobia and Arbuscular Mycorrhizal Fungi on Yield, Size Distribution and Fatty Acid of Soybean Seeds Grown under Drought Stress. *Microbiol. Res.* **2021**, *242*, 126640.
4. Jalgaonwala, R.E.; Mohite, B.V.; Mahajan, R.T. A Review: Natural Products from Plant Associated Endophytic Fungi. *J. Microbiol. Biotechnol. Res.* **2011**, *1*, 21–32.

5. Omojate Godstime, C.; Enwa Felix, O.; Jewo Augustina, O.; et al. Mechanisms of Antimicrobial Actions of Phytochemicals against Enteric Pathogens—A Review. *J. Pharm. Chem. Biol. Sci.* **2014**, *2*, 77–85.
6. Tusharbhai, N.; Amar, P.; Pandey, N. Salinity Tolerance of *Aegiceras corniculatum* (L.) Blanco from Gujarat Coasts of India. *An. Biol. Fax* **2009**, *31*, 93–104.
7. Vinh, L.B.; Phong, N.V.; Ali, I.; et al. Identification of Potential Anti-Inflammatory and Melanoma Cytotoxic Compounds from *Aegiceras Corniculatum*. *Med. Chem. Res.* **2020**, *29*, 2020–2027. https://doi.org/10.1007/s00044-020-02613-5.
8. Janmanchi, H.; Raju, A.; Degani, M.S.; et al. Antituberculosis, Antibacterial and Antioxidant Activities of *Aegiceras Corniculatum*, a Mangrove Plant and Effect of Various Extraction Processes on Its Phytoconstituents and Bioactivity. *S. Afr. J. Bot.* **2017**, *113*, 421–427.
9. Roome, T.; Razzak, A.; Ali, P.; et al. Therapeutic Effect of *Aegiceras Corniculatum* in Chronic Granulomatous Inflammation and Arthritis. *J. Dow Univ. Heal. Sci*. **2014**, *8*, 98–103.
10. Gurudeeban, S.; Satyavani, K.; Ramanathan, T.; et al. Antidiabetic Effect of a Black Mangrove Species *Aegiceras Corniculatum* in Alloxan-Induced Diabetic Rats. *J. Adv. Pharm. Technol. Res.* **2012**, *3*, 52.
11. Sopalun, K.; Strobel, G.A.; Hess, W.M.; et al. A Record of *Muscodor albus*, an Endophyte from *Myristica fragrans* in Thailand. *Mycotaxon* **2003**, *88*, 239–247.
12. Wei, J.C. *Handbook of Fungal Identification*; Shanghai Scientific and Technical Publishers: Shanghai, China, 1979; pp. 1–802.
13. Lin, Z.-J.; Lu, Z.-Y.; Zhu, T.-J.; et al. Penicillenols from *Penicillium* sp. GQ-7, an Endophytic Fungus Associated with Aegiceras Corniculatum. *Chem. Pharm. Bull.* **2008**, *56*, 217–221.
14. Pharamat, T.; Palaga, T.; Piapukiew, J.; Whalley, A.J.S.; Sihanonth, P. Antimicrobial and Anticancer Activities of Endophytic Fungi from Mitrajyna Javanica Koord and Val. *Afr. J. Microbiol. Res.* **2013**, *7*, 5565–5572.
15. Cui, J.-L.; Guo, T.-T.; Ren, Z.-X.; et al. Diversity and Antioxidant Activity of Culturable Endophytic Fungi from Alpine Plants of *Rhodiola Crenulata*, *R. Angusta*, and *R. Sachalinensis*. *PLoS ONE* **2015**, *10*, e0118204.
16. Anesini, C.; Ferraro, G.E.; Filip, R. Total Polyphenol Content and Antioxidant Capacity of Commercially Available Tea (*Camellia sinensis*) in Argentina. *J. Agric. Food Chem.* **2008**, *56*, 9225–9229.
17. Saeed, N.; Khan, M.R.; Shabbir, M. Antioxidant Activity, Total Phenolic and Total Flavonoid Contents of Whole Plant Extracts *Torilis leptophylla* L. *BMC Complement. Altern. Med.* **2012**, *12*, 221.
18. Amorim, E.L.C.; Nascimento, J.E.; Monteiro, J.M.; et al. A Simple and Accurate Procedure for the Determination of Tannin and Flavonoid Levels and Some Applications in Ethnobotany and Ethnopharmacology. *Funct. Ecosyst. Communities* **2008**, *2*, 88–94.
19. Rivera-Chávez, J.; Figueroa, M.; del Carmen González, M.; et al. α-Glucosidase Inhibitors from a *Xylaria feejeensis* Associated with *Hintonia latiflora*. *J. Nat. Prod.* **2015**, *78*, 730–735.
20. You, Q.; Chen, F.; Wang, X.; et al. Inhibitory Effects of Muscadine Anthocyanins on α-Glucosidase and Pancreatic Lipase Activities. *J. Agric. Food Chem.* **2011**, *59*, 9506–9511.
21. Masoko, P.; Picard, J.; Eloff, J.N. The Antifungal Activity of Twenty-Four Southern African *Combretum* Species (Combretaceae). *S. Afr. J. Bot.* **2007**, *73*, 173–183.
22. Sun, J.-F.; Lin, X.; Zhou, X.-F.; et al. Pestalols A–E, New Alkenyl Phenol and Benzaldehyde Derivatives from Endophytic Fungus *Pestalotiopsis* sp. AcBC2 Isolated from the Chinese Mangrove Plant *Aegiceras corniculatum*. *J. Antibiot.***2014**, *67*, 451–457.
23. Noshin, S.; Bairagi, R.D.; Airin, S.; et al. Synergistic Bioactivity of *Aegiceras corniculatum* (L.) Blanco and Its Endophytic Fungus Aspergillus: Antioxidant, Antimicrobial, and Cytotoxic Effects. *Cell Biochem. Biophys.* **2024**, 1–10.
24. Taware, A.S.; Chauhan, R.P.; Rajurkar, S.K. Screening of Secondary Metabolites from Endophytic Fungi of *Albizia lebbeck* from Various Regions of Aurangabad, Maharashtra (India). *Adv. Life Sci. Hum. Welf.* **2017**, 27–32.
25. da Silva, M.H.R.; Cueva-Yesquén, L.G.; Júnior, S.B.; et al. Endophytic Fungi from Passiflora Incarnata: An Antioxidant Compound Source. *Arch. Microbiol.* **2020**, *202*, 2779–2789.
26. Scioli, G.; Marinaccio, L.; Bauer, M.; et al. New Teixobactin Analogues with a Total Lactam Ring. *ACS Med. Chem. Lett.* **2023**, *14*, 1827–1832.
27. Singh, R.; Dubey, A.K. Endophytic Actinomycetes as Emerging Source for Therapeutic Compounds. *Indo Glob. J. Pharm. Sci.* **2015**, *5*, 106–116.

*Article*

# Exploring the Therapeutic Promise of *Drynaria coronans*: Phytochemical Analysis, Antioxidant Capacity, α-Amylase Inhibition with Safety Assessment

**Prabhat Kumar Jha [1,2,*], Sindhu KC [1], Ram Kishor Yadav [1], Bipindra Pandey [2], Sandesh Paudel [1], Ravindra Khadka [1], Kushal Subedi [1], Sushil Panta [1,*]**

[1] School of Health and Allied Sciences, Pokhara University, Kaski 33700, Nepal
[2] Department of Pharmacy, Madan Bhandari Academy of Health Sciences, Hetauda 44100, Nepal
* Correspondence: jha.prabhat9999@gmail.com.com (P.K.J.); sushilmax@hotmail.com (S.P.); Tel.: +977-9864512660 (P.K.J.); +977-9846032882 (S.P.)

Received: 1 December 2024; Revised: 9 January 2025; Accepted: 7 February 2025; Published: 10 February 2025

**Abstract:** Background: Secondary metabolites derived from plants have been identified as potential natural antioxidants, exhibiting robust capabilities in neutralizing oxidative stress that can emerge under various pathological conditions. Aim: The aim of this study was to analyze the phytochemical composition of ethanolic extract of *Drynaria coronans (D. coronans)*, and its antioxidant potential along with α-amylase inhibition and oral toxicity studies. Methods: Preliminary qualitative phytochemical screening was conducted for *D. coronans* while quantitative phytochemical analysis involved the estimation of total phenolic and flavonoid contents. In vitro antioxidant activities were evaluated by using ferrous reducing-antioxidant power (FRAP), hydrogen peroxide ($H_2O_2$) scavenging activity, nitric oxide scavenging activity and DPPH free radical scavenging assays. The in vitro antidiabetic activity was evaluated by using the α-amylase inhibition assay. In vivo oral acute toxicity studies were evaluated in the rats as per the Organization for Economic Cooperation and Development guidelines. Results: The extraction yield of *Drynaria coronans* was found to be 11.94% using ethanol as the primary solvent and plant sample with solvent in a 4:1 ratio. Phytochemical analysis of the rhizome revealed the presence of secondary metabolites, including alkaloids, phenols, tannins, glycosides, and carbohydrates, which were further validated by TLC profiling. The dried extract contained 56.38 ± 0.09 mg gallic acid equivalent (GAE/g) of total phenols and 202.54 ± 0.22 mg quercetin equivalents (QE/g) of flavonoids. The extract demonstrated notable antioxidant properties, with $IC_{50}$ values of 43.59 (DPPH), 758.94 (NO), and 715.60 ($H_2O_2$), while also exhibiting strong reducing power (0.164 ± 0.011) at 700 nm. Additionally, it showed effective α-amylase inhibition with an $IC_{50}$ of 889.84 µg/mL. When tested for acute oral toxicity at a dose of 5000 mg/kg, no behavioral changes, morbidity, or mortality were observed in Swiss albino rats. Conclusion: The ethanolic extract of *Drynaria coronans* rhizomes have a positive correlation of total phenolic and flavonoid contents with the antioxidant, and inhibitory potential of the α-amylase.

**Keywords:** α-amylase; antioxidant activity; *Drynaria coronans*; phytochemical analysis; toxicity studies

## 1. Introduction

Oxidative stress arises from an excess of reactive oxygen species (ROS) and reactive nitrogen species (RNS) [1]. These terms collectively describe free radicals and other non-radical reactive oxidants. ROS include dioxygen ($O2^{\bullet-}$), hydrogen peroxide ($H_2O_2$), and hydroxide ($^{\bullet}OH$), while RNS include nitrogen dioxide ($NO_2$), nitric oxide ($NO^{\bullet}$), and peroxynitrite ($ONOO^-$) [2,3]. An accumulation of ROS and RNS leads to oxidative damage to nearly all molecules. Under normal physiological conditions, ROS and RNS groups are not harmful [4,5]. However, when the body fails to adequately eliminate them, oxidative stress promotes atherosclerotic plaque formation, increasing the risk of atherosclerosis, cancer and even type 2 diabetes mellitus [6–8].

α-Amylase, a class of hydrolytic enzymes, is synthesized by various organisms including humans, animals, fungi, bacteria, and plants. In the human body, these enzymes are produced in the salivary glands and subsequently released into the oral cavity. Additionally, the pancreas secretes α-amylase directly into the small intestine [9]. The primary function of α-amylase is to catalyze the breakdown of starch molecules. Specifically, these enzymes

target and cleave the α-(1,4)-glycosidic bonds within starch, leading to the production of several compounds such as glucose, maltodextrins, maltotetraose, maltose, and maltotriose [9].

Oxidative stress has been linked to numerous health conditions, including diabetes, cancer, neurodegenerative disorders, and cardiovascular diseases [10,11]. Additionally, diabetes-related cardiovascular issues can develop through various mechanisms, with oxidative stress being a significant factor. Consequently, managing oxidative stress and blood sugar levels is crucial for overall health. The natural world has consistently provided a rich source of beneficial compounds that contribute to human well-being [12]. Substantial evidence demonstrates that plants and other natural food sources are primary providers of antioxidants and have been shown to inhibit α-amylase and α-glucosidase activities [13].

*Drynaria coronans* (Wall. ex Mett.) J.Sm. ex T.Moore (Family: Polypodiaceae), is one of the medicinal plants of Nepal which are commonly found densely on tree trunks at higher elevations of approximately 1500–2000 m altitude [14]. The rhizome of *D. coronans* is used to treat constipation, bone fractures and backaches [15–17]. There was a paucity of research on the *D. coronans* rhizome as per the literature review since the search for bioactive plant components from this plant is a deciding factor in the search for new bioactive phytoconstituents. Investigating the *D.coronans* rhizome will help to improve the proper use of this plant in various medical conditions as an alternative treatment plan and aiding in the discovery of therapeutic agents for specific diseases. The purpose of this study was to investigate the in vitro antioxidant activity, phytochemical profile, α-amylase inhibitory activity, and acute oral toxicity of the rhizome of *D. coronans.*

## 2. Material and Methods

### *2.1. Collection and Authentication of Plant Material*

*D. coronans* rhizomes (Figure S1) were collected from Pokhara-31, Kaski district of Nepal, with the help of the local people. The plant was authenticated by the taxonomist via macroscopical and microscopical evaluation methods from the National Herbarium and Plant Laboratories, Godawari, Nepal. The parts of plant were collected from their natural habitat, and plant samples were stored in the Pharmacognosy Laboratory of the School of Health and Allied Sciences, Pokhara University, Kaski, with the Herbarium Sheet voucher number PUH-2022-40.

### *2.2. Drugs and Chemicals*

Ethanol was purchased from Central Drug House (P.) Ltd.,Delhi, India, while Times Pharmaceutical Limited, Chitwan, Nepal, provided the standard reference drug, acarbose. Ascorbic acid, gallic acid, quercetin, and DPPH were purchased from HiMedia Pvt. Ltd., Maharashtra, India. All the reagents and chemicals used in this study were of analytical grade.

### *2.3. Plant Extract Preparation and Percentage Yield*

A modified version of the cold maceration method was used for extraction. The plant sample (100 g) was macerated twice for 24 h in ethanol (800 mL). A rotary evaporator was used to evaporate and dry the extract. The extracts were stored in glass vials with silica crystals for drying in vacuum desiccators operating at 60 mbar. The extract weights were recorded daily until a steady dry weight was obtained. The percentage yield was calculated from the weight of the dried extract. The percentage of extract yield was determined using the following formula:

$$\text{Extract Yield Percentage} = [(\text{Weight of dry extract} / \text{Weight of crude drug sample}) \times 100\%]$$

### *2.4. Phytochemical Screening*

Phytochemical screening was performed to identify the secondary metabolites present in the ethanolic extract of *D. coronans* using color reactions with different reagents [18]. The ethanolic extracts were tested for carbohydrates, flavonoids, glycosides, alkaloids, tannins, saponins, proteins, and steroids.

### *2.5. TLC Profiling of the Plant Extracts*

TLC profiling of the extracts was performed for qualitative analysis [19,20]. One gram of dried extract of *D. coronans* rhizome was mixed with ethanol to prepare sample. TLC profiling was then carried out by applying the sample into three different TLC plates, which were allowed to run separately in the mobile phase of a solvent mixture (chloroform:methanol:water = 7:3:0.5). The TLC plates were then observed under UV lamps at both 365 nm and 254 nm wavelengths. The spraying reagents were 10% $FeCl_3$, 10% $H_2SO_4$, and 100 µM DPPH solution.

### *2.6. Total Phenolic and Flavonoid Compound Analysis*

Using the Folin-Ciocalteu technique, the total phenolic content of extracts was determined [21] using certain adjustments, and the results were expressed as gallic acid (GA) equivalents in milligrams (mg) per gram of dry rhizome extract (mg GAE/g).

The total flavonoid content was evaluated using the aluminum chloride method [10] with a few minor adjustments, and the total flavonoid content was expressed as milligrams of quercetin equivalents (QE) per gram of extract from dried rhizome (mg QE/g).

### *2.7. Invitro Antioxidant Activity*

#### 2.7.1. DPPH Free Radical Scavenging Assays

The scavenging activity of plant extracts towards the stable free radical DPPH was measured using a previously established method with some modifications [22,23]. A small portion (2 mL) of different concentrations of the extract and ascorbic acid solutions (1, 2.5, 5, 7.5, 15, 30, and 60 µg/mL) in ethanol was mixed with 100 µM DPPH solution (2 mL). The mixture was allowed to stand in the dark for 30 min. After 30 min, the absorbance of each test sample was measured at 517 nm by using a UV Spectrophotometer. Ascorbic acid was used as the standard reference. All findings were expressed in the $IC_{50}$ values, which represent the 50% inhibition of the free radicals with the plant extract and standard reference. The radical scavenging activity of each sample was calculated using the following formula [24]:

$$\text{DPPH free radical scavenging activity (\%)} = [(A_{\text{control 517}} - A_{\text{Sample 517}}) / A_{\text{control 517}}] \times 100$$

where, $A_{\text{control 517}}$ is the absorbance control (sample was replaced with distilled water); $A_{\text{sample 517}}$ is the plant extract sample absorbance

#### 2.7.2. Nitric Oxide Scavenging Activity

The nitric oxide radical scavenging activity of the plant extracts was determined using a previous method [25]. The plant extract (1 mL) of different concentrations (0.1 µg/mL, 1 µg/mL, 10 µg/mL, 100 µg/mL, 250 µg/mL, 500 µg/mL, 1000 µg/mL) was mixed with SNP (1 mL) solution and incubated for 2.5 h at 29 °C. During incubation, the extract scavenges nitric oxide radicals, and the Griess reagent determines the quantity of remaining nitric oxide radicals. After 2.5 h, Griess (2 mL) reagent was added to the reaction mixture and absorption was measured at 548 nm using a UV spectrophotometer. Curcumin was used as the standard reference. The NO scavenging activity was calculated using the following formula:

$$\text{NO free radical scavenging activity (\%)} = [(A_{\text{control 548}} - A_{\text{Sample 548}}) / A_{\text{control 548}}] \times 100$$

where, $A_{\text{control 548}}$ is the absorbance control $A_{\text{sample 548}}$ is the plant extract sample absorbance

#### 2.7.3. Hydrogen Peroxide Scavenging Activity

The ability of the extracts to scavenge hydrogen peroxide was performed by the method of [26] with little modification 1 mL of the extracts from (0.1 µg/mL, 1 µg/mL, 10 µg/mL, 100 µg/mL, 250 µg/mL, 500 µg/mL, 1000 µg/mL) was added to 20 mM $H_2O_2$ (2 mL) solution prepared in 0.1 M phosphate buffer saline (pH 7.4). The mixture was incubated for 10 min and the absorbance was read at 230 nm using a UV spectrophotometer against a buffer blank. Ascorbic acid was used as the standard reference. The control was similar to that of the reaction mixture but without a test sample. Similarly, the plant control was a reaction mixture of the extract and buffer only. The scavenging activity was calculated using the following equation:

$$\text{Hydrogen Peroxide scavenging activity (\%)} = [(A_{\text{control 230}} - A_{\text{Sample 230}}) / A_{\text{control 230}}] \times 100$$

where, $A_{\text{control 230}}$ is the absorbance control $A_{\text{sample 230}}$ is the plant extract sample absorbance

#### 2.7.4. Ferrous Reducing-Antioxidant Power (FRAP)

The ability of the plant extracts to reduce antioxidant power was determined [27,28] with a slight modification. The ethanolic extract of varying concentrations (12.5 to 100 µg/mL) was mixed with 0.25 mL phosphate buffer (0.2 mM, pH 6.6) and 1% potassium ferricyanide (0.25 mL). The reaction system was closed and incubated at 50 °C (it's an o then superscripted, never use zero) in a water bath for 20 min. After the incubation

period, 10% trichloroacetic acid (0.25 mL) was added to the assay system, and the contents were mixed thoroughly. The mixture was then centrifuged at 5000 rpm for 5 min. The supernatant (0.5 mL) was mixed with equal volumes of distilled water and ferric chloride solution (0.1 mL, 0.1%, *w*/*v*). The developed color was read at 700 nm using a UV-visible spectrophotometer, and the results were compared with those of ascorbic acid as a standard reference. The readings were expressed as the mean absorbance value.

### *2.8. In-Vitro Antidiabetic Activity*

#### 2.8.1. α-Amylase Inhibitory Activity

An α-amylase inhibition assay was performed using the 3, 5-dinitrosalicylic acid (DNSA) method [29]. The α-amylase solution (200 µL, 5 mg/100 mL) was mixed with of the extract (200 µL) and incubated for 10 min at 30 °C. Next, the starch solution (200 µL, 1%) was added to each tube and incubated for 3 min. The reaction was terminated by adding DNSA (200 µL) reagent and boiling for 10 min in a water bath at 85–90 °C. The mixture was cooled to ambient temperature, diluted with distilled water (5 mL), and the absorbance was measured at 540 nm using a UV-visible spectrophotometer. A blank with 100% enzyme activity was prepared by replacing the plant extract with buffer (200 µL, mention buffer details, pH). The plant control was similarly prepared using the plant extract at each concentration in the absence of the enzyme solution. A positive control sample was prepared using acarbose (50 µg/mL, 100 µg/mL, 250 µg/mL, 500 µg/mL, 1000 µg/mL), and the reaction was performed similarly to the reaction with the plant extract, as mentioned above. The α-amylase inhibitory activity was expressed as a percentage inhibition and calculated using the following equation: % α-amylase inhibition was plotted against the extract concentration, and the $IC_{50}$ values were obtained from the graph.

#### 2.8.2. Acute Toxicity Studies

The Organization for Economic Co-operation and Development Guidelines (OECD) No. 425 were used to test the acute oral toxicity of *D. coronans* extracts [30]. Healthy non-pregnant and nulliparous female Swiss albino rats (180–240 g) were used for this purpose (Figure S4). All rats were fasted for 18 h with free access to water before and 3–4 h after the administration of the ethanolic extract. For the oral acute toxicity study, *D. coronans* ethanolic extract was dissolved in distilled water and treated as three doses at 2000 mg/kg, 3000 mg/kg, and 5000 mg/kg dose orally for three female healthy Swiss albino rats in the first phase by oral gavage tube. The treated rats were observed continuously for 4 h at half-hour intervals and then for 2 weeks at 1 d for general signs and symptoms of toxicity (diarrhea, weight loss, lethargy, tremors, and paralysis), food and water consumption, and mortality. In the second phase for the confirmation of the further toxicity study *D. coronans* ethanolic extract at a dose of 5000 mg/kg was administered to two female rats and observed for the signs and symptoms. If no mortality is observed in any of the tested animals, the $LD_{50}$ of the test substance is said to be greater than 5000 mg/kg and hence has a high degree of safety

#### 2.8.3. Statistical Analysis

A standard calibration curve for gallic acid and quercetin was created using the regression line equation in Microsoft Excel (2019) to estimate the concentrations of the phenolic and flavonoid compounds. All the in vitro and in vivo study was performed in triplicate and all results data are presented as mean ± standard error of mean.

## 3. Results

### *3.1. Phytochemical Screening*

Numerous phytoconstituents, including tannins, flavonoids, saponins, carbohydrates, alkaloids, phenol, and glycosides, were found in the ethanolic extract using qualitative phytochemical screening (Table 1).

Phenol, flavonoids, and antioxidants were confirmed by applying extracts and chemicals to TLC plates, as shown in Figure 1, Figure S2, Figure S3

**Table 1.** Phytochemical Screening of Hydro-ethanolic extract of *D. coronans*.

| Phytochemical Test | | Inference |
|---|---|---|
| Alkaloid | Wagner test | + |
| Flavonoid | Alkaline reagent test | + |
| Phenol | Sodium hydroxide test | + |
| Tannin | Ferric chloride Test | + |
| Glycoside | Salkowski's Test | + |
| Saponin | Lead acetate test | − |
| Carbohydrate | Fehling test | + |
| '+' and '−' sign indicates the presence and absence, respectively | | |

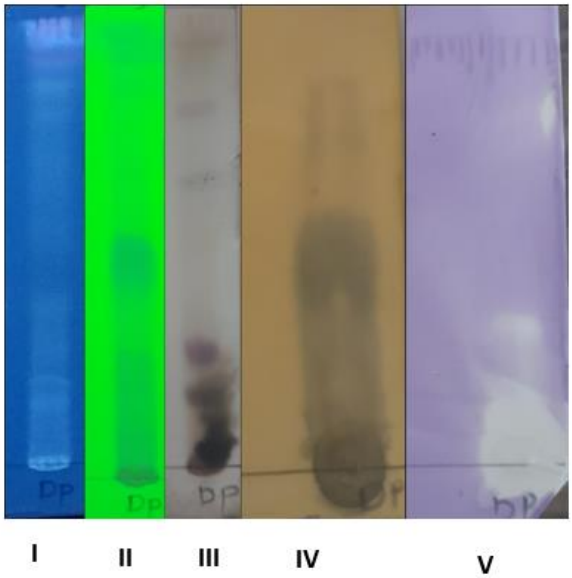



**Figure 1.** TLC plate observation. *Drynaria coronans* rhizome extract TLC spot observation under (I) short UV 254 nm, (II) long UV 365 nm, and TLC spot sprayed with (III) 10% $H_2SO_4$ /Heat, (IV) 10% $FeCl_3$, (V) 100 µM DPPH.

### *3.2. Total Phenolic and Flavonoid Content Estimation*

The extract obtained from the rhizome of *D. coronans* was a golden yellow solid (11.94%). The phenol content for the *D. coronans* ethanolic extract was (56.38 ± 0.09) mg GAE/gm dry extract weight while flavonoid content was obtained as (202.54 ± 0.22) mg QE/gm dry extract weight.

### *3.3. Antioxidant Assay*

#### 3.3.1. DPPH Radical Scavenging Activity

Compared with conventional ascorbic acid, the ethanol extracts of *D. coronans* rhizome ($IC_{50}$ = 43.59 µg/mL) demonstrated good DPPH free radical scavenging activity (Table 2).

**Table 2.** Antioxidant activity of hydroethanolic extract of *D. coronans* using DPPH, NO, $H_2O_2$, and FRAP assays.

| S.N. | Antioxidant Assay | Sample | Concentration (µg/mL) | | | | | $IC_{50}$ (µg/mL) |
|---|---|---|---|---|---|---|---|---|
| | | | 2.5 µg/mL | 5 µg/mL | 15 µg/mL | 30 µg/mL | 60 µg/mL | |
| 1 | DPPH scavenging (%) | *D. coronans* | 1.57 ± 0.04 | 3.86 ± 0.09 | 27.04 ± 2.44 | 36.86 ± 1.26 | 63 ± 2.43 | 43.59 |
| | | Ascorbic acid | 22.2 ± 0.5 | 48.4 ± 2.1 | 61.34 ± 0.34 | 76.28 ± 2.09 | 98.25 ± 0.13 | 3.96 |
| | | | 10 µg/mL | 100 µg/mL | 250 µg/mL | 500 µg/mL | 1000 µg/mL | |
| 2 | NO scavenging (%) | *D. coronans* | 3.7 ± 0.06 | 9.28 ± 0.6 | 22.11 ± 0.6 | 37.33 ± 2.9 | 62.49 ± 0.1 | 758.94 |
| | | Curcumin | 33.89 ± 0.74 | 43.23 ± 0.68 | 61.66 ± 1.75 | 81.74 ± 0.64 | 91.09 ± 1.34 | 162.79 |
| | | | - | 100 µg/mL | 250 µg/mL | 500 µg/mL | 1000 µg/mL | |
| 3 | $H_2O_2$ Scavenging (%) | *D. coronans* | - | 22.7 ± 0.1 | 29.89 ± 0.3 | 42.29 ± 2.07 | 61 ± 6.03 | 715.6 |
| | | Ascorbic acid | - | 28.7 ± 0.2 | 42.12 ± 0.3 | 61.89 ± 4.4 | 94.3 ± 1.7 | 353.96 |
| | | | 12.5 µg/mL | 25 µg/mL | 50 µg/mL | 75 µg/mL | 100 µg/mL | |
| 4 | FRAP ($Abs_{700\ nm}$) | *D. coronans* | 0.005 ± 0.008 | 0.084 ± 0.005 | 0.127 ± 0.006 | 0.145 ± 0.008 | 0.164 ± 0.011 | - |
| | | Ascorbic acid | 0.234 ± 0.007 | 0.388 ± 0.008 | 0.545 ± 0.005 | 0.797 ± 0.006 | 1.07 ± 0.006 | - |

All experiments were performed in triplicate (n = 3) and expressed as the mean ± standard error of the mean.

3.3.2. NO Free Radical Scavenging Activity

Compared with conventional curcumin, the ethanol extract of *D. coronans* rhizome ($IC_{50}$ = 758.94 µg/mL) demonstrated good NO free radical scavenging activity (Table 2).

3.3.3. Hydrogen Peroxide Scavenging Activity

Compared to conventional ascorbic acid, the ethanol extracts of *D. coronans* rhizomes ($IC_{50}$ value 715.60 µg/mL) demonstrated better hydrogen peroxide scavenging activity (Table 2).

3.3.4. Ferric ion Reducing Antioxidant Assay (FRAP)

The *D. coronans* extract demonstrated moderate ferric-reducing activity at 100 µg/mL (0.164 ± 0.011) compared to the ascorbic acid standard (Table 2).

3.3.5. α-Amylase Inhibitory Activity

Compared to conventional drugs acarbose, *D. coronans* rhizome extract performed comparable α-amylase inhibitory activity ($IC_{50}$ = 889.84 µg/mL) as shown in (Table 3, Figure S5).

**Table 3.** The α-amylase inhibitory activity of hydro-ethanolic extract of *D. coronas*.

| S. No. | SampleName | % α-Amylase Inhibitory Activity | | | | | $IC_{50}$ Value (µg/mL) |
|---|---|---|---|---|---|---|---|
| | | 50 µg/mL | 100 µg/mL | 250 µg/mL | 500 µg/mL | 1000 µg/mL | |
| 1 | *D. coronans* | 11.83 ± 6.98 | 14.31 ± 0.36 | 21.88 ± 0.58 | 35.04 ± 0.248 | 53.75 ± 1.39 | 889.84 |
| 2 | Acarbose | 16.06 ± 0.49 | 24.84 ± 0.15 | 29.47 ± 0.34 | 50.86 ± 0.27 | 83.08 ± 0.31 | 511.51 |

All data are expressed as mean ± standard error of the mean (SEM) (n = 3).

*3.4. Oral Acute Toxicity Study*

Oral administration of the ethanolic extract of *D. coronans* in rats did not cause any behavioral changes such as locomotion, activity, hair texture, pupil size, or feeding. No morbidity or mortality was observed at 5000 mg/kg. Hence, the $LD_{50}$ of both ethanolic extracts was estimated to be greater than 5000 mg/kg and was considered the Globally Harmonized System (GHS) category 5.

## 4. Discussion

It is generally acknowledged that using natural products to identify potential lead compounds for drug development is beneficial [31]. Isolation of morphine for the first time in 1806 paved the way for the development of many natural lead compounds such as strychnine, atropine, and colchicine [32]. Plant-derived natural products have been essential to the world's healthcare systems for thousands of years. They offer various biological activities and active components, making them useful for developing complementary or alternative therapies in contemporary medicine [33]. Based on traditional and ethno-medicinal usage and a scant scientific study of the plant, *D. coronans* rhizomes were chosen for this study. The *D. coronans* rhizome extract was subjected to various antioxidant tests, α-amylase inhibitory activity tests, and oral acute toxicity studies.

Phytochemical analysis of the *D. coronans* extract showed the presence of phytochemicals, such as phenols, flavonoids, tannins, saponins, alkaloids, terpenoids, coumarins, anthracene, and glycosides (Table 1). The presence of several *D. coronans* compounds was confirmed by separation of several bands in the chromatograms visualized under UV light at 254 and 365 nm (Figure 1, Figures S2, S3). The chromatogram after spray/heating with sulfuric acid (10%) showed yellow, black, and reddish-brown spots, suggesting the presence of flavonoids, carbohydrates, and terpenoids, respectively [34]. The presence of phenolic compounds in the chromatogram could also be visualized by spraying with ferric chloride (10%) [35]. The spots at the bottom of the *D. coronans* chromatogram suggested the presence of a phenolic compound. Similarly, the presence of antioxidants was confirmed by a white/yellow band on a violet or purple background on the *D. coronans* chromatogram [34]. Because the plant contains phenols, flavonoids, and antioxidants, they can favorably correlate with antidiabetic activity [36].

The TPC (mg/g) was determined from the regression equation of the calibration curve ($y = 0.01x + 0.093$, $R^2 = 0.9976$) and expressed as GAE 56.38 ± 0.09. TFC (mg/g) was determined from the regression equation of the calibration curve ($y = 0.0008x + 0.0324$, $R^2 = 0.99$) and expressed as QE 202.54 ± 0.22. The ethyl acetate and methanol extract of *D. coronans* carried out by Koirala et al., showed a phenolic content of 292.14 ± 1.82 and

37.48 ± 5.41 mg GAE/g extract respectively while the flavonoid content of the ethyl acetate and methanol fraction was obtained as 322.90 ± 3.94 and 9.87 ± 1.00 mg CE/g extract respectively [37,38]. Because we experimented with the ethanolic extract, the data may vary.

The $IC_{50}$ values for the free radical scavenging activities were determined using the linear regression method, which involves plotting the percentage of the radical scavenging activity against the tested drug concentrations. The DPPH scavenging assay was carried out following the method described in the literature [38]. It showed a higher value ($IC_{50}$ = 93.3 µg/mL) than the experimental result ($IC_{50}$ = 43.59 µg/mL). Similarly, DPPH activity carried in ethyl acetate and methanol extract of the same species showed a DPPH scavenging activity with $IC_{50}$ values (37.54 ± 1.25 µg/mL) and (80.25 ± 1.37 µg/mL) [37]. The ethanolic extract showed DPPH scavenging value similar to that of the ethyl acetate extract of *D. coronans.* This could be due to polar compounds in the ethanolic and ethyl acetate extracts.

A free radical scavenging method using NO, $H_2O_2$, and FRAP was performed for the first time using an ethanolic extract of *D. coronans.* NO or reactive species are responsible for altering the structural and functional nature of many cellular components [39]. Plant antioxidants can mitigate or inhibit the chain reactions triggered by excessive NO production, which is detrimental to human health. [40]. The NO radical scavenging activity in the *D. coronans* rhizome extract was $IC_{50}$ value of 758.94 µg/mL against the standard curcumin with $IC_{50}$ value of 162.79 µg/mL. The NO-scavenging activity of the extract was lower than that of the standard, suggesting that the extract may not be able to scavenge reactive species. The hydrogen peroxide scavenging activity of the extract showed a pattern similar to that of the NO scavenging activity. *D. coronans* showed moderate scavenging activity against $H_2O_2$, with an $IC_{50}$ Value of 715.60 µg/mL. The reducing capacity of a sample is an important indicator of its antioxidant activity. FRAP was used to determine the reducing potential of the extract. The reducing value of *D. coronans* extract was 0.164 ± 0.011 at a concentration of 100 µg/mL. The reducing capacity of the extract also increased with increasing extract concentrations (Table 2).

An α-Amylase inhibitory assay was performed according to the modified 3,5-Dinitrosalicyclic Acid (DNSA) method to determine the in vitro antidiabetic activity of the ethanolic extract of *D. coronans* rhizome. An inhibition assay was performed for the first time in this species. In this study, the extract showed α-amylase inhibitory activity ($IC_{50}$ Value 889.84 µg/mL), while we compared it with standard acarbose ($IC_{50}$ Value 511.51 µg/mL). The enzyme-inhibitory activity of plant extracts is attributed to various classes of phytochemicals such as alkaloids, flavonoids, tannins, and phenolic acids [34]. The α-amylase inhibitory activity of *D. coronans* rhizome extracts depend on the total phenol content, as phenols have been reported as potential antidiabetic agents. Data obtained on the phenol content of the extract also suggest that the α-amylase inhibitory activity could be due to the highest amount of phenolic compounds in this plants [41]. Since α-amylase activity and total phenolic and flavonoid contents and their respective antioxidant activity were correlated.

None of the treatment groups showed any mortality or notable behavioral changes related to acute toxicity. The ethanolic extract of *D. coronans* had an oral $LD_{50}$ of greater than 5000 mg/kg. It is unlikely that the plant will kill people because anything with an $LD_{50}$ above this threshold is thought to be harmless [42]. The limitation of thisstudy was the inability to perform the in vivo antidiabetic activity and evaluation of the biochemical parameters and histopathology examination for the further mechanism of the antidiabetic properties.

## 5. Conclusions

In conclusion, the ethanolic extract of *D. coronans* yielded significant amounts of extract and a considerable quantity of flavonoids and phenolic compounds, exhibiting antioxidant properties due to its high free radical scavenging activity in terms of DPPH, NO, FRAP, and $H_2O_2$. This study also demonstrates that *D. coronans* extracts shows high α-amylase inhibitory activity, since such plant could be potential hypoglycemic agents. The ethanolic extract of *D. coronans* rhizome did not show any toxicity effect up to 5000 mg/kg, which confirms its safety for oral use. The findings of this study warrant further scientific validation, exploration, and analysis to ascertain possible therapeutic applications of the *Drynaria coronans* ethanolic extract as oral hypoglycemic agents.

**Supplementary Materials:** The following are available online at https://www.sciltp.com/journals/jmnp/2025/1/748/s1, Figure S1: *Drynaria coronans* grown as an epiphytic plant in *Shorea robusta*; Figure S2: TLC of Drynaria coronans spot (10% $H_2SO_4$/Heat sprayed); Figure S3: Observation of Phenolic and antioxidant activity using the TLC method; Figure S4: Swiss albino rats used for toxicity study; Figure S5: Alpha-amylase activity of standards used for the research; Figure S6: Filtrate of *Drynaria coronans* rhizome; Figure S7: Ethical approval letter.

**Author Contributions:** P.K.J. and S.P. (Sushil Panta) conceived and designed the experiments. P.K.J., SK, R.K.Y., S.P2. (Sandesh Paudel) and R.K. performed experiments. P.K.J., B.P. and S.P2. (Sandesh Paudel) analyzed the data. P.K.J., B.P., R.K.Y., and K.S. wrote the manuscript. P.K.J., B.P., K.S. and S.P. (Sushil Panta) have reviewed the manuscript. S.P2. (Sandesh Paudel) and B.P. critically revised the manuscript and provided intellectual support. S.P. (Sushil Panta) supervised the project. All authors have read and agreed to the published version of the manuscript

**Funding:** No funding was allocated for this project.

**Institutional Review Board Statement:** The study was conducted with extreme care and showed no signs of endangering people or the environment according to an ethical clearance certificate obtained from the Institute Review Committee of Pokhara University, Nepal (Reference Number: 105-079/80). The documentation for ethical approval is attached in a supplementary file.

**Informed Consent Statement:** Not applicable.

**Data Availability Statement:** All relevant data are in the paper, and upon request, the corresponding author can answer any questions regarding this study's findings.

**Acknowledgments:** The authors are grateful to the School of Health and Allied Sciences, Pokhara University, Kaski, Nepal, for providing the laboratory and research facilities during the study. We sincerely thank Hari Prasad Devkota, Graduate School of Pharmaceutical Sciences, Kumamoto University, Japan and Nabin Pathak, Madan Bhandari Academy of Health Sciences, Nepal for constructive feedback on the manuscript. The manuscript writing part has been assisted by the AI software Grammarly. It was only used for grammar correction and the necessary selection of words.

**Conflicts of Interest:** The authors declare no conflicts of interest.

## References

1. Hayes, J.D.; Dinkova-Kostova, A.T.; Tew, K.D. Oxidative stress in cancer. *Cancer Cell* **2020**, *38*, 167–197.
2. Oguntibeju, O.O. Type 2 diabetes mellitus, oxidative stress and inflammation: examining the links. *Int. J. Physiol. Pathophysiol. Pharmacol.* **2019**, *11*, 45.
3. Radi, R. Peroxynitrite, a stealthy biological oxidant. *J. Biol. Chem.* **2013**, *288*, 26464–26472.
4. Halliwell, B. The wanderings of a free radical. *Free. Radic. Biol. Med.* **2009**, *46*, 531–542.
5. Pandey, B.; Baral, R.; Kaundinnyayana, A.; et al. Promising hepatoprotective agents from the natural sources: A study of scientific evidence. *Egypt. Liver J.* **2023**, *13*, 14.
6. Pandey, B.; Thapa, S.; Biradar, M.S.; et al. LC-MS profiling and cytotoxic activity of Angiopteris helferiana against HepG2 cell line: Molecular insight to investigate anticancer agent. *PloS ONE* **2024**, *19*, e0309797.
7. Poznyak, A.V.; Grechko, A.V.; Orekhova, V.A.; et al. Oxidative stress and antioxidants in atherosclerosis development and treatment. *Biology* **2020**, *9*, 60.
8. Rawal, P.; Pandey, B.; Yadav, R.K.; et al. Antioxidant, alpha-amylase inhibitory and hypoglycemic activity of Smallanthus sonchifolius leaves from Nepal: An integrated in vitro, in vivo, and in silico approach. *Food Sci. Nutr.* **2024**, *13*, e4672.
9. Robyt, J.F. Starch: Structure, properties, chemistry, and enzymology. *Glycoscience* **2008**, 1437. https://doi.org/10.1007/978-3-540-30429-6_35
10. de Araújo, R.F.; Martins, D.B.G.; Borba, M.A.C. Oxidative Stress and Disease. In *A Master Regulator of Oxidative Stress-The Transcription Factor nrf2*; IntechOpen: London, UK, 2016.
11. Wang, Y.; Xiang, L.; Wang, C.; et al. Antidiabetic and antioxidant effects and phytochemicals of mulberry fruit (*Morus alba* L.) polyphenol enhanced extract. *PloS ONE* **2013**, *8*, e71144.
12. Beidokhti, M.N.; Jäger, A.K. Review of antidiabetic fruits, vegetables, beverages, oils and spices commonly consumed in the diet. *J. Ethnopharmacol.* **2017**, *201*, 26–41.
13. Mustafa, I.; Faisal, M.N.; Hussain, G.; et al. Efficacy of Euphorbia helioscopia in context to a possible connection between antioxidant and antidiabetic activities: a comparative study of different extracts. *BMC Complement. Med. Ther.* **2021**, *21*, 62.
14. Rajbhandary, S. Fern and fern allies of Nepal. *Front. Bot.* **2016**, *36*, 124–150.
15. Gewali, M.B.; Awale, S. *Aspects of Traditional Medicine in Nepal*; Institute of Natural Medicine University of Toyama: Toyama, Japan, 2008; pp. 140–142.
16. Manandhar, N.P. *Plants and People of Nepal*; Timber Press: Portland, OR, USA, 2002.
17. Subba, B.; Srivastav, C.; Kandel, R.C. Scientific validation of medicinal plants used by Yakkha community of Chanuwa VDC, Dhankuta, Nepal. *Springerplus* **2016**, *5*, 1–14.
18. Shaikh, J.R.; Patil, M. Qualitative tests for preliminary phytochemical screening: An overview. *Int. J. Chem. Stud.* **2020**, *8*, 603–608.
19. Gwatidzo, L.; Dzomba, P.; Mangena, M. TLC separation and antioxidant activity of flavonoids from Carissa bispinosa, Ficus sycomorus, and Grewia bicolar fruits. *Nutrire* **2018**, *43*, 3.
20. Yadav, R.K.; Shrestha, P.; Timilsina, K.; et al. Antioxidant, antibacterial activity, in silico molecular docking, and admet-toxicity study of lactone from rhizome of Angiopteris helferiana. *J. Chem.* **2024**, *2024*, 5623028.
21. Singleton, V.L.; Rossi, J.A. Colorimetry of total phenolics with phosphomolybdic-phosphotungstic acid reagents. *Am. J. Enol. Vitic.* **1965**, *16*, 144–158.

22. Aryal, S.; Baniya, M.K.; Danekhu, K.; et al. Total phenolic content, flavonoid content and antioxidant potential of wild vegetables from Western Nepal. *Plants* **2019**, *8*, 96.
23. Villaño, D.; Fernández-Pachón, M.S.; Moyá, M.L.; et al. Radical scavenging ability of polyphenolic compounds towards DPPH free radical. *Talanta* **2007**, *71*, 230–235.
24. Gurung, S.; KC, H.; Budhathoki, S.; et al. Analysis of antibacterial effects of methanolic extract of Drynaria propinqua on different bacterial strains. *Pharm. Sci.* **2024**, *4*, 193–200.
25. Jagetia, G.C.; Rao, S.K.; Baliga, M.S.; et al. The evaluation of nitric oxide scavenging activity of certain herbal formulations in vitro: a preliminary study. *Phytother. Res.* **2004**, *18*, 561–565.
26. Sroka, Z.; Cisowski, W. Hydrogen peroxide scavenging, antioxidant and anti-radical activity of some phenolic acids. *Food Chem. Toxicol.* **2003**, *41*, 753–758.
27. Oh, J.; Jo, H.; Cho, A.R.; et al. Antioxidant and antimicrobial activities of various leafy herbal teas. *Food Control* **2013**, *31*, 403–409.
28. Sanjeevkumar, C.; Londonkar, R.L.; Kattegouda, U.M. In vitro antioxidant anti-inflammatory and cytotoxicity activities from Hexane extract of Bryonopsis laciniosa fruits. *Int. J. Phytomedicine* **2018**, *10*, 23–29.
29. Miller, G.L. Use of dinitrosalicylic acid reagent for determination of reducing sugar. *Anal. Chem.* **1959**, *31*, 426–428.
30. Schlede, E. Oral acute toxic class method: OECD Test Guideline 423. *Rapp. Istisan* **2002**, *41*, 32–36.
31. Mir, R.H.; Mohi-ud-din, R.; Mir, P.A.; et al. Chapter 18—Therapeutic potential of plant-derived flavonoids against inflammation. In *Recent Developments in Anti-Inflammatory Therapy*; Academic Press: Cambridge, MA, USA, 2023; pp. 279–293. Available online: https://www.sciencedirect.com/science/article/pii/B978032399988500019X (accessed on 9 June 2024).
32. Khazir, J.; Mir, B.A.; Mir, S.A.; et al. Natural products as lead compounds in drug discovery. *J. Asian Nat. Prod. Res.* **2013**, *15*, 764–788.
33. Zhang, L.; Ravipati, A.S.; Koyyalamudi, S.R.; et al. Antioxidant and anti-inflammatory activities of selected medicinal plants containing phenolic and flavonoid compounds. *J. Agric. Food Chem.* **2011**, *59*, 12361–12367.
34. Yadav, R.K.; Dhakal, A.; Timilsina, K.; et al. Antioxidant and antibacterial activities evaluation, phytochemical characterisation of rhizome from Angiopteris helferiana and barks from Saurauia fasciculata in Nepal. *Sci. World J.* **2024**, *2024*, 1119165.
35. Devkota, H.; Adhikari-Devkota, A.; Takano, A.; et al. HPLC and TLC fingerprints of selected Nepalese natural medicines and medicinal plants. *J. Nepal Pharm. Assoc.* **2017**, *28*, 1–11.
36. Wairata, J.; Fadlan, A.; Purnomo, A.S.; et al. Total phenolic and flavonoid contents, antioxidant, antidiabetic and antiplasmodial activities of Garcinia forbesii King: A correlation study. *Arab. J. Chem.* **2022**, *15*, 103541.
37. Koirala, A.; Devkota, A.; Rajbhandari, M. Phytochemical evaluation of Aglaomorpha coronans from Nepal. *Sci. World* **2024**, *17*, 91–98.
38. Khanal, L.N.; Sharma, K.R.; Pokharel, Y.R.; et al. Assessment of phytochemical, antioxidant and antimicrobial activities of some medicinal plants from Kaski District of Nepal. *Am. J. Plant Sci.* **2020**, *11*, 1383.
39. Parul, R.; Kundu, S.K.; Saha, P. In vitro nitric oxide scavenging activity of methanol extracts of three Bangladeshi medicinal plants. *Pharma Innov.* **2013**, *1*, 83.
40. Moncada, S. The 1991 Ulf von Euler Lecture: The l-arginine: nitric oxide pathway. *Acta Physiol. Scand.* **1992**, *145*, 201–227.
41. Ali Asgar, M.D. Anti-Diabetic potential of phenolic compounds: A review. *Int. J. Food Prop.* **2013**, *16*, 91–103.
42. Zhang, R.; Niu, Y.; Li, Y.; et al. Acute toxicity study of the interaction between titanium dioxide nanoparticles and lead acetate in mice. *Environ. Toxicol. Pharmacol.* **2010**, *30*, 52–60.

*Journal of Medicinal Natural Products*

Scilight

*Article*

# Investigation of In-Vitro Antioxidant, Antimicrobial, Thrombolytic and In-Vivo Anti-Inflammatory and Antidepressant Potential of *Holigarna Longifolia* Leaf Extract

**Md Mizan [1,2,*], Md Shawon Miah [1] and Md Khairul Islam [1]**

[1] Department of Pharmacy, Dhaka International University, Satarkul, Badda, Dhaka 1212, Bangladesh
[2] Pharmacy Discipline, School of Life Science, Khulna University, Khulna 9208, Bangladesh
* Correspondence: hellotomizan@gmail.com; Tel.: +88-1746469198
Received: 11 January 2025; Revised: 9 February 2025; Accepted: 18 February 2025; Published: 20 February 2025

**Abstract:** Discovery of genus *Holigarna longifolia* and its traditional uses were obtained by living in forest or semi forest areas by close observations of the indigenous populations. The main aim of this study was to evaluate phytochemical screening, antioxidant and anti-microbial activity, thrombolytic activity, in vivo anti-inflammatory and neuropharmacological activity of *H. longifolia* leaf extract. In vitro antioxidant was evaluated by DPPH and ferric reducing power assay, Disc diffusion method was used to evaluate anti-microbial activity, thrombolytic activity was evaluated by clot lysis, determine the ability of the extracts to suppress inflammation was done using the formaldehyde-induced paw oedema test influence of the sedative activity of the drug on the forced swimming and tail flick-suspension tests were studied by using diazepam. The phytochemical screening of *H. longifolia* extract revealed the presence of reducing sugars, tannins, gums, steroids, glycosides, xanthoprotein and terpenoids. In DPPH the extract exhibited strong antioxidant with an $IC_{50}$ of 0.877 µg/mL, comparable to ascorbic acid $IC_{50}$ 0.839 µg/mL and in ferric reducing power assay the extract showed significant $IC_{50}$ value about 43.736 µg/mL. In anti-microbial tests, the highest inhibition, 19 mm was observed against *Escherichia coli* at 500 µg/disc, while *Bacillus magaterium* showed the lowest inhibition 9 mm at 250 µg/disc. In the MIC test, the extract leaves showed highest activity against *E. coli* (MIC = 4 mg/mL) and lowest against *B. cereus*, *B. megaterium*, and *A. niger* (MIC = 128 mg/mL). The extract demonstrates clot-lytic activity of 20.45 ± 1.42% versus 31.17 ± 0.65 for streptokinase. *H. longifolia* extract was determined to be safe in the acute toxicity trial, with no toxicity, mortality, or behavioral abnormalities in the mice. In the anti-inflammatory test, paw thickness was significantly reduced with inhibition rates of 54.22% by the dose of 250 mg/kg and 60.27% by 500 mg/kg) compared to 71.71% for the standard. Neuropharmacological tests revealed reduced immobility periods of 126.2 s in forced swimming and 145.8 s in tail suspension tests at dose of 200 mg/kg. Further studies will be needed to evaluate the full pharmacological potential *H. longifolia.*

**Keywords:** phytochemicals; anti-oxidant; anti-microbial; thrombolytic; anti-inflammatory; antidepressant

## 1. Introduction

The plant *Holigarna longifolia* grows natively across South and Southeast Asia as it occurs in India together with Bangladesh and many other nearby locations. *H. longifolia* extends its growth best within semi-evergreen and evergreen forests of hilly environments under warm and humid climatic conditions. Evaluations show that this species exists in Western Ghats plus northeastern India areas and particular sections of forest located in Bangladesh [1]. The plant is from the Annonaceae family and has been used for ages in traditional medicine because of its wide vast therapeutic effects. Several studies have been reported on the occurrence of a number of bioactive compounds in the *H. longifolia*, namely alkaloids, flavonoids, terpenoids and phenolic compounds, probably being the causative of its therapeutic potential. Recalling this work, the present research aimed at exploring the phytochemical composition and determining its antioxidant, antimicrobial, thrombolytic and neuropharmacological activity, in order to explore *H. longifolia* potential as a good source for discovery of drugs [2].

Phytochemical screening is important as a first step in identifying the bio active constituents that may be responsible for medicinal value in plants. *H. longifolia*, was reported to possess, among others, flavonoids, tannins,

glycosides, and alkaloids. Many biological activities such as antioxidant, antimicrobial as well as anti-inflammatory have been reported to these compounds. To understand the plant's therapeutic mechanisms, the identification and quantification of these bioactive compounds are important. Previous studies have shown that in *H. longifolia*, several phytochemicals are present and therefore it should be subjected to further pharmacological investigations [3].

This property of plants regarding its antioxidant actions is import in eliminating reactive oxygen species (ROS) and preventing oxidative stress, which is linked to the pathogenesis of diverse chronic diseases such as cancer, diabetes, or cardiovascular disorders. Studies on *H. longifolia* have shown its antioxidant activity as scavenging of free radical and reducing oxidative damage. To measure the plant's antioxidant capacity using various in vitro assays, DPPH (2,2-diphenyl-1-picrylhydrazyl) and ABTS (2,2′-azino-bis (3-ethylbenzothiazoline-6-sulfonic acid)) assays have been used. Results from these studies indicate that *H. longifolia* may be a natural source of antioxidants and could be valuable in avoiding oxidative diseases resulting from oxidation stress [4].

Drug resistant diseases have aroused great interest as a global health problem and the search for 'natural alternatives' to synthetic antibiotics is one of them. *H. longifolia* has been screened for its antimicrobial activity against different bacterial and fungal strains. However, extracts from the plant showed strong inhibitory effects against both Gram positive and Gram-negative bacteria and fungal species. It is believed that antimicrobial properties of the plant are due to its bioactive compounds present in the plant, i.e., flavonoids and alkaloids. *H. longifolia* can also be a promising natural solution to fight against microbial infections, especially due to increasing rate of antibiotic resistance [5].

Thrombolytic drugs are a well-established medical therapy for thromboembolic diseases, including stroke and myocardial infarction. In recent days, investigations have been conducted on the ability of *H. longifolia* to promote clot dissolution in vitro. Collectively, the plant's extracts have been demonstrated to promote fibrinolytic activity (the ability to break down blood clots) and to inhibit the formation of new clots. It is likely that this thrombolytic activity results, in part, by virtue of the presence of various bioactive compounds (including flavonoids and tannins) affecting the coagulation cascade. With an increasing need for natural thrombolytic agents, *H. longifolia* could be a potential resource for the development of new cardiovascular diseases therapies [6].

Researchers have gained interest in neuropharmacological investigations of *H. longifolia* on basis its possible role as a neuroprotective and cognitive enhancing agent. The plant's extracts have been shown to modulate neurotransmissions of the cholinergic, adrenergic and dopaminergic pathways. Importantly, these effects may be an avenue to its potential therapeutic application in neurodegenerative diseases e.g., Alzheimer's disease or Parkinson's disease. Additionally, the plant's role in maintaining mental health has been added as well, as it has anxiolytic and sedative properties. *H. longifolia* contains rich chemical composition, including alkaloids, flavonoids and other secondary metabolites, and the neuropharmacological properties associated with it are possibly due to these ingredients [7].

Finally, pharmacological activities of various nature have been described for *H. longifolia:* antioxidant, antimicrobial, thrombolytic and neuropharmacological. The rich phytochemical profile presents the potential of this species as a source of bioactive compounds for future development of novel therapeutic agents. It is necessary to further research these compounds and more fully understand the mechanisms of action for these compounds. For this study, the findings may pave the way to the use of *H. longifolia* for the modern medicine in the treatment of various diseases and health conditions [8].

Future aims of this research include further isolating and characterizing the bioactive compounds of *H. longifolia* to attempt to identify their specific mechanisms of action. The in vivo validations include studies in life, to confirm antioxidant, antimicrobial, thrombolytic and neuropharmacological activities noted in vitro. Furthermore, it will examine the possibility of synergy when combining bioactive compounds found in the plant for enhanced therapeutic efficacy. The study also evaluates the safety and toxicity profile of *H. longifolia* and evaluates its potential feasibility for clinical applications.

## 2. Materials and Methods

### *2.1. Chemicals and Reagents*

Analytical grade chemicals, including 2,2-diphenyl-1-picryl hydrazyl (DPPH) from Sigma, St. Louis, MO, USA, $AlCl_3$, NaOH, $Na_2HPO_4 \cdot 2H_2O$ and $NaH_2PO_4 \cdot 2H_2O$ from Loba, Mumbai, India, as well as $H_2O_2$ from Merck, Darmstadt, Germany, and phenazine methosulfate (PMS) from Sigma, USA, were utilized. All required chemicals were acquired from Department of Pharmacy, Dhaka International University, Bangladesh

### *2.2. Collection and Preparation of Plant Extract*

Plants of *H. longifolia* were collected from Bhola, Barisal, Bangladesh. The identification of the plant specimen was conducted by Khondokar Kamrul Islam, a senior scientific officer at the national Herbarium in Mirpur, Dhaka, Bangladesh and the authentication number was DACB-87893. With the exception of the root, the entire plant was collected, thoroughly cleaned with fresh water to get rid of any dirt, and then allowed to dry in the shade for a few days. Next, a grinding mill was used to break the plant into a coarse powder. To remove moisture, the plant's leaves sample were dried at room temperature. Then leaves were subjected to size reduction to get coarse powder was then stored in a clean dry airtight container. Next, in an amber glass container, 350 g of the dried-crushed materials were steeped in approximately 1150 mL of ethanol. For seven days, this mixture was periodically shaken and agitated before being filtered through a piece of fresh white cotton. Next, Whatman No. 1 filter paper was used to filter it once again. With constant air flow, the filtrate was evaporated and dried at room temperature. Then, the extract was stored in refrigerator (4 °C) for further use.

### *2.3. Animals*

Swiss albino mice of four to six weeks old with an average weight of twenty to twenty-five grams were bought from Jahangirnagar University, Bangladesh. Due to the need for acclimatization, the animals were housed in the pharmacology lab of the Pharmacy Department's animal house immediately after purchase for two to three weeks. This was done in Bangladesh in Dhaka International University. Every trial took place in an isolated, reduced acoustic, and reverberant environment. All animals used in this study were handled strictly following the ethical principles endorsed by the Department of Pharmacy, Dhaka International University, Satarkul, Badda, Dhaka-1212, Committee of Clinical Pharmacy & Pharmacology [Source: EC/009/CPP/DIU].

### *2.4. Phytochemical Screening*

Phytochemical Screening as described by Ghani [9]. A few positive control techniques were used to establish the chemical constituents of the plant extract. Chemical reagents used for the classification include Fehling's Solution and Benedict's solution for Reducing sugar, Mayer's and Dragendroff's reagents for alkaloids, saponins reagent for saponins reactivity test, glycoside reagent for glycosides, and steroid and $H_2SO_4$ for steroids, tannin with ferric chloride and potassium dichromate solution, and Molish's reagent Specifically, the chemical constituents of *H. longifolia* were classified and analyzed qualitatively through these methods [10].

### *2.5. 1,1-Diphenyl-2-Picrylhydrazyl-Free Radical Scavenging Activity (DPPH)*

Plant extract and ascorbic acid in ten test tubes with the following concentrations: 500, 250, 125, 62.5, 31.25, 15.625, 7.813, 3.906, 1.953, and 0.977 μg/mL. The plant extract and ascorbic acid were measured three times before being dissolved in ethanol to create these amounts. The positive control in this case was ascorbic acid. To create a 0.004% (*w*/*v*) solution, a precise amount of DPPH was weighed and dissolved in ethanol. A Sonicator was then used to mix the solution. The sonicator was sourced from Shenzhen Boda Ultrasonic Engineering Co. Ltd., headquartered in Shenzhen, Guangzhou, China. One milliliter of the different ascorbic acid and plant extract concentrations was added to each test tube. The 0.004% DPPH solution was then pipetted into each test tube in an amount of 3 mL. The flasks were then moved to incubator and kept at room temperature and in dark for 30 min in order to enhance the intensity of the reaction. Moreover, control test tubes, including only ethanol with DPPH, were also prepared. Subsequently, after this incubation, the absorbance of each test tube was measured at 517 nm using UV Spectro-photometer [11]. The percentage of inhibition was determined with the following formula: % inhibition = [(Blank absorbance − Sample absorbance)/Blank absorbance] × 100.

### *2.6. Ferric Reducing Antioxidant Power Assay*

To perform the FRAP assay, the same working reagent, standards, controls, and test samples are used; reagent and sample volumes are simply increased pro rata to give a volume large enough for manual handling/transfer of reaction mixtures. For example, 3.0 mL of working FRAP reagent is mixed with 100/mL. test sample or standard in a test tube; this is vortex mixed, and the absorbance at 593 nm is read against a reagent. Aqueous ascorbic acid solutions at 100, 250, 500, and 1000/mM (equivalent to 200, 500, 1000, and 2000/mM FRAP) prepared fresh daily and aged QC serum freshly spiked with ascorbic acid are recommended as quality control samples. These should be run in parallel with test samples to actively monitor the performance of the test and to ensure comparability with previous results. A standard solution or 0.2 mL of plant extract incorporate with 3 milliliters of FRAP reagent. For 30 min, reactions are incubated at 37 °C in an $H_2O$ bath. Measured the absorbance at 593 nanometers [12].

### *2.7. Determination of Antimicrobial Activity*

To evaluate the extract's antimicrobial activity disc diffusion method was used which was outlined by Fakruddin et al. [13]. Tests were performed on the extract against a range of eight gram-negative microorganisms, which included *Shigella boydii*, *Shigella dysenteriae*, *Pseudomonas aeruginosa*, *Salmonella paratyphi*, *Salmonella typhi*, *Vibrio parahaemolyticus*, *Vibrio mimicus*, and *Escherichia coli*. Additionally, there were eight gram-positive bacteria tested as well, *comprising Sarcina lutea*, *Bacillus cereus*, *Bacillus megaterium*, *Bacillus subtilis*, *Saccharomyces cerevisiae*, *Candida albicans*, *Aspergillus niger*, and *Staphylococcus aureus*. To prepare test plates, Mueller-Hinton agar medium was used. Five-millimeter filter paper discs (Whatman No. 1) were loaded with 25 and 50 micrograms per microliter of crude leaf extract. The discs were completely dry after that. The discs that had been imbued were incubated on sterilized agar plates for 24 h at a temperature of 37 °C, during which they were covered with 100 µL of the culture. For the positive control, discs containing ciprofloxacin (5 µg) were utilized. After the incubation period, diameter of the inhibition zone was measured in millimeters. A sterilized blank disc was used as a control reference.

### *2.8. Determination of Minimum Inhibitory Concentration (MIC)*

The agar dilution method was chosen to identify the MIC [14]. Sensitization was done in 10 cm experimental tubes containing SD broth for fungi and Muller Hilton broth for bacteria in different concentrations (20, 40, 80, 160, 320, 640, 1280, 2560 mg/mL). Nine milliliters of autoclave sterilized SDA for fungi and Muller Hilton for bacteria are put in each tube. To prepare the final concentrations of 2, 4, 8, 16, 32, 64, 128, 256 and 512 mg/mL, 1 mL of each extract concentrations was added on each tube after it had cooled. In laminar flow cabinet, the SDA/Muller Hilton and extract combination was aseptically placed into plates. Therefore, when the agar medium had solidified, 2 µL of the modified spore suspension was inoculated onto each plate by micropipette and plates were placed in incubators at temperature optimal for fungi, at 27 °C and bacteria at 35 °C. In order to control, the SDA and Muller Hilton had no addition of any herbal extract in their formulation. The MIC of the extract was describe after 12 days of incubation, as the lowest concentration of the extract at which no appreciable growth was observed as compared to the control.

### *2.9. Thrombolytic Activity*

This test was conducted in accordance with the procedure outlined by Prasad et al. [15]. Following the addition of 5 mL of sterile distilled water, the commercially available lyophilized streptokinase vial (1,500,000 IU) was thoroughly mixed. An adequate dilution was made from this suspension, which served as the stock solution. To create the clot, 5 mL of venous blood was drawn from 10 healthy volunteers who had never taken oral contraceptives or anticoagulants. The blood was then divided into 10 sterile micro centrifuge tubes, each 0.5 mL in size, and incubated for 45 min at 37 °C. Following this clot formation, the serum was extracted entirely without causing any disruption to the clot, and the weight of the clot was determined by weighing each tube containing the clot once more. Every micro centrifuge tube containing a pre-weighed clot received 100 µL of ethanol extract (10 mg/mL). To the designated control tube, 100 µL of streptokinase was added as a positive control, and 100 µL of distilled water was added as a negative control [16]. After 90 min of incubation at 37 °C, each tube was examined for clot lysis. Following incubation, the discharged fluid was withdrawn, and tubes were weighed once more to determine the weight difference following clot disruption. The percentage of clot lysis was determined by comparing the weight acquired before and after clot lysis.

### *2.10. Evaluation of Acute Toxicity*

All the animals were kept at overnight fasting before to the experiment with free access to water. Group I was administered orally normal saline solution (0.9% NaCl). The mice of this group were administered (0.01 × body weight) ml solution on 1st day. Groups II and III, IV were orally administered 300, 2000 and 5000 mg/kg body weight of the extract (dissolved in distilled water). The mice of these group were administered (0.01 × body weight) ml solution on 1st day. The animals were observed for any toxic effect for first 4 h after the treatment period. Further animals were investigated for a period of 3 days for any toxic effect [17]. Behavioral changes and other parameters such as body weight, urinations, food intake, temperature, changes in eye and skin colors were noted.

### *2.11. Evaluation of Anti-Inflammatory Activity*

Reducing inflammation potential of *H. longifolia* was also tested by Garrido et al. using formaldehyde induced paw edema method in mice [18]. The positive control group of four animal groups was administered with 100 mg/kg body weight of the routine drug ibuprofen. The doses of the plant material were 250 and 500 mg/kg body weight, given per oral route. Linear circumference of right hind paw was measured after 30 min by placing two slide calipers at the proximal and distal ends of paw. After that, the mice were injected with a 0.2% formaldehyde solution in to the right hind paw. Paw volume of the injected limb was measured with a plethysmometer at one, two, three, and four hours after the formaldehyde injection with paw size differences determined.

### *2.12. Neuropharmacological Test*

#### 2.12.1. Forced Swimming Test

This approach was described by Rd Bertin [19]. As a result of random sampling five groups of animals were selected for observation. Each mouse was acclimatized for 30 min, daily in an open cylindrical container measuring 45 cm long by 20 cm in diameter, with 17 cm deep water at 25 °C for five minutes. The mice were then administered with distilled water (0.1 mL/mouse, oral), extract in different concentrations (Low = 50 mg/kg/Kg, Medium = 100 mg/kg, High = 200 mg/kg), diazepam (1 mg/kg, intraperitoneally) or regular drug. Any mice which simply bobbed on the water or those that swam only slightly to keep their heads elevated were considered to be "still".

#### 2.12.2. Tail Suspension Test

This technique was named by (Steru Raymond; Thierry, Bernard; Simon, Pierre, 1985). All together five groups of mice were prepared. After 30-min test groups were administered oral doses of 50, 100 and 200 mg/kg of the extract. The control group was treated with distilled water (0.1 mL/mouse orally); the standard treatment was diazepam (1 mg/kg/case, IP). Sterile adhesive tape was applied one centimeter from the extremity of tails of mice and these were hanged at 50 cm height above the floor [20].

### *2.13. Statistical Analysis*

All experimental results were reported using the means ± standard errors of the means. Statistical significance was determined by one-way analysis of variance using Dunnett's test. Analysis of the data was done using Prism 6.0 (Graph Pad Software Inc., San Diego, CA, USA), and Microsoft Excel. The findings of the research were considered statistically significant if $p < 0.05$.

The least squares mean ± standard errors of the means were used to report all experimental results. To compare for significance, the ANOVA test was conducted at 0.5 level of significance using Dun net test. The results were analyzed statistically using computer basics soft wares that include, Prism 6.0 (Graph Pad Software Inc., San Diego, CA, USA) and Microsoft Excel. All statistical tests used employed significance level of 5% was used, that is the *p* value must be below 0.05.

## 3. Results and Discussion

### *3.1. Phytochemical Screening*

The phytochemical analysis of the *H. longifolia* extracts is presented in Table 1. The extracts contained reducing sugar, tannin, flavonoids, saponins, alkaloids, glycosides, terpenoids and acidic compounds.

Phytochemical screening of the prepared extracts was conducted to identify the presence of various chemical constituents with various qualitative tests. The tests were performed by different reagents for different chemicals were also used. These were identified by characteristic color changes using standard procedures [9]. Phytochemicals found in plant leaves may have a role in their therapeutic properties. The therapeutic effect of medicinal plants is attributed to their secondary metabolites, or phytochemicals, as well as other chemical elements, according to Varadarajan et al. [21] Saponins, for instance, are glycosides of steroids and triterpenes with hypotensive and cardiodepressant effects. Congestive heart failure and cardiac arrhythmia are treated with naturally cardioactive medications called cardiac glycosides [22]. Numerous studies suggested that different types of polyphenolic chemicals, such as tannins, flavonoids, and phenolic acids, have a wide range of biological effects, including antioxidant activity [23].

**Table 1.** Phytochemical activity of ethanol extract of *H. longifolia.*

| Phytochemicals Group | Result |
|---|---|
| Reducing sugar (Benedict's test) | − |
| Reducing sugar (Fehling's test) | + |
| Combined reducing sugar | − |
| Tannins (Ferric chloride test) | + |
| Tannins (Potassium dichromate test) | + |
| Flavonoids | + |
| Saponins | + |
| Gums | − |
| Steroids | − |
| Alkaloids | + |
| Glycoside | + |
| Xantho Proteins | − |
| Terpenoids | + |
| Acidic compounds | + |

Note: Present = +, Absent = −.

### *3.2. 1,1-Diphenyl-2-Picrylhydrazyl-Free Radical Scavenging Activity (DPPH)*

The DPPH free radical scavenging assay was conducted to assess the quantitative antioxidant activity, revealing that the $IC_{50}$ values for the ethanol crude extract of *H. longifolia* was measured at 517 nm. the $IC_{50}$ values for the ethanol crude extract of *H. longifolia* 0.877 µg/mL, while ascorbic acid, the standard reference, had a value of 0.839 µg/mL (Figure 1). *H. longifolia*, plant extract was evaluated for its capacity to neutralize free radicals by reacting with DPPH free radicals. The extract's effectiveness in scavenging DPPH was compared to that of ascorbic acid, which is recognized as a potent antioxidant. The ability to act as an antioxidant is a crucial pharmacological characteristic of plants. DPPH is commonly used to evaluate the free radical scavenging or antioxidant potential of plant extracts since it is easily neutralized by antioxidants [24]. This indicates that the crude extract has strong antioxidant properties compared to the standard. Additionally, the findings imply that the plant contains phytoconstituents that can donate hydrogen, offering protection to cells against potential harm.

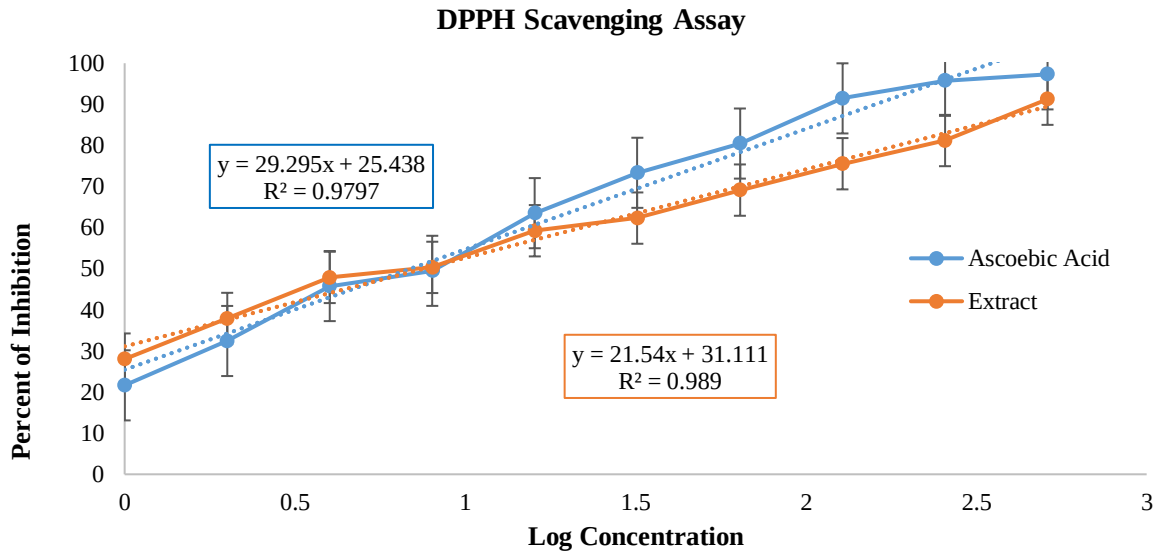



**Figure 1.** Comparison of absorbance vs. log concentration graph for ascorbic acid vs. *H. longifolia.*

### *3.3. Ferric Reducing Power Assay*

The $IC_{50}$ of ascorbic acid (standard) was found to be 12.763 µg/mL, while the $IC_{50}$ of *H. longifolia* (extract) was 43.736 µg/mL (Figure 2). Observations were conducted to determine the ferric ion reducing activities of extracts at different concentrations. The results showed that extract has a high ferric ion reducing activity compared to the standard Ascorbic acid. This suggests that the extract has the ability to donate hydrogen. During the FRAP assay, a deep blue color was observed, which occurs due to a non-specific reaction that converts the ferric tripyridyl

triazine (Fe III TPTZ) complex to the ferrous form. This assay is commonly used for determining hydrophilic antioxidants [25].

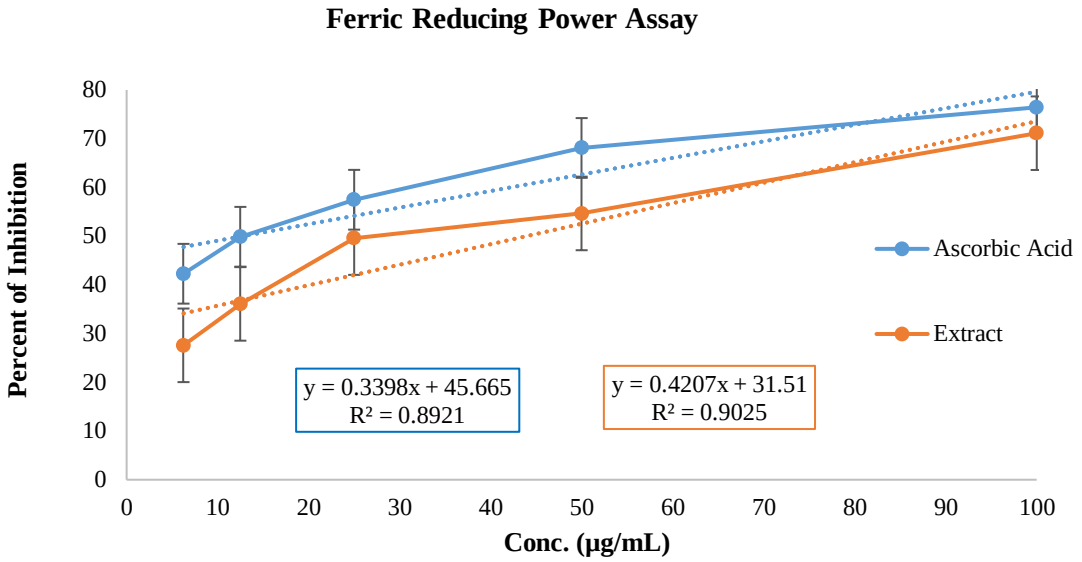



**Figure 2.** Comparison of percent inhibition vs. concentration graph for ascorbic acid and extract.

### *3.4. Determination of Antimicrobial Activity*

*H. longifolia* shown antimicrobial efficacy against both gram-positive and gram-negative by utilizing the disk diffusion method (*Bacillus subtilis, Shigella dysenteriae, Pseudomonas aureus, and Sarcina lutea*) etc. in disk diffusion assay.

The results showed in that the extracts from *H. longifolia* inhibited the growth of those microbes with 19-mm zone of inhibition and an amount of extract employed of 500 µg/disc, *Escherichia coli*, a gram-negative bacterium, had the highest inhibition rate. *Aspergillus niger* a gram-positive bacterium, has the lowest inhibition rate; the zone of inhibition is just 6 mm, and 250 µg/disc of extract is utilized (Figure 3).

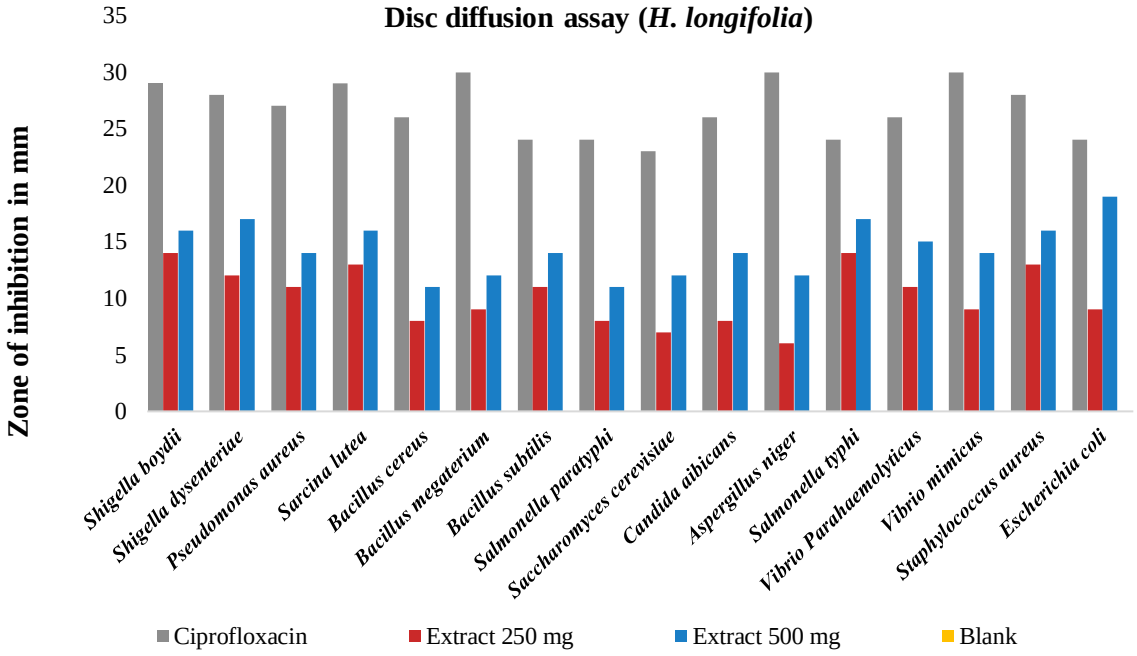



**Figure 3.** In vitro antibacterial activity of ethanolic extract of *H. longifolia* by disc diffusion assay.

### *3.5. Evaluation Minimum Inhibitory Concentration (MIC)*

Table 2 displays the MIC values of the ethanolic extract of *H. longifolia* leaves, which show differing degrees of efficacy against various bacteria. With a MIC of 4 mg/mL, E. coli showed the maximum sensitivity. The lowest

sensitivity was shown by the MIC of 128 mg/mL for *B. cereus*, *B. megaterium*, and *A. niger*. The MIC for *Salmonella typhi* was 8 mg/mL, and the MIC for *V. parahaemolyticus* was 16 mg/mL. These results imply that *H. longifolia* leaf ethanolic extract is least effective against *B. cereus*, *B. megaterium*, and *A. niger* and most effective against *E. coli*.

Gram-positive bacteria have more mucopeptide in their cell wall composition than gram-negative bacteria, which have a thin layer of mucopeptide and a cell structure primarily composed of lipoprotein and lipopolysaccharides. This is because the two types of bacteria differ in their cell structure. Gram-negative bacteria have more resistance as a result [26]. As Kotzekidou et al. [27] hypothesized, this data add credence to the notion that antimicrobial substances from plant extracts alter bacterial membrane permeability and consequently release intracellular biomolecules. Cell wall and membrane disruption is highlighted in many investigations as a way through which bacterial survival is compromised due to disruptions in electron transport, enzyme activity in food uptake.

**Table 2.** Minimum inhibitory concentration (MIC) of *H. longifolia* extract.

| Microorganisms | Concentration mg/mL | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | **512** | **256** | **128** | **64** | **32** | **16** | **8** | **4** | **2** | **Control** |
| *Shigella boydii* | + | + | + | + | + | − | − | − | − | − |
| *Shigella dysenteriae* | + | + | + | + | + | − | − | − | − | − |
| *Pseudomonas aureus* | + | + | + | + | − | − | − | − | − | − |
| *Sarcina lutea* | + | + | + | + | + | − | − | − | − | − |
| *Bacillus cereus* | + | + | + | − | − | − | − | − | − | − |
| *Bacillus megaterium* | + | + | + | − | − | − | − | − | − | − |
| *Bacillus subtilis* | + | + | + | + | + | − | − | − | − | − |
| *Salmonella paratyphi* | + | + | + | + | − | − | − | − | − | − |
| *Saccharomyces cerevisiae* | + | + | + | + | − | − | − | − | − | − |
| *Candida aibicans* | + | + | + | + | − | − | − | − | − | − |
| *Aspergillus niger* | + | + | + | − | − | − | − | − | − | − |
| *Salmonella typhi* | + | + | + | + | + | + | + | − | − | − |
| *Vibrio Parahaemolyticus* | + | + | + | + | + | + | − | − | − | − |
| *Vibrio mimicus* | + | + | + | + | + | − | − | − | − | − |
| *Staphylococcus aureus* | + | + | + | + | + | + | − | − | − | − |
| *Escherichia coli* | + | + | + | + | + | + | + | + | − | − |

(+): Positive inhibition; (−): Negative inhibition.

### *3.6. Thrombolytic Activity*

In Figure 4, clot lysis was demonstrated by 37.42% in the ethanolic extracts and 64.17% by streptokinase. Distilled water, on the other hand, demonstrated very little clot lysis (3.05%). There was a significant difference ($p$ = 0.0013) in the mean clot lysis percentage between the extract and negative control.

Generally, thrombin forms blood clots from fibrinogen. Medication classified as thrombolytic or antithrombotic can obstruct the thrombus formation process. Plasmin, which can be activated by activators from inactive plasminogen, is the primary mechanism via which thrombolytic therapy breaks down fibrin [28]. Staphylokinase and streptokinase, two cofactor molecules involved in bacterial plasminogen activator, help generate exosite and improve the enzyme's ability to provide substrate to the enzyme. In addition to destroying the extracellular matrix (ECM) and fibrin fibers that keep cells together, staphylokinase stimulates plasminogen to dissolve clots. When the clots were treated with aqueous and ethanol extract, a considerable thrombolytic activity was seen when compared to this positive and negative control. However, the results of the *H. longifolia* ethanol extract indicates less potential to lyse the clot. The extract from *H. longifolia* may have thrombolytic activity (Clot lysis) due to the individual compounds or to the combined action of all the active compounds present.

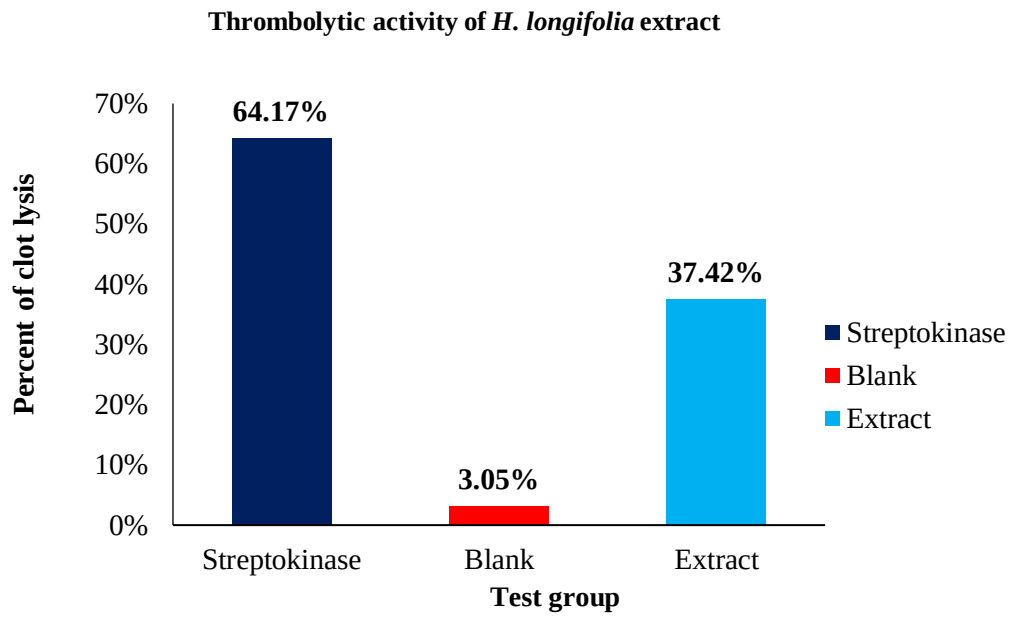



**Figure 4.** Percent of clot lysis of ethanol extracts of *H. longifolia.*

### *3.7. Evaluation of Acute Toxicity*

In this acute toxicity investigation, the extract of *H. longifolia* leaves had no effects on mice's appearance or behavior, nor did it show any indications of toxicity or mortality. By the end of the experiment, every rat was in good health and continued to exhibit its typical behaviors and gait. Given the assumption that *H. longifolia* is a safe medical plant with no harmful side effects, its $LD_{50}$ was calculated to be greater than 5000 mg/kg b.wt (Figure 5 and Table 3).

**Table 3.** Effects of ethanolic extract of *H. longifolia* leaves on body weight of mice.

| Group | Avg. Weight ± SEM | Avg. Weight ± SEM |
|---|---|---|
| Group-I (control) | 26.6 ± 1.76 | 28.3 ± 1.82 |
| Group-II (300 mg/kg) | 18.2 ± 1.67 | 21.2 ± 1.56 |
| Group-III (2000 mg/kg) | 15.8 ± 0.92 | 21.8 ± 0.82 |
| Group-IV (5000 mg/kg) | 22 ± 1.48 | 24.45 ± 1.51 |

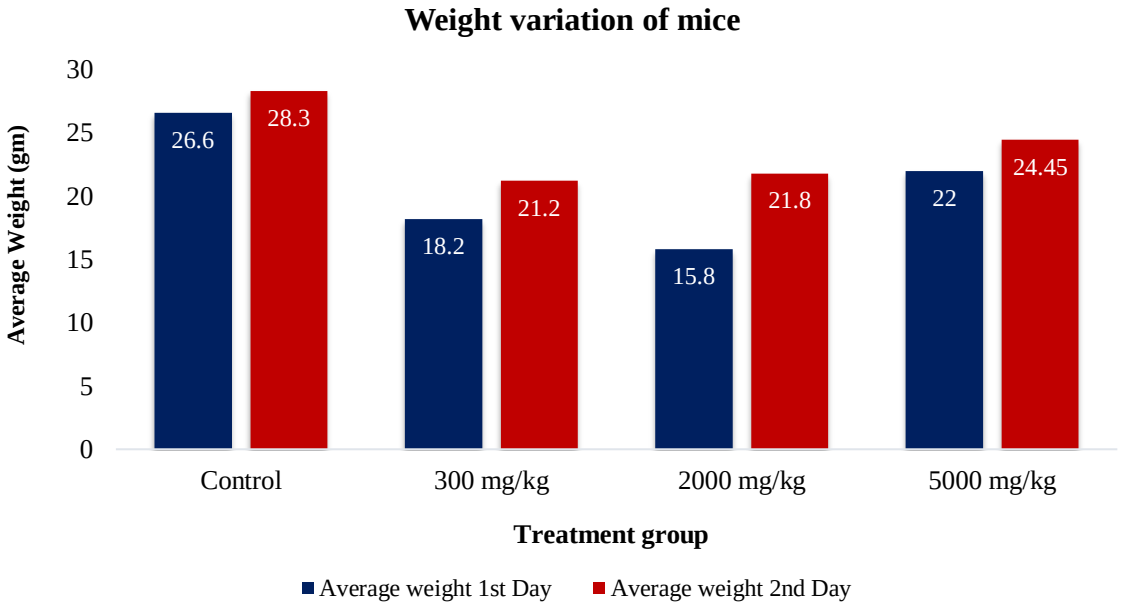



**Figure 5.** Effects of ethanolic extract of *H. longifolia* leaves on body weight of mice.

### *3.8. Anti-Inflammatory Activity*

Inflammation, pain and pyrexia produce similar compulsive environments in our body [29]. So, following the evaluation of the analgesic activity of the plant, we have tested its anti-inflammatory potential. In the anti-inflammatory test, the *H. longifolia* extract showed significant inhibition in the paw edema in mice within the observation period at both doses of 250 mg/kg and 500 mg/kg b.wt. The values are shown in (Figure 6). This tissue paw thickness measurements revealed that the animals have shown dose- dependent anti-inflammatory effects. The mice of the ibuprofen group (100 mg/kg) exhibited the highest decrease in paw thickness that reduce by 0.56 ± 0.110 mm after 4 h and the inhibition percentage was 71.71%. The extract at 250 mg/kg had the III% of inflammation reduced at 54.22% on 4 h whiles the 500 mg/kg had more marked activity with III% of inflammation reduced at 60.27% after 4 h of administration. Substantial inhibition of paw edema demonstrated by the extract can be due to the presence of anti-inflammatory compounds like 4H-Pyran-4–one, catechin, gallocatechin, etc., [30]. The study also justifies the use of plant parts as an anti-inflammatory agent in folklore medicine [30].

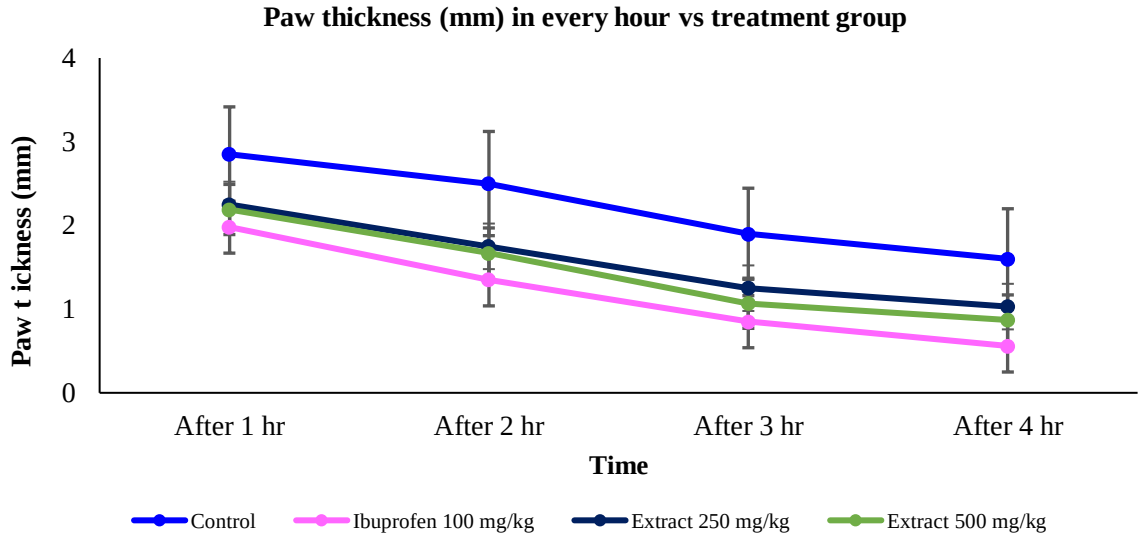



**Figure 6.** Paw thickness (mm) in every hour for negative control, positive control and *H. longifolia* extract.

### *3.9. Neuropharmacological Activity*

#### 3.9.1. Forced Swimming Test

*H. longifolia* extract significantly increased immobility times when given orally at dosages of 50, 100, and 200 mg/kg in comparison to the control group ($p < 0.05$). This finding indicates that the extract may have a major effect on immobility at the dosages of 50, 100, and 200 mg/kg it showed the immobility time significantly 132.4, 112.2 and 126.2 s respectively while standard showed 180.2 s. Further investigation is required to determine any possible therapeutic advantages. Similarly, as expected, a significant increase in immobility durations ($p < 0.05$) was also observed with the usual medication diazepam (1 mg/kg, intraperitoneal). These findings highlight how crucial it is to carry out more studies and create efficient remedies for this illness (Figure 7).

The tail suspension test is one of the widely used tests for appraisal of the antidepressant-like effect in medicine [31] noting that based on the duration of immobility time, this model has CNS depression-like evidence which is manifested as an extended duration of immobility while a short duration of immobility in this model has antidepressant effect. Nevertheless, in the forced swimming test, CNS depressive effect was observed. The experiment was also generalized to know whether this tail suspension test could be employed to provide a fast and sensitive method for establishment of the psychotropic nature of the substance. Consequently, the energy provided by mice to escape from suspension forms the measuring principle in total. During this test, the mice's movements were observed in concern with the energy and certain power that they built gradually. It significantly increased the extent of the mice's motor rest period, which has indicated CNS depressive effect of the extract.

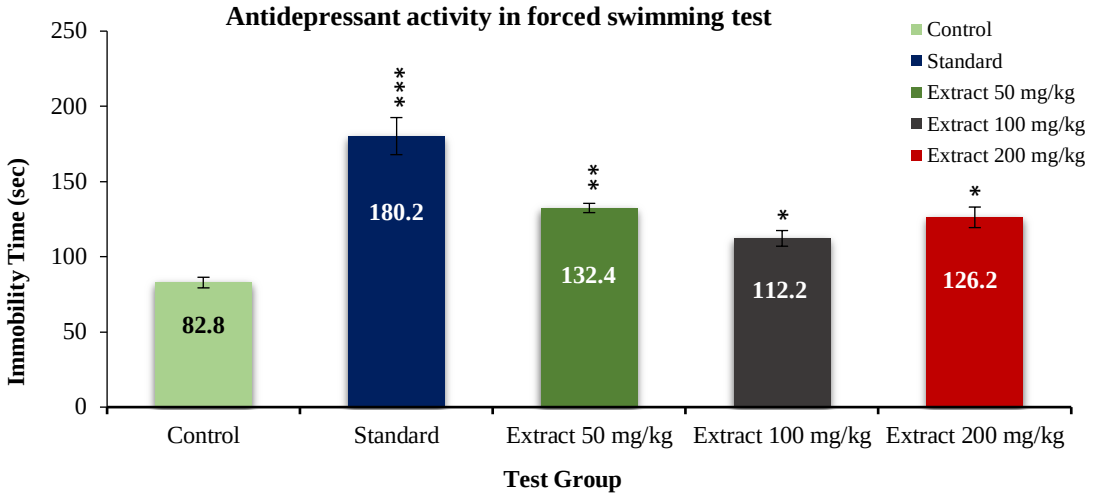



**Figure 7.** Effects of extract administration on the immobility time in the Forced Swim Test *H. longifolia* extract and Diazepam. Significance: *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$.

3.9.2. Tail-Suspension Test

Comparing the results of the test sessions with the control group the extract at the doses of 50, 100 and 200 mg/kg increased the immobility times significantly ($p < 0.05$). The result of the extract on behavior in this test is presented in the (Figure 8) below. This finding indicates that the extract has a major effect on immobility at the dosages of 50, 100, and 200 mg/kg it showed the immobility time significantly 113.2, 128.4 and 145.8 s respectively while standard showed 152.6 s. In the present study similar to that of the control group after diazepam (1 mg/kg, i.p), the duration of immobility time was significantly increased ($p < 0.05$).

In order to evaluate whether animal models exhibit antidepressant-like behavior, the forced swimming test is frequently employed. In this concept, a reduction in immobility time signifies antidepressant action, whereas a longer immobility time represents a CNS depression-like impact [31]. However, in the forced swimming test, a CNS depressive effect was noted. The experiment was also expanded to see if the tail suspension test might be used as a quick and accurate way to determine whether a substance has psychotropic qualities. In essence, the energy generated by mice attempting to break free of their suspension serves as the basis for the measuring principle. The mice's motions throughout this test were examined in terms of the energy and power that they gradually developed. The extract dramatically lengthened the mice's immobility period, suggesting that it had CNS depressive effects. In a mouse model, the common medication diazepam also showed CNS depressive effects. It has been suggested that the tail suspension test has higher pharmacological sensitivity and is less taxing than the force swimming test [32,33].

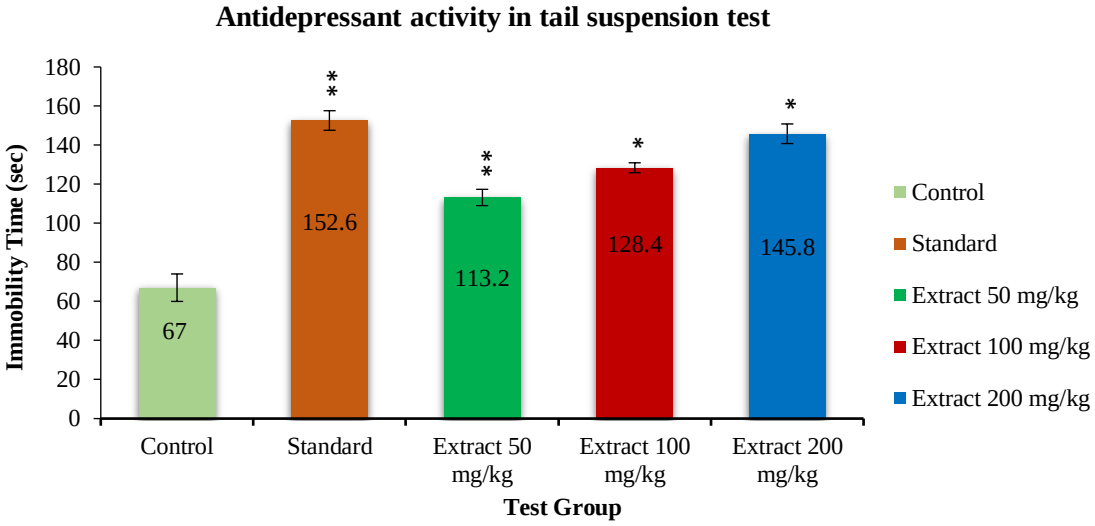



**Figure 8.** Effects of ethanolic leaves extract administration on the immobility time in the Tail suspension Test. *H. longifolia* Extract and Diazepam. Significance: *$p < 0.05$, ** $p < 0.01$.

## 4. Conclusions

The therapeutic value of plant extracts is directly related to phytochemical compositions they contain. Several bioactive components present in *H. longifolia* include flavonoids, glycosides, alkaloids, terpenoids, acidic compounds, tannins etc. The extract proved more efficient in DPPH and FRAP scavenging than ascorbic acid because its $IC_{50}$ value. The bioactive substances present in the plant form a strong basis for examining the pharmacological prospects of *H. longifolia* especially in its antimicrobial properties. In the antimicrobial assessment *E. coli* demonstrated maximum susceptibility to the antibacterial effect and it displayed the highest susceptibility to the microorganism while, *B. megaterium* demonstrated minimal inhibitory capacity. The thrombolytic test revealed that extract has ability to clot lysis. The extract exhibited no toxicity and behavioral changes in addition to no recorded fatalities. Among the treatment groups the inhibitory effect on inflammation reached 54.22% with 250 mg/kg and 60.27% with 500 mg/kg at the four-hour mark. The tail suspension and forced swimming tests demonstrated that the extract showed psychotropic features in addition CNS depressive properties. Future research will employ modern technology to identify and analyze the bio-compounds responsible for the evaluation of drug mechanisms and performance through in silico methodology will establish their potential use in medical drug development to address multiple healthcare conditions.

**Author Contributions:** M.K.I., M.M. and M.S.M. carried out writing the manuscript, literature review and data analysis. M.M., M.S.M. and M.K.I., helped in manuscript writing and data analysis. M.S.M. and M.M. helped in conceptualization, writing, review, and editing the manuscript. M.M. supervised the project. All authors have read and agreed to the published version of the manuscript.

**Funding:** There was no external funding provided for the project.

**Institutional Review Board Statement:** This research project has received ethical approval from the Ethical Committee of the Department of Pharmacy, Dhaka International University, Dhaka 1212, Bangladesh according to their ethical guideline (Ref No: CPP/DIU/EC/008).

**Informed Consent Statement:** Not applicable.

**Data Availability Statement:** Not applicable.

**Acknowledgments:** We are very grateful to the Department of Pharmacy, Dhaka International University, Bangladesh for providing the laboratory facilities to carry out the research.

**Conflicts of Interest:** The authors declare no conflict of interest.

## References

1 Biswas, S.R.; Misbahuzzaman, K. Tree species diversity and regeneration traits of the dominant species in a dipterocarp forest in Bangladesh: Implications for conservation. *Int. J. Biodivers. Sci. Manag.* **2008**, *4*, 81–91.

2 Singh, D.; Sharma, R.; Kumar, V. Phytochemical and pharmacological investigations of *Holigarna longifolia*: A review. *J. Med. Plants Res.* **2017**, *11*, 421–432.

3 Rani, M.; Kumar, S.; Yadav, S. Phytochemical constituents and therapeutic potentials of *Holigarna longifolia*. *Phytomedicine J.* **2020**, *29*, 1–10.

4 Patel, P.; Desai, S.; Mehta, M. Evaluation of antioxidant activity *of Holigarna longifolia*. *Asian J. Pharm. Clin. Res.* **2018**, *11*, 15–19.

5 Kumar, P.; Yadav, R.; Mishra, P. Antimicrobial activity of *Holigarna longifolia* against clinical isolates. *Int. J. Drug Dev. Res.* **2019**, *11*, 45–50.

6 Verma, P.; Meena, S.; Saini, N. Thrombolytic activity of *Holigarna longifolia* and its potential as an anticoagulant agent. *Thromb. Res.* **2021**, *198*, 35–42.

7 Das, S.; Gupta, A.; Sharma, P. Neuropharmacological effects *of Holigarna longifolia*: A review. *Neuropharmacol. Res.* **2022**, *52*, 12–18.

8 Soni, S.; Choudhury, M.; Gupta, R. Therapeutic potential of *Holigarna longifolia*: Antioxidant, antimicrobial, thrombolytic, and neuropharmacological activities. *J. Herb. Med.* **2023**, *45*, 107–116.

9 Ghani, A.; Ahmad, S.B. Determination and identification of phytochemical properties of medicago sativa l.(alfalfa) leaf, stem and root extracts against various pathogens. *Pak. J. Phytopathol.* **2019**, *31*, 97–103.

10 Baliyan, S.; Mukherjee, R.; Priyadarshini, A.; et al. Determination of Antioxidants by DPPH Radical Scavenging Activity and Quantitative Phytochemical Analysis of Ficus religiosa. *Molecules* **2022**, *27*, 1326.

11 Govindarajan, R.; Rastogi, S.; Vijayakumar, M.; et al. Studies on the Antioxidant Activities of *Desmodium gangeticum*. *Biol. Pharm. Bull.* **2003**, *26*, 1424–1427.

12 Benzie, I.F.F.; Strain, J.J. Ferric reducing/antioxidant power assay: Direct measure of total antioxidant activity of biological fluids and modified version for simultaneous measurement of total antioxidant power and ascorbic acid concentration. *Methods Enzymol.* **1999**, *299*, 15–27.

13 Fakruddin, M.; Mannan, K.S.B.; Mazumdar, R.M.; et al. Antibacterial, antifungal and antioxidant activities of the ethanol extract of the stem bark of *Clausena heptaphylla*. *BMC Complement. Altern. Med.* **2012**, *12*, 1–9.

14 Behbahani, B.A.; Shahidi, F.; Yazdi, F.T.; et al. Antifungal effect of aqueous and ethanolic mangrove plant extract on pathogenic fungus "in vitro". *Int. J. Agron. Plant Prod.* **2013**, *4*, 1652–1658.

15 Prasad, S.; Kashyap, R.S.; Deopujari, J.Y.; et al. Development of an in vitro model to study clot lysis activity of thrombolytic drugs. *Thromb. J.* **2006**, *4*, 14.

16 Watson, L.; Broderick, C.; Armon, M.P. Thrombolysis for acute deep vein thrombosis. *Cochrane Database Syst. Rev.* **2016**, *11*, 1465–1858.

17 Manaharan, T.; Chakravarthi, S.; Radhakrishnan, A.K.; et al. In vivo toxicity evaluation of a standardized extract of *Syzygium aqueum* leaf. *Toxicol. Rep.* **2014**, *1*, 718–725.

18 Garrido, G.; González, D.; Delporte, C.; et al. Analgesic and anti-inflammatory effects of *Mangifera indica* L. extract (Vimang). *Phytother.Res. Int. J. Devoted Pharmacol. Toxicol. Eval. Nat. Prod. Deriv.* **2001**, *15*, 18–21.

19 Rd Bertin, M.; Jalfre, P.A. Behavioral despair in mice: A primary screening test for antidepressants. *Arch. Int. Pharmacodyn. Ther.* **1977**, *229*, 327–336.

20 Steru, L.; Chermat, R.; Thierry, B.; et al. The tail suspension test: A new method for screening antidepressants in mice. *Psychopharmacology* **1985**, *85*, 367–370.

21 Varadharajan, R.; Palani, S. Phytochemical screening, in-vitro antioxidant and cardioprotective potential of monochoria vaginalis on doxorubicin induced cardiotoxicity. *J. Pharm. Biol. Sci.* **2017**, *5*, 56.

22 Agbafor, K.N.; Nwachukwu, N. Phytochemical Analysis and Antioxidant Property of Leaf Extracts of *Vitex doniana* and *Mucuna pruriens*. *Biochem. Res. Int.* **2011**, *2011*, 459839.

23 Prakash, O.; Kumar, R.; Mishra, A.; et al. *Artocarpus heterophyllus* (Jackfruit): An overview. *Pharmacogn. Rev.* **2009**, *3*, 353.

24 Hossain, A.; Hossain, A.; Mannan, S.J. Evaluation of antioxidant and analgesic activities of three medicinal plants. *Pharmacogn. Res.* **2019**, *11*, 248–253.

25 Mukherjee, M.; Bhaskaran, N.; Srinath, R.; et al. Anti-ulcer and antioxidant activity of GutGard™. *Indian J. Exp. Biol.* **2010**, *48*, 269–274.

26 Ghalem, B.R.; Mohamed, B. Antibacterial activity of leaf essential oils of Eucalyptus globulus and *Eucalyptus camaldulensis*. *Afr. J. Pharm. Pharmacol.* **2008**, *2*, 211–215.

27 Kotzekidou, P.; Giannakidis, P.; Boulamatsis, A. Antimicrobial activity of some plant extracts and essential oils against foodborne pathogens in vitro and on the fate of inoculated pathogens in chocolate. *LWT—Food Sci. Technol.* **2008**, *41*, 119–127.

28 Yuan, J.; Yang, J.; Zhuang, Z.; et al. Thrombolytic effects of *Douchi* Fibrinolytic enzyme from *Bacillus subtilis* LD-8547 in vitro and in vivo. *BMC Biotechnol.* **2012**, *12*, 36.

29 Afsar, T.; Khan, M.R.; Razak, S.; et al. Antipyretic, anti-inflammatory and analgesic activity of Acacia hydaspica R. Parker and its phytochemical analysis. *BMC Complement. Altern. Med.* **2015**, *15*, 1–12.

30 Powar, P.; Gaikwad, D. GC-MS Analysis of bioactive compounds of *Aegiceras corniculatum* bark. *Indian J. Plant Sci.* **2016**, *5*, 13–17.

31 Roome, T.; Razzak, A.; Ali, P.; et al. Therapeutic effect of *Aegiceras corniculatum* in chronic granulomatous inflammation and arthritis. *J. Dow Univ. Health Sci.* **2014**, *8*, 98–103.

32 Subarnas, A.; Tadano, T.; Nakahata, N.; et al. A possible mechanism of antidepresant activity of beta-amyrin palmitate isolated from *lobelia inflata* leaves in the forced swimming test. *Life Sci.* **1993**, *52*, 289–296.

33 Dhingra, D.; Sharma, A. Evaluation of antidepressant-like activity of glycyrrhizin in mice. *Indian J. Pharmacol.* **2005**, *37*, 390–394.

*Review*

# Ethnopharmacological and Medicinal Properties of *Allophylus dimorphus* Radlk.: An Update

**Azazahemad A. Kureshi [1,*], Emran Habibi [2,3], Satyajit D. Sarker [3] and Lutfun Nahar [4,*]**

[1] Analytical Development and Innovation Center, Pharmanza Herbal Pvt. Ltd., Anand 388430, Gujarat, India
[2] Medicinal Plants Research Centre, Institute of Herbal Medicines and Metabolic Disorders, Mazandaran University of Medical Sciences, Sari 48157-33971, Iran
[3] Centre for Natural Products Discovery (CNPD), School of Pharmacy and Biomolecular Sciences, Liverpool John Moores University, James Parsons Building, Byrom Street, Liverpool L3 3AF, UK
[4] Laboratory of Growth Regulators, Palacký University and Institute of Experimental Botany, The Czech Academy of Sciences, Šlechtitelů 27, 78371 Olomouc, Czech Republic
[*] Correspondence: kureshi.azaz90@gmail.com (A.A.K.); nahar@ueb.cas.cz (L.N.)
Received: 2 February 2025; Revised: 7 March 2025; Accepted: 10 March 2025; Published: 12 March 2025

**Abstract:** *Allophylus dimorphus* Radlk., a member of the Sapindaceae family, is a shrub or tree primarily found in the Nicobar Islands, Malaysia, and the Philippines. Despite its potential relevance in traditional medicine, research on the ethnopharmacological and medicinal properties of this species is currently limited. However, a few closely related species, such as *Allophylus cobbe* and *Allophylus serratus*, have been studied for their bioactive compounds with antioxidant, antimicrobial, and anti-inflammatory properties. The World Health Organization (WHO) recognizes the significance of traditional medicine, which remains a vital healthcare source for many communities worldwide. The rapid loss of plant diversity and the potential for discovering novel medicinal compounds highlight the necessity of documenting and investigating *A. dimorphus* and related species. This update consolidates existing knowledge on the phytochemistry, ethnomedicinal uses, and pharmacological properties of *A. dimorphus*, providing a foundation for future research and conservation efforts.

**Keywords:** *Allophylus dimorphus*; ethnopharmacology; medicinal properties; phytochemicals; pharmacological activity

## 1. Introduction

Nature has been an excellent source of therapeutic agents for thousands of years, and several modern drugs have originated from natural sources, many based on their use in traditional medicine [1]. Medicinal plants have served as an essential source of therapeutic agents for centuries, with approximately 80% of the global population relying on them for disease prevention and treatment [2]. Over 40% of pharmaceutical formulations are derived from natural sources, underscoring their significant role in drug development [3]. The Sapindaceae family, comprising over 1000 species, includes *Allophylus dimorphus*, a plant with potential medicinal value that remains underexplored [4,5].

The importance of medicinal plants in traditional healthcare systems cannot be overstated, particularly in regions where access to modern healthcare is limited. Indigenous communities have utilized plant-based treatments for generations, and many of these traditional remedies are now being scientifically validated [5]. The growing interest in ethnomedicine is driven by the increasing need for novel bioactive compounds that can serve as alternatives to synthetic pharmaceuticals, which often come with adverse side effects [1]. The *Allophylus* L. genus has been extensively used in traditional medicine in various cultures, with *Allophylus cobbe* (L.) Forsyth fil. and *Allophylus serratus* (Roxb.) Kurz. showing promising pharmacological activities, including antioxidant, antimicrobial, and anti-inflammatory properties [6,7].

*Allophylus dimorphus* remains relatively unexplored in terms of its chemical composition and medicinal potential, despite its similarity to well-studied species, *A. cobbe* and *A. serratus*. This lack of research is concerning, given that other members of the genus have been shown to contain phytochemicals with therapeutic benefits. Moreover, the loss of biodiversity due to habitat destruction, climate change, and unsustainable harvesting practices poses a threat to the availability of medicinal plants, making conservation efforts imperative [8].

The study of *A. dimorphus* is particularly significant in the context of sustainable medicine, as it could contribute to the development of natural drug alternatives while supporting ecological conservation. This review highlights the need for further research into this species to validate its traditional uses and explore its potential applications in modern medicine. By synthesizing available data and identifying research gaps, this work aims to encourage future studies on *A. dimorphus*, ensuring that its medicinal potential is fully realized while promoting conservation efforts to protect this valuable plant species. In addition, it provides insights into potential research directions, facilitating comprehensive studies that could lead to novel drug discoveries and the sustainable utilization of this species. Therefore, the primary objective of this update is to consolidate and analyze existing knowledge on *A. dimorphus*, covering its botanical characteristics, phytochemical composition, ethnomedicinal applications, pharmacological activities, and conservation status, while also proposing future research directions.

## 2. Methods

A systematic approach was adopted to gather information on *Allophylus dimorphus* Radlk. Relevant data were collected from various scientific databases using specific search keywords, including "*Allophylus dimorphus* Radlk". + medicinal properties", "*Allophylus dimorphus* Radlk. Radlk. + biological activities", "*Allophylus dimorphus* Radlk. + phytochemicals", and "*Allophylus dimorphus* Radlk. + ethnomedicinal uses". The search focused on compiling insights into the botanical description, ethnobotanical significance, phytochemical composition, and pharmacological properties of the species. Taxonomic classification was verified using authoritative botanical references, and relevant studies were critically analyzed to identify gaps in existing knowledge and potential areas for future research. A schematic representation (Figure 1) was developed to summarize the findings and guide the update. Moreover, the study emphasized the necessity of exploring *A. dimorphus* for its therapeutic potential, given its status as a vulnerable species and the scarcity of scientific reports. This methodological framework ensures a comprehensive and structured analysis of the available literature, contributing to a deeper understanding of this underexplored species.


**Figure 1.** Schematic representation for a review on *Allophylus dimorphus* Radlk.

## 3. Botanical and Morphological Characteristics

### *3.1. Botanical Characteristics*

*Allophylus dimorphus* is a small to medium-sized tree or shrub that thrives in humid tropical climates, particularly in the Nicobar Islands, Bangladesh, China, Malaysia, Thailand, and the Philippines [9–11]. *Allophylus dimorphus* is found in coastal forests, mangrove ecosystems, and tropical evergreen forests, where it plays a vital role in maintaining ecological stability by preventing soil erosion and supporting biodiversity [11]. It is a medium-sized shrub or tree typically ranging from 2 to 4 m in height, characterized by slender, few-branched stems that are green and pubescent. The taxonomy and classification of *A. dimorphus* is as follows: Kingdom: Plantae; Phylum: Tracheophyta; Class: Magnoliopsida; Order: Sapindales; Family: Sapindaceae; Genus: *Allophylus* L.; Species: *Allophylus dimorphus* Radlk. [11–13].

### *3.2. Morphological Characteristics*

Morphologically, *A. dimorphus* is characterized by pinnate leaves that are alternately arranged, with elliptical to oblong leaflets possessing serrated margins [12]. The leaflets have petiolules of 1–1.5 cm, with the outermost one being nearly sessile. The blades are papery and display tufts of hairs in the lateral vein axils, with 9 to 10 prominent lateral veins on both sides [12,13]. The terminal leaf blade is broadly lanceolate or narrowly elliptic, measuring 8–18 × 3–6.5 cm, with a cuneate base, sharply serrulate margins above the middle, and a caudate-acuminate apex (Figure 2) [14].

**Figure 2.** The specimen diagram of *Allophylus dimorphus* leaves [12] and its flower [14].

The plant produces small, greenish-white flowers that are borne in panicles (Figure 2), which later develop into bright red, fleshy berries that serve as a food source for birds and small mammals [14]. The species exhibits high adaptability to various soil conditions and is often found thriving in nutrient-poor sandy or lateritic soils, making it a resilient species in disturbed habitats.

## 4. Geographical and Habitat Distribution

Geographically, *A. dimorphus* is primarily found in the tropical belts of Southeast Asia, extending beyond Bangladesh and India to other regions within its native range. This species thrives in tropical forests, particularly in the Sylhet and Chittagong regions of Bangladesh and the Sundarbans of West Bengal, India [11]. Locally known as "Si Sa Calaai" or "Kro Kaya Dung" among the Marma people, *A. dimorphus* adapts well to diverse soil types, contributing to tropical forest biodiversity. Its fruits serve as a food source for birds and animals, facilitating seed dispersal [11,12]. However, habitat destruction poses a significant threat, highlighting the need for conservation efforts. A detailed geographical map provides comprehensive information on the habitats where *Allophylus* species occur (Figure 3) [8,12]. The insights from the mapping resources enhance the understanding of the ecological context in which *A. dimorphus* exists and emphasize the urgency of conservation strategies to preserve this vulnerable species and its habitat [13]. Access to such geographical data is crucial for guiding future research initiatives and conservation efforts aimed at protecting biodiversity.

**Figure 3.** A detailed geographical map of *Allophylus* species [13].

Several members of the *Allophylus* genus, including *A. cobbe* and *A. serratus*, have been studied for their medicinal potential due to their similar morphological characteristics and phytochemical compositions [6,7,15]. The documented ethnomedicinal uses of these related species suggest that *A. dimorphus* may also possess bioactive compounds with therapeutic properties [15]. Future studies should prioritize mapping the full distribution range of *A. dimorphus*, assessing its ecological roles, and evaluating the impact of environmental changes on its populations. The synthesis of a variety of phytochemicals is greatly affected by several factors including genetics, climate, and soil [16]. In the past few decades, rising global temperatures, changes in rainfall patterns, increased carbon dioxide ($CO_2$) levels, and frequent extreme weather events have altered the growth, distribution, and levels of phytochemicals in medicinal plants. These changes have implications for global health [17]. Moreover, sustainable harvesting practices and habitat restoration initiatives must be implemented to prevent further decline of this valuable species [10].

## 5. Ethnobotanical and Traditional Uses

The ethnobotanical uses of *A. dimorphus* are deeply rooted in local traditions and practices. Among the Gor tribe in northeastern Bangladesh, the fruits of *A. dimorphus*, when combined with the crushed tubers of *Alocasia macrorrhizos* (Giant taro) and juices from *Calotropis gigantea* (Giant calotrope) and *Cynodon dactylon* (Scutch grass), are used as a remedy for erectile dysfunction [5,11]. The roots exhibit notable astringent properties, making them effective for treating piles and managing nasal bleeding. In some regions in India, the roots are reputed for their ability to enhance lactation, while a hot infusion made from the root barks plays a role in preventing both diarrhea and rheumatic pain [7,11]. In addition, the leaves are utilized to induce lactation, and a paste derived from the leaves is applied topically to treat ulcers [18,19]

*Allophylus dimorphus* has been utilized in traditional medicine across various indigenous cultures, particularly in Southeast Asia and the Nicobar Islands. The plant has been used by tribal communities for treating gastrointestinal ailments, and inflammatory conditions, and as a general health tonic [18,19]. Several reports indicate that decoctions made from the leaves and bark are commonly used for their analgesic and anti-inflammatory effects, particularly in treating joint pain and rheumatism [7,18,19]. In traditional Indian and Bangladeshi medicine, *A. dimorphus* and closely related species such as *A. cobbe* have been prescribed for managing diarrhea, dysentery, and stomach ulcers. The leaves are often chewed raw or boiled into herbal infusions to soothe digestive discomfort [5]. The presence of tannins and flavonoids, is known for their astringent and anti-inflammatory properties.

Among the indigenous Nicobarese communities, *A. dimorphus* has been used as a natural remedy for skin infections and wound healing. The crushed leaves are applied topically to cuts, burns, and ulcers to promote faster recovery and prevent microbial infections [18,19]. The antimicrobial potential of *Allophylus* species has been documented, and preliminary studies suggest that bioactive compounds such as phenolic acids and alkaloids may contribute to their antibacterial activity [7]. The plant is also valued in Ayurvedic medicine as a blood purifier and detoxifying agent. In India and Bangladesh, traditional practitioners prepare a concoction using roots and bark to treat fever, colds, and respiratory ailments [11]. The antioxidant-rich composition of *A. dimorphus* aligns with its traditional use in immune-boosting formulations Ethnopharmacological surveys conducted in Malaysian and Indonesian villages have highlighted the use of *A. dimorphus* in reproductive health. Women consume infusions made from the leaves to alleviate menstrual pain and hormonal imbalances [9–11].

In African traditional medicine, related *Allophylus* species have been used in the treatment of malaria, and it is speculated that *A. dimorphus* may share similar therapeutic effects [4]. The presence of alkaloids and saponins in the genus suggests potential anti-malarial activity, which warrants further scientific investigation. Despite its extensive traditional applications, scientific validation of many of these claims remains limited. Future studies should focus on conducting rigorous pharmacological evaluations, including in vitro and in vivo assessments, to establish the efficacy and safety of *A. dimorphus* in treating these ailments. The integration of scientific inquiry with traditional knowledge surrounding *A. dimorphus* strengthens the case for further exploration of its therapeutic potential, highlighting the need for comprehensive studies to validate its applications in modern healthcare. The ethnopharmacological uses of *A. dimorphus* are highlighted in Table 1. Scientific validation for many of these traditional uses remains limited, necessitating further pharmacological research to establish efficacy and safety

**Table 1.** Ethnopharmacological uses of *Allophylus dimorphus.*

| Part Used | Traditional Use (s) | Region | References |
|---|---|---|---|
| Fruits | Combined with *Alocasia macrorrhizos*, *Calotropis gigantea*, and *Cynodon dactylon* for erectile dysfunction | Bangladesh (Gor tribe) | [5,11] |
| Roots | Astringent properties used for treating piles and nasal bleeding | Various regions | [7,11] |
| Roots | Enhancing lactation | India | [7,11] |
| Root Bark | Hot infusion used to prevent diarrhea and rheumatic pain | India | [7,11] |
| Leaves | Inducing lactation and applied as a paste for ulcers | Various regions | [18,19] |
| Leaves & Bark | Decoctions for analgesic and anti-inflammatory effects (joint pain, rheumatism) | Southeast Asia, Nicobar Islands | [7,18,19] |
| Leaves | Chewed raw or boiled into herbal infusions for digestive issues (diarrhea, dysentery, stomach ulcers) | India, Bangladesh | [5] |
| Leaves | Applied topically for skin infections, wound healing, burns, and ulcers | Nicobarese communities | [18,19] |
| Roots & Bark | Concoctions for fever, colds, and respiratory ailments | India, Bangladesh | [11] |
| Leaves | Infusions consumed for menstrual pain and hormonal imbalance | Malaysia, Indonesia | [9–11] |
| Various parts | Speculated anti-malarial properties based on related *Allophylus* species | Africa | [4] |

## 6. Phytochemical Constituents

Phytochemical studies on *A. dimorphus* are limited and have remained unexplored for new chemical entities. There are no recent reports on the isolation and characterization of compounds from the various parts (fruits, seeds, leaves, stem) of *A. dimorphus* available. Previous reports indicate the presence of benzylamide, phenylacetamide, and phytosterols in *Allophylus* species, supporting their potential medicinal applications. It has also been reported that leaves of other species such as *A. cobbe* contain an alkaloid-enriched fraction [17].

In another study, the bark of this species along with the leaves also showed the presence of alkaloids in trace amounts [20]. These compounds are associated with antimicrobial, anti-inflammatory, and antioxidant activities, which justify their traditional medicinal use [18,19].

However, research on related species suggests the presence of flavonoids, phenolics, alkaloids, and terpenoids, which are known for their diverse therapeutic properties [6,7]. β-Sitosterol is a common phytosterol found in *Allophylus* species and has been linked to cholesterol-lowering and cardioprotective effects [18]. Further phytochemical investigations using advanced chromatographic techniques such as high-performance liquid chromatography (HPLC) and gas chromatography-mass spectrometry (GC-MS) could enhance the understanding of its potential for pharmaceutical applications. [21]. Since the species is underutilized and other species have been explored thoroughly the scientific community could and investigate each part of this species qualitatively and quantitatively using modern analytical and computational tools such as mass spectrometry combined with metabolomics and chemometrics. The chemical structures of identified phytochemicals in various *Allophylus* species are depicted in Figure 4.

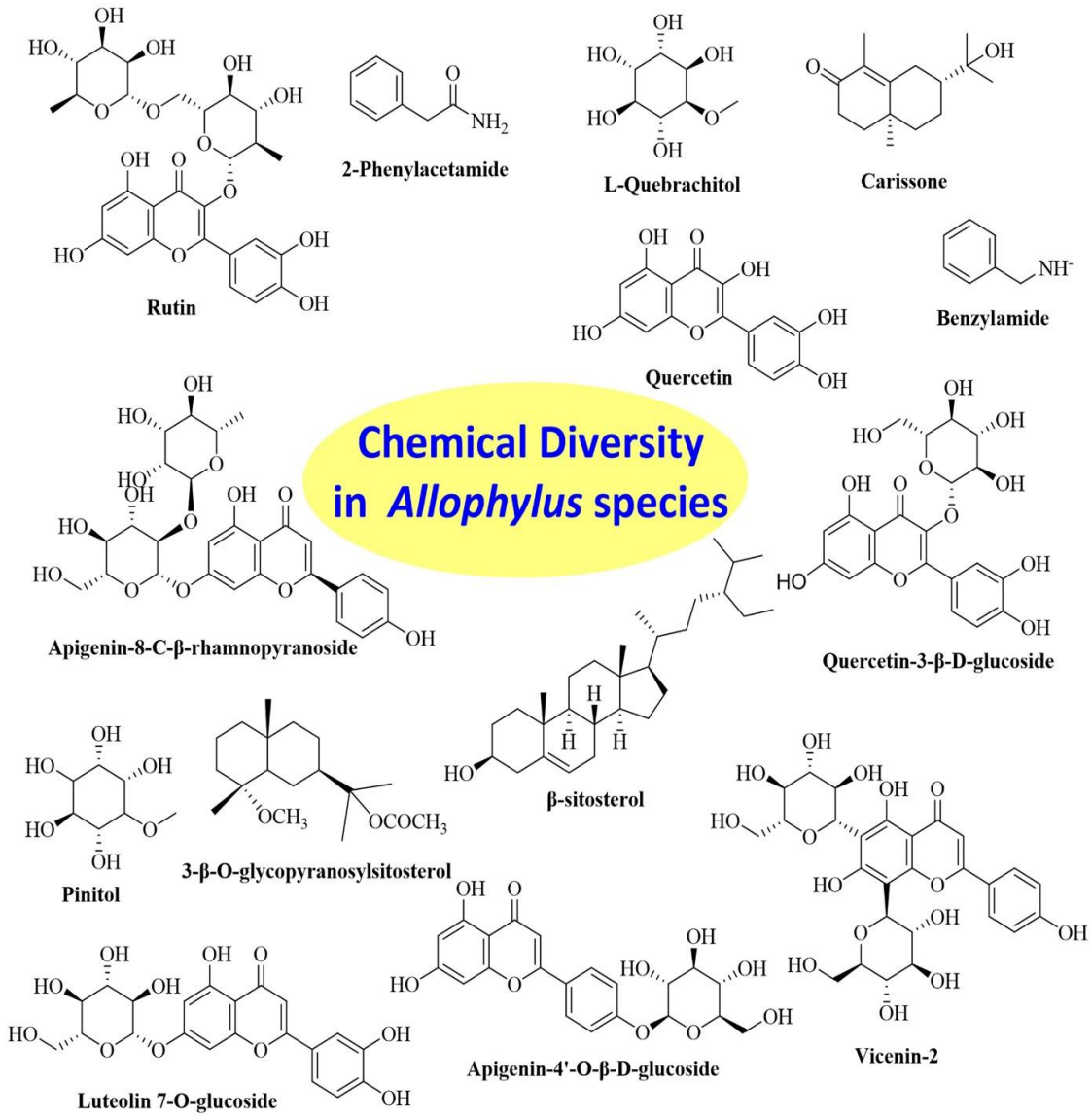



**Figure 4.** Chemical structures of identified phytochemicals in various *Allophylus* species.

## 7. Medicinal Properties

For millennia, humans have utilized plants for medicinal purposes, and a significant portion of modern pharmaceuticals are either derived from or inspired by natural products [1]. While direct pharmacological studies on *Allophylus dimorphus* are limited, research on related *Allophylus* species reveals a broad spectrum of bioactivities, suggesting similar potential for *A. dimorphus*. *A. serratus* exhibits notable anti-inflammatory and antioxidant properties [7], while *A. cobbe* demonstrates significant antimicrobial and antidiabetic effects [6]. Moreover, *A. africanus* displays anticancer and antiulcer properties highlighting the potential for discovering novel therapeutic agents within this genus [22]. The pharmacological properties of some of the common *Allophylus* species are shown in Figure 5.

The antioxidant potential is attributed to polyphenols and flavonoids, which neutralize oxidative stress and prevent chronic diseases such as cancer, cardiovascular disorders, and diabetes [1]. *A. cobbe* has demonstrated radical scavenging activity, suggesting that *A. dimorphus* may exhibit similar properties [6]. The presence of rutin, quercetin, and catechin in related species supports this assumption [7]. The antioxidant properties observed in related *Allophylus* species are likely due to their flavonoid and phenolic content. These compounds neutralize free radicals and reduce oxidative damage linked to chronic diseases like cancer, diabetes, and cardiovascular disorders [1]. In vitro and in vivo studies are necessary to confirm the extent of antioxidant efficacy. The presence of β-sitosterol in *A. serratus* [7], also points towards possible benefits in managing hyperlipidemia and improving cardiovascular health [18].

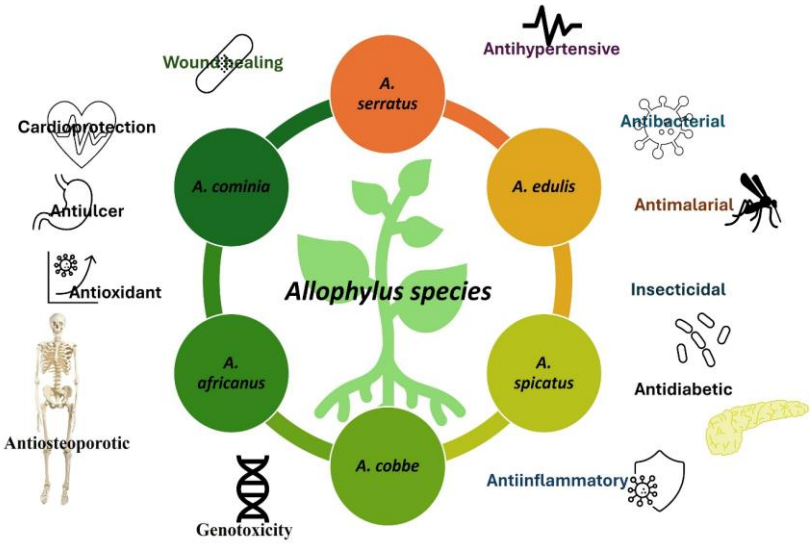



**Figure 5.** Pharmacological properties of some of the common *Allophylus* species.

Antimicrobial screening of *A. cobbe* and *A. serratus* has shown activity against Gram-positive bacteria such as *Staphylococcus aureus* and *Bacillus subtilis*, but limited efficacy against Gram-negative bacteria [6,7]. The anti-inflammatory properties of *Allophylus* species are due to flavonoids, which inhibit TNF-α and IL-6. The presence of alkaloids and terpenoids in *Allophylus* species further suggests potential antimicrobial and anti-inflammatory properties, with applications in treating infectious diseases and inflammatory conditions [6,7]. The medicinal properties of *A. dimorphus* and its related species are shown in Table 2.

Research suggests that *Allophylus dimorphus* extracts may have similar effects because of their chemical similarities. Further studies should use bioassay-guided fractionation to identify active compounds. While *A. dimorphus* has demonstrated promising medicinal applications, its toxicity profile remains unassessed. Related species have been found to exhibit mild cytotoxicity at high concentrations [7]. Long-term safety studies, including acute and chronic toxicity assessments, are necessary before clinical application. Further pharmacological studies, including in vitro and in vivo experiments, are needed to confirm the medicinal potential of *Allophylus dimorphus*. Long-term safety and toxicity assessments should be prioritized before clinical applications. To investigate the medicinal and pharmacological properties of *A. dimorphus*, research should first focus on its phytochemical aspects. A thorough inspection on biological-guided fractionation for each part and various polarity extracts may provide some potential leads to understanding the phyto-pharmacological profile of this species.

**Table 2.** Medicinal properties of *A. dimorphus* and its related species.

| Property | Observed in Species | Bioactive Compounds | Potential Applications | References |
|---|---|---|---|---|
| Antioxidant Activity | *A. cobbe*, *A. serratus* | Polyphenols, flavonoids (rutin, quercetin, catechin) | Protection against oxidative stress, cardiovascular disorders, diabetes | [1,7] |
| Anti-inflammatory | *A. cobbe*, *A. serratus* | Flavonoids, alkaloids, terpenoids | Inhibition of TNF-α and IL-6, treatment of inflammatory diseases | [6,7] |
| Antimicrobial | *A. cobbe*, *A. serratus* | Alkaloids, terpenoids | Activity against Gram-positive bacteria (*S. aureus*, *B. subtilis*) | [6,7] |
| Anticancer | *A. africanus* | Unknown | Potential for novel cancer therapies | [22] |
| Antiulcer | *A. africanus* | Unknown | Protection against gastric ulcers | [22] |
| Antidiabetic | *A. cobbe* | Unknown | Regulation of blood sugar levels | [6] |
| Cardiovascular Health | *A. serratus* | β-sitosterol | Management of hyperlipidaemia, heart health | [7,18] |

## 8. Conservation Concerns

Many plant species are threatened by habitat transformation, over-exploitation, invasive alien species, pollution, and climate change, and are now in danger of extinction [24]. The conservation status of *A. dimorphus* is a critical concern. The primary causes of its threat include land conversion and habitat degradation due to commodity-driven desertification, fluctuating agricultural practices, urbanization, and forestry activities such as logging [8]. Its limited distribution and vulnerability to habitat loss [8] necessitate immediate conservation efforts [23,24]. The reliance of many populations on this species for food and medicine highlights its ecological and socio-economic importance. Habitat destruction, driven by deforestation and agricultural expansion poses a significant threat to its continued survival [23,24]. The vulnerability of *A. dimorphus* necessitates the development and implementation of sustainable management strategies including habitat protection, cultivation, and the integration of traditional knowledge with modern scientific practices [3]. To stop population decline, the interventions are highly recommended together with the introduction of species to botanic gardens for *ex-situ* conservation to safeguard its continued survival [8,24].

## 9. Future Research Directions

Future research on *Allophylus dimorphus* should prioritize several key areas. A comprehensive phytochemical analysis is critical to fully characterize its bioactive compounds [1]. Advanced techniques such as metabolomics and proteomics will provide detailed information on its biochemical pathways and the interactions of its various components. This knowledge will provide a more comprehensive understanding of the mechanisms underlying its observed bioactivities and will aid in the identification of specific lead compounds for the development of potential therapeutics [1].

Future research should prioritize comprehensive phytochemical analyses to isolate and characterize the bioactive compounds present in *A. dimorphus* and its relatives. Pharmacological investigations should focus on evaluating the antioxidant, antimicrobial, anti-inflammatory, and anticancer activities of *A. dimorphus*, emphasizing unique properties that distinguish it from other *Allophylus* species. Integrating traditional knowledge with scientific methodologies will not only validate its therapeutic uses but also promote the sustainable utilization of this valuable species in natural product research and drug development. Integrating sustainable harvesting practices with conservation efforts is critical [23,24]. The available information on the morphology, geographical distribution, and ecological context of *A. dimorphus* can help with the selection of appropriate cultivation methods and promote successful propagation efforts [8,13–17].

## 10. Conclusions

*Allophylus dimorphus*, a vulnerable species with a long history of ethnomedicinal use, represents a promising yet underexplored source of bioactive compounds for modern medicine. This review synthesizes existing knowledge on the botanical, ethnopharmacological, and pharmacological aspects of *A. dimorphus* and related species, highlighting its potential therapeutic applications. While direct research on *A. dimorphus* remains limited, the promising bioactivities demonstrated by related *Allophylus* species, along with its ethnomedicinal uses and rich phytochemical profile, warrant further investigation. Future studies focusing on comprehensive phytochemical analysis, rigorous bioactivity testing, and the development of sustainable cultivation practices are urgently needed to realize the full therapeutic potential of *A. dimorphus* while simultaneously safeguarding this valuable species and its associated biodiversity. A multidisciplinary approach integrating traditional knowledge with modern scientific methods is key to achieving this goal. The phytochemical studies on other *Allophylus* species show a wide variety of compounds, including flavonoids and other phenolics, that are responsible for a broad range of bioactivities. To develop novel drugs and nutraceuticals, detailed investigations into their phytochemistry using conventional and advanced tools may help to enrich the bioactive fraction followed by the purification of newer chemical entities [21]. This approach could facilitate the discovery of novel or known compounds that may aid in the treatment of certain diseases [1]. Most importantly, failing to implement effective conservation and sustainable use strategies will jeopardize the future availability of this and other valuable medicinal plant resources.

**Author Contributions:** All authors contributed equally. L.N., and A.A.K. finalized the manuscript. All authors reviewed the final version. All authors have read and agreed to the published version of the manuscript.

**Funding:** This research received no external funding.

**Institutional Review Board Statement:** Not applicable.

**Informed Consent Statement:** Not applicable.

**Data Availability Statement:** Not applicable.

**Acknowledgments:** Lutfun Nahar gratefully acknowledges the support from the European Regional Development Fund (Project ENOCH #CZ.02.1.01/0.0/0.0/16_019/0000868) and the Czech Science Foundation (Projects #23-05474S and #23-05389S).

**Conflicts of Interest:** The authors declare no conflict of interest.

## References

1. Sarker, S.D.; Nahar, L. Medicinal natural products: A disease-focused approach. In *Annual Reports in Medicinal Chemistry*; Academic Press: Cambridge, MA, USA, 2020; pp. 1–44.
2. The World Health Organization (WHO). Integrating Traditional Medicine in Health Care. 2023. Available online: https://www.who.int/southeastasia/news/feature-stories/detail/integrating-traditional-medicine (accessed on 30 January 2025).
3. The World Health Organization (WHO). WHO Global Traditional Medicine Centre. Why Is It needed? 2024. Available online: https://www.who.int/initiatives/who-global-traditional-medicine-centre/about-us (accessed on 30 January 2025).
4. Menzel, C.M. Fruits of tropical climates | fruits of the Sapindaceae. *Encycl. Food Sci. Nutr.***2003**, 2786–2790.
5. Azam MN, K.; Ahmed, M.N.; Rahman, M.M.; et al. Ethnomedicines used by the Oraon and Gor tribes of Sylhet district, Bangladesh. *AEJSA* **2013**, *7*, 391–402.
6. Ankad, G.M.; Upadhya, V.; Pai, S.R.; et al. Erratum to: Total polyphenols, antioxidant and antimicrobial activity of leaves and stem bark extracts of *Allophylus cobbe* (L.) Raeusch. *Proc. Natl. Acad. Sci. India Sec. B Biol. Sci.* **2016**, *86*, 145–149.
7. Chavan, R.B.; Gaikwad, D.K. The ethnobotany, phytochemistry and biological properties of *Allophylus* species used in traditional medicine: A review. *World J. Pharm. Pharm. Sci.* **2016**, 6, 664–682. https://doi.org/10.20959/wjpps201611-8039.
8. IUCN. The IUCN Red List of Threatened Species. 2020. Available online: https://www.iucnredlist.org/species/197827067/203233498 (accessed on 27 January 2025).
9. Mao, A.A.; Dash, S.S. *Flowering Plants of India: An Annotated Checklist (Dicotyledons)*; Botanical Survey of India: Kolkata, India, 2020; Volume 2.
10. Wu, H.M. *Flora of China*; Science Press: Beijing, China; Missouri Botanical Garden Press: St. Louis, MO, USA, 2007; Volume 12.
11. Medicinal Plants of Bangladesh (MPBD). *Allophylus dimorphus* Radlk. 2024. Available online: https://mpbd.cu.ac.bd/details.php?id=704 (accessed on 19 November 2024)
12. Davis, C.C; Choisy, P. Medicinal plants meet modern biodiversity science. *Curr. Biol.* **2024**, *34*, R158–R173.
13. Kew Royal Botanic Gardens *Allophylus dimorphus* Radlk. 2024. Available online: https://powo.science.kew.org (accessed on 30 January 2025).
14. World Flora Online (WFO). *Allophylus dimorphus* Radlk. 2024. Available online: http://www.worldfloraonline.org/taxon/wfo-0000526648 (accessed on 30 January 2025).
15. Ogwu, M.C.; Izah, S.C.; Joshua, M.T. Ecological and environmental determinants of phytochemical variability in forest trees. *Phytochem. Rev.* **2025**. https://doi.org/10.1007/s11101-025-10066-0.
16. Ebi, K.L.; Vanos, J.; Baldwin, J.W.; et al. Extreme weather and climate change: Population health and health system implications. *Annu. Rev. Public Health* **2021**, *42*, 293–315.
17. Leenhouts, P.W. A conspectus of the genus *Allophylus* (Sapindaceae). *Regnum Veg.* **1963**, *27*, 3
18. Rastogi, R.; Mehrotra, B. *Compendium of Indian Medicinal Plants*; Central Drug Research Institute, Lucknow, India, 1990; Volume 1, pp. 388–389.
19. Johns, S.R.; Lamberton, J.A. Isolation of simple acid amides from *Allophylus cobbe* (Sapindaceae), *Hamalium foetidum* (Flacourtceae) and from a *Aglabia species* (Meliaceae). *Aus J. Chem.* **1969**; *22*, 1315–1316.
20. Asolkar, L.V.; Kakkar, K.K.; Chakre, O.J. *Second Supplement to Glossary of Indian Medicinal Plants with Active Principles Part-1 (A-K)*; CSIR: New Delhi, India, 1992.
21. Sarker, S.D.; Nahar, L. (Eds.). *Natural Products Isolation*; Humana Press: Totowa, NJ, USA, 2012.
22. Ikenna, C.; Mba, T.C.; Odoh, U.E.; et al. Phytochemical and anti-ulcer properties of *Allophylus africanus* P Beauv. (Sapindaceae) leaf. *Asian J. Res Medical and Pharm. Sci.* **2023**, *12*, 38–45. https://doi.org/10.9734/ajrimps/2023/v12i1208.
23. Corlett, R.T. Plant diversity in a changing world: Status, trends, and conservation needs. *Plant Divers.* **2016**, *38*, 10–16.
24. The Plant Conservation Report (2024). Available online: https://www.cbd.int/doc/publications/plant-conservation-report-en.pdf (Accessed on 27 Jan 2025).

*Review*

# Phytotherapy Targeting Rheumatoid Arthritis: A Clinically Based Approach

**Georgeta Stefanovici Zugravu [1,2] and Anca Miron [1,*]**

[1] Faculty of Pharmacy, Grigore T. Popa University of Medicine and Pharmacy, 16, Universitatii Street, 700115, Iasi, Romania
[2] Clinical Rehabilitation Hospital, 14, Pantelimon Halipa Street, 700661, Iasi, Romania
***** Correspondence: anca.miron@umfiasi.ro
Received: 1 January 2025; Revised: 17 March 2025; Accepted: 19 March 2025; Published: 21 March 2025

**Abstract:** Rheumatoid arthritis is a chronic autoimmune-inflammatory disease characterized by joint destruction and physical disability. The present treatment options in rheumatoid arthritis include nonsteroidal anti-inflammatory drugs, glucocorticoids, and synthetic and biological disease-modifying antirheumatic drugs. However, all these classes of medications have disadvantages associated with severe adverse reactions, patients' low adherence to treatment, and numerous drug interactions. These drawbacks emphasize the need to identify novel anti-inflammatory agents to replace or support standard therapy and improve treatment compliance. This mini-review focuses on herbal preparations whose efficacy was evaluated in clinical trials. Extracts of various plant species (*Tripterygium wilfordii* Hook F, *Paeonia lactiflora* Pallas, *Olea europea* L., *Silybum marianum* (L.) Gaertn., *Hippophaë rhamnoides* L., *Punica granatum* L., *Vaccinium macrocarpon* Aiton) and powdered plant parts (*Allium sativum* L., *Rosa canina* L.) significantly improved the clinical parameters, disease activity indices, and biochemical markers in rheumatoid arthritis patients when they were administered as supportive therapy alongside the standard medication or, more rarely, as monotherapy. The bioactive compounds have been only partially identified and further research is required to fully elucidate the phytochemical profile of these herbal preparations. Although the clinical studies performed up to now support the benefits of herbal supplementation in rheumatoid arthritis, there is a strong need for more human trials to validate the efficacy and safety of herbal preparations.

**Keywords:** rheumatoid arthritis; herbal preparations; disease activity indices; ACR response; EULAR response

## 1. Introduction

Rheumatoid arthritis (RA) is a complex, systemic, autoimmune, and inflammatory condition characterized by progressive multiple joint destruction and deformity [1]. RA is also defined by numerous extra-articular manifestations such as rheumatoid nodules, pleuro-pulmonary involvement, renal impairment, vasculitis, and accelerated atherosclerosis. Patients experience morning stiffness, fatigue, functional disability, and reduced quality of life [2–4]. The treatment of RA has evolved remarkably in recent decades, with the therapeutic arsenal now including nonsteroidal anti-inflammatory drugs (NSAIDs), glucocorticoids, and synthetic and biological disease-modifying antirheumatic drugs (DMARDs). The systemic adverse effects caused by the chronic use of NSAIDs (gastric, renal, hepatic, cardiac, and hematologic side effects) [5–11], glucocorticoids (increased risk of infections, osteoporosis, myopathy, diabetes mellitus, peptic ulcer, hypertension, increased cardiovascular risk, obesity, Cushing syndrome, cataracts, glaucoma, psychosis, depression, insomnia) [12–15], and conventional synthetic DMARDs (hepatic and pulmonary fibrosis, leukopenia, anemia, thrombocytopenia, increased risk of infections, lymphomas, teratogenic effects) [16–18], as well as the numerous drug interactions and patients' low adherence to the prescribed treatments, call for the discovery of effective and less toxic anti-inflammatory agents to replace/support standard therapy and improve treatment compliance [19–21]. In recent years, there has been an increased interest from both patients and physicians in herbal preparations. The benefits of herbal preparations in patients with RA were confirmed in numerous clinical studies. Most of them were randomized trials evaluating the benefits of herbal supplementation in RA patients undergoing various treatments compared to control or placebo [22–25]. The clinical assessment was mainly based on a set of indicators established by the American College of Rheumatology (ACR) and validated by the Outcome Measures in Rheumatoid Arthritis Clinical Trials (OMERACT): tender and swollen joint count in 68 and 66 joints, respectively/28 joints (TJC68, SJC66, TJC28,

SJC28), morning stiffness (in min.), patient's and physician's global assessments of disease activity and patient's assessment of pain (on visual analogue scale), health assessment questionnaire – disability index (HAQ-DI), disease activity score in 28 joints (DAS28), simplified disease activity index (SDAI), clinical disease activity index (CDAI), ACR and European League Against Rheumatism (EULAR) responses [26–30]. ACR20/50/70 response indicates at least 20/50/70% improvement in TJC68 and SJC66 and in three of the following five variables: patient's and physician's global assessments of disease activity, HAQ-DI, patient's assessment of pain, and erythrocyte sedimentation rate (ESR)/C-reactive protein (CRP) [30]. EULAR responses were assessed according to improvements in DAS score [28]. In addition, inflammatory, oxidative stress, and immunological markers were also considered when evaluating the clinical benefits of herbal preparations in RA patients.

## 2. Herbal Preparations in Clinical Studies

Various herbal preparations have undergone clinical investigation for their potential to treat RA. Extracts of the roots of *Tripterygium wilfordii* Hook F (Pin Yin name lei gong teng, also known as thunder god vine) have been used in the Traditional Chinese Medicine (TCM) for centuries to treat autoimmune and inflammatory conditions. The extracts demonstrated clinical efficacy in patients with RA both as monotherapy and in combination with various DMARDs. In a 6-month randomized, controlled clinical trial, patients with active RA experienced significantly greater improvements with *Tripterygium wilfordii* root extract (60 mg × 3/day) than with sulfasalazine (1 g × 2/day), a widely used conventional DMARD [31]. The extract, obtained by sequential extraction of powdered roots with ethanol and ethyl acetate, was standardized in triptolide and tripdiolide (60 mg of extract contained a total of 30 μg from both compounds combined) [32]. The improvements were evaluated based on the percentages of patients achieving ACR20, ACR50, and ACR70 responses, HAQ score, number of tender and swollen joints, pain intensity, patient's and physician's global assessments of disease activity, ESR, CRP, interleukin (IL)-6, and rheumatoid factor (RF) [31]. It is worth emphasizing that *Tripterygium wilfordii* root extract caused more pronounced reductions compared to sulfasalazine in the plasma levels of IL-6, an indicator of cartilage loss and RF, an indicator of autoimmune conditions [31,33,34]. Moreover, patients treated with the extract reported significantly fewer adverse effects compared to those receiving sulfasalazine; the most commonly mentioned adverse effects by extract-treated patients were gastrointestinal events [31]. Combining *Tripterygium wilfordii* root extract with methotrexate boosted the clinical efficacy of the latter. In a 6-month multicentre, open-label, randomized, controlled clinical study, a combination of *Tripterygium wilfordii* root extract (not further specified) (20 mg × 3/day) and methotrexate (from 7.5 to 12.5 mg/week within one month, then 12.5 mg/week) was better than methotrexate in terms of patients attaining ACR20, ACR50, ACR70, good or moderate EULAR responses and improvements in tender and swollen joints, patient's and physician's global assessments of disease activity, ESR, CRP, DAS28, and CDAI. In addition, extract-based monotherapy was more effective than methotrexate monotherapy (at the doses stated above) [35]. Other clinical studies demonstrated the superiority of *Tripterygium wilfordii* root extract to placebo [32] and clinical efficacy associated with the topical application of the root extract (tincture) [36]. Diterpenoids (triptolide, tripdiolide, triptonide) are the main anti-inflammatory and immunoregulatory compounds in *Tripterygium wilfordii* roots [35]. Triptolide, for example, decreased inflammatory markers (tumor necrosis factor (TNF)-alpha, IL-1beta, IL-6, matrix metalloproteinase (MMP)-3, -9) in RA-fibroblast-like synoviocytes and collagen-induced arthritis rats [37], reduced the number of osteoclasts in the inflamed joints, reduced the activation of nuclear factor kappa-B (NF-kB), and up-regulated osteoprotegerin in collagen-induced arthritis mice [38]. *Tripterygium wilfordii* extracts can induce hepatotoxicity, nephrotoxicity, gastrointestinal, reproductive, and skin toxicity. These side effects, some of them reversible when the treatment is stopped, can be mitigated by dose adjustment [38].

Total glucosides of paeony (TGP), extracted from the roots of *Paeonia lactiflora* Pallas and containing 90% paeoniflorin, is extensively used to treat RA in China. In 1998, China Food and Drug Administration approved TGP as a disease-modifying drug for RA [39]. Eight randomized controlled trials enrolling 522 RA patients evaluated the efficacy and safety of TGP in combination with methotrexate (0.3/0.6 g TGP × 3/day, 7.5–15 mg methotrexate once a week, 12/24 weeks). TGP combined with methotrexate demonstrated superior therapeutic effects compared to methotrexate alone regarding the decrease in SJC and ESR. The rate of adverse effects (mild to moderate digestive events, mild liver abnormalities) was lower in patients treated with the combination therapy [40]. Other randomized controlled trials (463 RA patients, 465 controls) support the efficacy and safety of TGP co-administration with methotrexate and leflunomide therapy in active RA. The addition of TGP (0.6–1.8 g/day) to methotrexate (7.5–15 mg/week) and leflunomide (10–20 mg/day) therapy for 12–24 weeks was more efficient in reducing ESR, CRP, and RF than methotrexate and leflunomide therapy. Both methotrexate and leflunomide are hepatotoxic and increase the risk of dyslipidemia in RA patients; the addition of TGP had positive effects on

the lipid profile and liver function [41]. Paeoniflorin, a monoterpene glucoside and the major active component of TGP, is a potent anti-inflammatory, immunomodulating, and antioxidant agent: it regulates the proliferation and activation of T and B lymphocytes, macrophages, dendritic cells, and synoviocytes, suppresses the production of inflammatory mediators (TNF-alpha, IL-1, prostaglandin E2 (PG E2)), regulates signaling pathways associated with inflammation (G protein-coupled receptor, mitogen-activated protein kinases (MAPKs), Janus kinase/signal transducers and activators of transcription (JAK/STAT) signaling pathways) and oxidative stress (reactive oxygen species (ROS)/p38/p53 pathway) [42].

Combined administration of a dry olive (*Olea europea* L.) leaf ethanolic (80% m/m) extract with methotrexate was more effective than methotrexate monotherapy in patients with early phase RA, but not in patients with long-term RA. The extract (190 mg of standardized extract with approximately 35 mg of oleuropein/capsule, 2 capsules × 2/day, 6 weeks) acted synergistically with methotrexate (10–17.5 mg/week) in counteracting oxidative stress (indicated by lipid peroxidation level in erythrocytes), DNA damage (expressed as number of lymphocytes with damaged DNA), and inflammatory status (assessed *via* plasma IL-6 level). This study demonstrates the capacity of the olive leaf ethanolic extract to modulate the effects of the standard methotrexate therapy and highlights the necessity of early intervention in the disease progression, before the onset of irreversible cellular damage [43]. Oleuropein, the major polyphenol in the dry olive leaf extract, is endowed with remarkable antioxidant and anti-inflammatory effects. Due to its ortho-diphenolic group, oleuropein effectively scavenges ROS. In addition, it enhances the level of both enzymatic and non-enzymatic endogenous antioxidants and significantly reduces various inflammatory mediators (IL-1beta, IL-6, NF-kB, TNF-alpha, inducible nitric oxide synthase (iNOS), cyclooxygenase-2 (COX-2), MMP-9) [44].

Garlic supplementation (500 mg dried garlic powder/tablet × 2/day equivalent to 2.5 g of fresh garlic, 2–3 mg allicin/garlic tablet, 8 weeks) in patients with active RA under treatment with DMARDs caused significant decreases in the serum levels of CRP and TNF-alpha, TJC and SJC, pain intensity, DAS28, and fatigue as compared with the placebo group. Garlic (*Allium sativum* L.) contains a wide variety of bioactive phytochemicals including organosulfur compounds (allicin, ajoene, diallyl sulphide, S-methylcysteine, S-allylcysteine, alliin), phenolic compounds (beta-resorcylic, protocatechuic, and gallic acids, pyrogallol, rutin, quercetin), amino acids, and polysaccharides [3]. Allicin, generated from alliin *via* the enzymatic activity of aliinase, has numerous pharmacological activities including anti-inflammatory activity. Allicin was reported to reduce the release of TNF-alpha, IL-1alpha, -1beta, -6, -8 and downregulate the expression of COX-2, iNOS, Cd68, and HLA-B2704 proteins both *in vitro* and *in vivo* [45].

Silymarin, a mixture of flavonolignans isolated from milk thistle (*Silybum marianum* (L.) Gaertn.) fruits with silybin (silibinin) as major component (50–70%), is extensively used for liver protection and regeneration due to its remarkable anti-inflammatory and antioxidant effects. In patients with active RA under treatment with conventional DMARDs, silymarin supplementation (300 mg/day, 8 weeks) caused significant improvements in the tender and swollen joints, pain, morning stiffness, disease activity and disability indices (DAS28, CDAI, SDAI, HAQ-DI), EULAR responses, fatigue, depression, and anxiety compared to patients treated solely with DMARDs [46]. This clinical trial confirmed the anti-inflammatory potential of silymarin demonstrated in various cell-based and animal experimental models. In IL-1beta-stimulated human primary chondrocytes, silymarin decreased pro-inflammatory cytokines (IL-1beta, TNF-alpha) and MMP-3, -9) and increased the production of tissue inhibitor of MMP-1 [47]. Silymarin showed anti-inflammatory activity in carrageenan and papaya latex-induced rat paw edema and arachidonic acid-induced mouse ear edema models and anti-arthritic activity in mycobacterial adjuvant-induced arthritis rat model [48]. In Freund's adjuvant-induced arthritis rat model, silymarin not only attenuated the increase in rat paw circumference but reduced seric RF and pro-inflammatory cytokines (TNF-alpha, IL-1beta, -17, PG E2) and increased seric anti-inflammatory cytokines (IL-4) [49].

Sea buckthorn (*Elaeagnus rhamnoides* (L.) A. Nelson syn. *Hippophaë rhamnoides* L.) fruit oil offers therapeutic benefits for various skin, mucosal, cardiovascular, liver, and metabolic conditions and ailments. It is also well-known for its capacity to attenuate the side effects of radiotherapy and chemotherapy in cancer patients [50–52]. Sea buckthorn fruit oil supplementation (900 mg × 2/day, 12 weeks) in patients with active RA under treatment with a combination of methotrexate (15 mg/week) and diclofenac sodium (50 mg/day) resulted in significant improvements in the TJC and SJC, pain, morning stiffness, patient's and physician's global assessments of disease activity, SDAI, CDAI, HAQ-DI, ACR20, ACR50, EULAR (ESR) and EULAR (CRP) responses, and serum malondialdehyde (MDA), an oxidative stress marker, compared to patients treated only with methotrexate and diclofenac sodium. Overall, sea buckthorn fruit oil supplementation significantly alleviated the swelling, pain, stiffness, and physical disability, improving the general condition of RA patients [53,54]. These benefits are undoubtedly linked to the compounds in sea buckthorn fruit oil that attenuate inflammation and oxidative stress by various mechanisms: oleic acid inhibits NF-kB, TNF-alpha, IL-6, and -12 release, COX-2, iNOS and activates

heme oxygenase-1 (HO-1), glutathione peroxidase (GPx), superoxide dismutase (SOD), and IL-10 release [55], palmitoleic acid reduces the production of TNF-alpha, interferon (IFN)-gamma, IL-2, -6, -17A and inhibits NF-kB and lipid peroxidation [56,57], carotenoids, tocopherols and sitosterols suppress iNOS, COX-2, NF-kB, TNF-alpha, IL-1beta, -6, -8, and lipid peroxidation while upregulating IL-10 [58–63].

In a double-blind randomized clinical trial, pomegranate (*Punica granatum* L.) extract (solvent not specified) supplementation (2 capsules of 250 mg extract/day, 8 weeks) significantly reduced the SJC and TJC, pain intensity, morning stiffness, DAS28 and HAQ scores, ESR, and increased GPx level compared with placebo in RA patients receiving standard medications (methotrexate, prednisolone, hydroxychloroquine, sulfasalazine, NSAIDs). The extract contained 40% ellagic acid [64]. The latter exhibits anti-inflammatory activity mediated mainly through the inhibition of NF-kB pathway with consequent downregulation of pro-inflammatory iNOS, COX-2, TNF-alpha, and IL-6 [65]. The anti-inflammatory activity of the investigated pomegranate extract is not solely attributed to ellagic acid and other polyphenols. Pomegranate has a positive impact on the gut microbiota, the latter playing a key role in the pathogenesis of RA [64]. RA patients have reduced fecal load of *Bifidobacterium* and *Bacteroides* species [66]. Pomegranate extract was reported to increase the abundance of bifidobacteria in the cecum [64].

A 90-day clinical study, including 41 women diagnosed with RA and treated with steroids and one or two DMARDs, demonstrated clear benefits (significant reduction in DAS28 score and anti-cyclic citrullinated peptide (anti-CCP) antibodies - diagnosis and prognosis markers in RA) for the ingestion of 500 mL/day of low calorie cranberry (*Vaccinium macrocarpon* Aiton) juice. Among cranberry juice components, quercetin and resveratrol play a major role in its anti-inflammatory activity. Both compounds downregulate NF-kB pathway. In addition, quercetin inhibits pro-inflammatory enzymes (COX, lipoxygenase) while resveratrol modulates JAK STAT3 pathway [67].

Administration of a highly purified aqueous extract from the pentacyclic chemotype of cat's claw (*Uncaria tomentosa* (Willd.) DC.) demonstrated favorable effects in RA patients under treatment with sulfasalazine or hydroxychloroquine. The extract (14.7 mg/g pentacyclic oxindole alkaloids, no tetracyclic oxindole alkaloids) was administered at a daily dose of 60 mg (20 mg × 3/day). In this 2-phase study (52 weeks), the extract reduced the number of painful and swollen joints and Ritchie index, the latter assessing joint tenderness and disease severity. The plant has been traditionally used to treat rheumatic diseases in Peru. The therapeutic properties of the plant are partly attributed to its pentacyclic oxindole alkaloids. These compounds have immunomodulatory effects (inhibition of activated lymphocytes proliferation and activation of resting/weakly activated lymphocytes proliferation, both effects being antagonized by the tetracyclic oxindole alkaloids). In addition, pentacyclic oxindole alkaloids suppress the production of TNF-alpha and mitigate oxidative stress [68].

A standardized rose hip (*Rosa canina* L.) powder showed benefits in patients with RA undergoing various treatments (NSAIDs, steroids, DMARDs). More specifically, 5 g of rose hip powder daily (5 capsules × 2/day, 0.5 g of powder/capsule, 6 months) substantially improved the physical ability and general condition of the patients as indicated by HAQ-DI, patient's and physician's global assessments of disease activity, short form (SF)-12 and RA-specific quality of life (RAQoL) scores. These positive outcomes were mainly attributed to a galacto-lipid that has structure similarities with gamma-linolenic acid, a well-known anti-inflammatory agent [69]. The rose hip powder was obtained from mature fruits of plants grown in standardized fields. Optimal fruits were dried below 40 °C, powdered, and controlled regarding mineral and vitamin content [70].

The main outcomes of the referenced clinical studies are given in detail in Table 1.

**Table 1.** Evidence from clinical studies on herbal preparations as alternative to or support for standard therapy in rheumatoid arthritis.

| Nr. | Type of Clinical Study | Nr. Patients | Part of Plant/Extract | Administration | Outcomes | Ref. |
|---|---|---|---|---|---|---|
| 1. | multicentre, double-blind, randomized, controlled | 121 | *Tripterygium wilfordii* ethanol/ethyl acetate root extract | extract (60 mg × 3/day) *vs*. sulfasalazine (1 g × 2/day); 6 months | ACR20 response: 65.0% *vs*. 32.8%;<br>ACR50 response: 33.3% *vs*. 4.9%;<br>ACR70 response: 16.7% *vs*. 1.6%;<br>decrease in HAQ score: 0.60 *vs*. 0.22;<br>decrease in IL-6 (pg/mL): 24.81 *vs*. 4.63;<br>decrease in RF (IU/mL): 483.77 *vs*. 152.59 | [31] |
| 2. | multicentre, open-label, randomized, controlled | 207 | *Tripterygium wilfordii* root extract | extract (20 mg × 3/day) *vs*. methotrexate (from 7.5 to 12.5 mg/week within one month, then 12.5 mg/week) *vs*. extract plus methotrexate (same doses mentioned above); 6 months | ACR20 responses: 72.5% *vs*. 63.8% *vs*. 92.8%;<br>ACR50 responses: 55.1% *vs*. 46.4% *vs*. 76.8%;<br>ACR70 responses: 30.4% *vs*. 23.2% *vs*. 43.5%;<br>CDAI good responses: 65.2% *vs*. 52.2% *vs*. 87.0%;<br>EULAR good responses: 47.8% *vs*. 26.1% *vs*. 58.0%;<br>ESR (mm/h): 21.2 *vs*. 27.6 *vs*. 17.4;<br>CRP (mg/L): 9.4 *vs*. 15.2 *vs*. 7.8;<br>DAS28: 3.57 *vs*. 4.03 *vs*. 3.19 | [35] |
| 3. | randomized, controlled (meta-analysis of 8 studies) | 522 | TGP | TGP (0.3/0.6 g × 3/day) plus methotrexate (7.5-15 mg once a week) *vs*. methotrexate (same doses mentioned above); 12/24 weeks | more significant decrease in ESR and SJC in TGP plus methotrexate group *vs*. methotrexate group | [40] |
| 4. | randomized, controlled (meta-analysis of 8 studies) | 928 | TGP | TGP (0.6-1.8 g/day) plus methotrexate (7.5-15 mg once a week) plus leflunomide (10-20 mg/day) *vs*. methotrexate plus leflunomide (same doses mentioned above); 12-24 weeks | more significant decrease in ESR, CRP and RF in TGP plus methotrexate plus leflunomide group *vs*. methotrexate plus leflunomide group | [41] |
| 5. | randomized, controlled | 32 | *Olea europea* leaf ethanolic (80% m/m) extract | extract (380 mg × 2/day) plus methotrexate (10-17.5 mg/week) *vs*. methotrexate (same doses mentioned above); 6 weeks | lymphocytes with damaged DNA: 7.33 *vs*. 22.84 (in early phase RA patients) | [43] |
| 6. | randomized, double-blind, placebo controlled | 70 | *Allium sativum* dried powder | dried powder (500 mg × 2/day) plus DMARDs *vs*. placebo plus DMARDs; 8 weeks | CRP (mg/L): 8.62 *vs*. 14.23;<br>TNF-alpha (ng/L): 19.04 *vs*. 32.10;<br>pain intensity (mm): 59.35 *vs*. 69.19;<br>TJC28: 3.61 *vs*. 5.55;<br>DAS28: 3.80 *vs*. 4.45;<br>fatigue: 30.90 *vs*. 37.64 | [3] |
| 7. | randomized, controlled | 122 | silymarin | silymarin (300 mg/day) plus DMARDs *vs*. DMARDs; 8 weeks | TJC28: 9.58 *vs*. 21.38;<br>SJC28: 3.97 *vs*. 13.57;<br>pain (cm): 3.56 *vs*. 7.88;<br>morning stiffness (min): 27.97 *vs*. 38.93;<br>DAS28 (ESR): 4.82 *vs*. 6.82;<br>DAS28 (CRP): 4.42 *vs*. 6.41;<br>CDAI: 20.37 *vs*. 50.77;<br>SDAI: 21.44 *vs*. 52.17;<br>HAQ-DI: 1.18 *vs*. 2.17;<br>EULAR good responses: 83.05% *vs*. 1.79%;<br>fatigue (VAS-F): 37.80 *vs*. 75.36;<br>depression (BDI-II): 8.90 *vs*. 21.21;<br>anxiety (GAD-7): 3.54 *vs*. 12.12 | [46] |

| | | | | | | |
|---|---|---|---|---|---|---|
| 8. | randomized, controlled | 80 | *Hippophaë rhamnoides* fruit oil | fruit oil (900 mg × 2/day) plus methotrexate (15 mg/week) plus diclofenac sodium (50 mg/day) *vs*. methotrexate plus diclofenac sodium (same doses mentioned above); 12 weeks | TJC28: 12.21 *vs*. 20.43;<br>SJC28: 5.81 *vs*. 13.05;<br>pain (cm): 3.16 *vs*. 7.35;<br>morning stiffness (min): 21.62 *vs*. 45.12;<br>DAS28 (ESR): 5.31 *vs*. 6.68;<br>DAS28 (CRP): 4.66 *vs*. 6.21;<br>CDAI: 24.35 *vs*. 48.17;<br>SDAI: 25.13 *vs*. 49.56;<br>HAQ-DI: 1.05 *vs*. 1.91;<br>EULAR (ESR) good responses: 35.1% *vs*. 0%;<br>EULAR (CRP) good responses: 59.5% *vs*. 0% | [53, 54] |
| 9. | randomized, double-blind, placebo controlled | 55 | *Punica granatum* extract | extract (250 mg × 2/day) plus standard medications *vs*. placebo plus standard medications; 8 weeks | swollen joints: decrease by 2.6 *vs*. increase by 0.08;<br>tender joints: decrease by 2.1 *vs*. increase by 0.9;<br>pain (mm): decrease by 17.6 *vs*. decrease by 1.6;<br>morning stiffness (min): decrease by 36.01 *vs*. increase by 0.0;<br>DAS28: decrease by 0.9 *vs*. increase by 0.1;<br>HAQ: decrease by 0.4 *vs*. decrease by 0.1;<br>ESR (mm/h): decrease by 4.3 *vs*. increase by 3.5;<br>GPx (nmoL/mL/min): increase by 18.3 *vs*. decrease by 1.6 | [64] |
| 10. | randomized, controlled | 41 | *Vaccinium macrocarpon* juice | juice (500 mL/day) plus standard medications *vs*. standard medications; 90 days | DAS28: 2.99 *vs*. 3.52;<br>anti-CCP antibodies (U/mL): 0.9 *vs*. 5.55 | [67] |
| 11. | randomized, double-blind, placebo-controlled, two-phase | 40 | *Uncaria tomentosa* aqueous extract | 1-phase: extract (20 mg × 3/day) plus sulfasalazine or hydroxychloroquine *vs*. placebo plus sulfasalazine or hydroxychloroquine; 24 weeks<br>2-phase: extract (20 mg × 3/day) plus sulfasalazine or hydroxychloroquine; 28 weeks | 1-phase, painful joints: decrease by 53.2% *vs*. decrease by 24.1%<br>2-phase, significant reductions in the painful and tender joints and Ritchie index in patients receiving extract only in phase-2 *vs*. patients receiving placebo in phase-1 | [68] |
| 12. | randomized, double-blind, placebo-controlled, parallel | 89 | *Rosa canina* standardized powder | dried powder (2.5 g × 2/day) plus standard medications *vs*. placebo plus standard medications; 6 months | HAQ-DI: 1.03 *vs*. 1.15;<br>DAS28: 3.93 *vs*. 4.42;<br>RAQol: 10.18 *vs*. 11.09;<br>SF-12 physical: 36.22 *vs*. 33.78 | [69] |

ACR - American College of Rheumatology; anti-CCP - anti-Cyclic Citrullinated Peptide antibodies; BDI-II - Beck Depression Inventory scale II; CDAI - Clinical Disease Activity Index; CRP - C-reactive Protein; DAS28 - Disease Activity Score (in 28 joints); DMARDs - Disease-Modifying Antirheumatic Drugs; DNA - Deoxyribonucleic Acid; ESR - Erythrocyte Sedimentation Rate; EULAR - European League Against Rheumatism; GAD-7 - Generalized Anxiety Disorder-7; GPx - Glutathione Peroxidase; HAQ - Health Assessment Questionnaire; HAQ-DI - Health Assessment Questionnaire – Disability Index; IL-6 – Interleukin-6; RA - Rheumatoid Arthritis; RAQol - Rheumatoid Arthritis - specific Quality of life; RF - Rheumatoid Factor; SDAI - Simplified Disease Activity Index; SF - Short Form; SJC – Swollen Joint Count; TGP - Total Glucosides of Paeony; TJC - Tender Joint Count; TNF-alpha - Tumor Necrosis Factor-alpha; VAS-F - Visual Analogue Scale-Fatigue.

## 3. Conclusion

The clinical studies completed so far demonstrate positive outcomes for the herbal preparations as alternative or complementary therapy in patients with RA. Further multicentre research enrolling larger patient cohorts is needed to confirm these findings and establish the optimal dosage regimen to achieve the best treatment outcomes with minimal side effects. Moreover, the mechanisms that contribute to the synergistic interaction between herbal preparations and conventional treatment should be explored.

**Author Contributions:** Writing—original draft preparation, G.S.Z. and A.M.; writing—review and editing, A.M.; supervision, A.M. All authors have read and agreed to the published version of the manuscript.

**Funding:** This research received no external funding.

**Institutional Review Board Statement:** Not applicable.

**Informed Consent Statement:** Not applicable.

**Data Availability Statement:** Not applicable.

**Conflicts of Interest:** The authors declare no conflict of interest.

## References

1. Aletaha, D.; Neogi, T.; Silman, A.J.; et al. Rheumatoid arthritis classification criteria: An American College of Rheumatology/European League Against Rheumatism collaborative initiative. *Arthritis Rheum.* **2010**, *62*, 2569–2581.
2. Smolen, J.S.; Aletaha, D.; McInnes, I.B. Rheumatoid arthritis. *Lancet* **2016**, *388*, 2023–2038.
3. Moosavian, S.P.; Paknahad, Z.; Habibagahi, Z.; et al. The effects of garlic (*Allium sativum*) supplementation on inflammatory biomarkers, fatigue, and clinical symptoms in patients with active rheumatoid arthritis: A randomized, double-blind, placebo-controlled trial. *Phytother. Res.* **2020**, *34*, 2953–2962.
4. Wang, Z.; Zhao, F.; Wei, P.; et al. Phytochemistry, health benefits, and food applications of sea buckthorn (*Hippophae rhamnoides* L.): A comprehensive review. *Front. Nutr.* **2022**, *9*, 1036295.
5. Trelle, S.; Reichenbach, S.; Wandel, S.; et al. Cardiovascular safety of non-steroidal anti- inflammatory drugs: Network meta-analysis. *Br. Med. J.* **2011**, *342*, c7086.
6. Gabriel, S.E.; Jaakkimainen, L.; Bombardier, C. Risk for serious gastrointestinal complications related to use of NSAIDs. A meta-analysis. *Ann. Intern. Med.* **1991**, *115*, 787.
7. Huerta, C.; Castellsague, J.; Varas-Lorenzo, C.; et al. NSAIDs and risk of ARF in the general population. *Am. J. Kidney Dis.* **2005**, *45*, 531.
8. Murray, M.D.; Brater, D.C. Renal toxicity of the NSAIDs. *Ann. Rev. Pharmacol. Toxicol.* **1993**, *33*, 435.
9. Edwards, J.E.; McQuay, H.J.; Moore, R.A. Efficacy and safety of valdecoxib for treatment of osteoarthritis and rheumatoid arthritis: Systematic review of randomised controlled trials. *Pain* **2004**, *111*, 286–296.
10. Scheiman, J.M.; Hindley, C.E. Strategies to optimize treatment with NSAIDs in patients at risk for gastrointestinal and cardiovascular adverse events. *Clin. Ther.* **2010**, *32*, 667–677.
11. Krug, H.; Broadwell, L.K.; Berry, M.; et al. Tolerability and efficacy of nabumetone and naproxen in the treatment of rheumatoid arthritis. *Clin. Ther.* **2000**, *22*, 40–52.
12. Del Rincón, I.; Battafarano, D.F.; Restrepo, J.F.; et al. Glucocorticoid dose thresholds associated with all-cause and cardiovascular mortality in rheumatoid arthritis. *Arthritis Rheumatol.* **2014**, *66*, 264–272.
13. Van Staa, T.P.; Laan, R.F.; Barton, I.P.; et al. Bone density threshold and other predictors of vertebral fracture in patients receiving oral glucocorticoid therapy. *Arthritis Rheumatol.* **2003**, *48*, 3224–3229.
14. Caramaschi, P.; Biasi, D.; Dal Forno, I.; et al. Osteonecrosis in systemic lupus erythematosus: An early, frequent, and not always symptomatic complication. *Autoimmune Dis.* **2012**, 725249.
15. Wilson, J.C.; Sarsour, K.; Gale, S.; et al. THU0172 Risk for Serious Adverse Events Associated with Oral Corticosteroid Therapy in Patients with Rheumatoid Arthritis: A Uk Population Based Study 2016. *Ann. Rheum. Dis.* **2016**, *75*, 246–247.
16. Juge, P.A.; Lee, J.S.; Lau, J.; et al. Methotrexate and rheumatoid arthritis associated interstitial lung disease. *Eur. Respir. J.* **2021**, *57*, 2000337.
17. Gilani, S.T.A.; Khan, D.A.; Khan, F.A.; et al. Adverse effects of low dose methotrexate in rheumatoid arthritis patients. *J. Coll. Physicians Surg. Pak.* **2012**, *22*, 101–104.
18. Shin, K.; Baek, H.J.; Kang, Y.M.; et al. Efficacy and safety of add-on tacrolimus versus leflunomide in rheumatoid arthritis patients with inadequate response to methotrexate. *Int. J. Rheum. Dis.* **2019**, *22*, 1115–1122.
19. Aletaha, D.; Stamm, T.; Kapral, T.; et al. Survival and effectiveness of leflunomide compared with methotrexate and sulfasalazine in rheumatoid arthritis: A matched observational study. *Ann. Rheum. Dis.* **2013**, *62*, 944–951.

20. Jiang, M.; Zha, Q.; He, Y.; et al. Risk factors of gastrointestinal and hepatic adverse drug reactions in the treatment of rheumatoid arthritis with biomedical combination therapy and Chinese medicine. *J. Ethnopharmacol*. **2012**, *141*, 615–621.
21. Chen, Z.; Li, X.P.; Li, Z.J.; et al. Reduced hepatotoxicity by total glucosides of paeony in combination treatment with leflunomide and methotrexate for patients with active rheumatoid arthritis. *Int. Immunopharmacol.* **2013**, *15*, 474–477.
22. Hänninen, O.; Kaartinen, K.; Rauma, A.L.; et al. Antioxidants in vegan diet and rheumatic disorders. *Toxicology* **2000**, *155*, 45–53.
23. Quinonez-Flores, C.M.; Gonzalez-Chavez, S.A.; Del Rio Najera, D.; et al. Oxidative stress relevance in the pathogenesis of the rheumatoid arthritis: A systematic review. *Biomed. Res. Int.* **2016**, 097417.
24. Fernández-Llanio Comella, N.; Fernández Matilla, M.; Castellano Cuesta, J.A. Have complementary therapies demonstrated effectiveness in rheumatoid arthritis? *Reumatol. Clin.* **2016**, *12*, 151–157.
25. Efthimiou, P.; Kukar, M.; MacKenzie, C.R. Complementary and alternative medicine in rheumatoid arthritis: No longer the last resort! *HSS J*. **2010**, *6*, 108–111.
26. Salaffi, F.; Ciapetti, A. Clinical disease activity assessments in rheumatoid arthritis. *Int. J. Clin. Rheumatol*. **2013**, *8*, 347–360.
27. Duarte-García, A.; Leung, Y.Y.; Coates, L.C.; et al. Endorsement of the 66/68 joint count for the measurement of musculoskeletal disease activity: OMERACT 2018 psoriatic arthritis workshop report. *J. Rheumatol*. **2019**, *46*, 996–1005.
28. Wells, G.; Becker, J.C.; Teng, J.; et al. Validation of the 28-joint Disease Activity Score (DAS28) and European League Against Rheumatism response criteria based on C-reactive protein against disease progression in patients with rheumatoid arthritis, and comparison with the DAS28 based on erythrocyte sedimentation rate. *Ann. Rheum. Dis*. **2009**, *68*, 954–960.
29. Seror, R.; Tubach, F.; Baron, G.; et al. Measure of function in rheumatoid arthritis: Individualised or classical scales? *Ann. Rheum. Dis*. **2010**, *69*, 97–101.
30. Felson, D.T.; LaValley, M.P. The ACR20 and defining a threshold for response in rheumatic diseases: Too much of a good thing. *Arthritis Res. Ther*. **2014**, *16*, 101–106.
31. Goldbach-Mansky, R.; Wilson, M.; Fleischmann, R.; et al. Comparison of *Tripterygium wilfordii* Hook F versus sulfasalazine in the treatment of rheumatoid arthritis: A randomized trial. *Ann. Intern. Med.* **2009**, *151*, 229–240.
32. Tao, X.; Younger, J.; Fan, F.Z.; et al. Benefit of an extract of *Tripterygium wilfordii* Hook F in patients with rheumatoid arthritis: A double-blind, placebo-controlled study. *Arthritis Rheum.* **2002**, *46*, 1735–1743.
33. Molnar, V.; Matišić, V.; Kodvanj, I.; et al. Cytokines and chemokines involved in osteoarthritis pathogenesis. *Int. J. Mol. Sci*. **2021**, *22*, 9208.
34. Ingegnoli, F.; Castelli, R.; Gualtierotti, R. Rheumatoid Factors: Clinical Applications. *Dis. Markers* **2013**, *35*, 727–734.
35. Lv, Q.W.; Zhang, W.; Shi, Q.; et al. Comparison of *Tripterygium wilfordii* Hook F with methotrexate in the treatment of active rheumatoid arthritis (TRIFRA): A randomised, controlled clinical trial. *Ann. Rheum. Dis*. **2015**, *74*, 1078–1086.
36. Cibere, J.; Deng, Z.; Lin, Y.; et al. A randomized double blind, placebo controlled trial of topical *Tripterygium wilfordii* in rheumatoid arthritis: Reanalysis using logistic regression analysis. *J. Rheumatol*. **2003**, *30*, 465–467.
37. Piao, X.; Zhou, J.; Xue, L. Triptolide decreases rheumatoid arthritis fibroblast-like synoviocyte proliferation, invasion, inflammation and presents a therapeutic effect in collagen induced arthritis rats via inactivating lncRNA RP11-83J16. 1 mediated URI1 and β-catenin signaling. *Int. Immunopharmacol*. **2021**, *99*, 108010.
38. Shan, Y.; Zhao, J.; Wei, K.; et al. A comprehensive review of *Tripterygium wilfordii* hook. f. in the treatment of rheumatic and autoimmune diseases: Bioactive compounds, mechanisms of action, and future directions. *Front. Pharmacol*. **2023**, *14*.
39. Luo, J.; Jin, D.-E.; Yang, G.-Y.; et al. Total glucosides of paeony for rheumatoid arthritis: A protocol for a systematic review. *BMJ Open* **2016**, *6*, e010116.
40. Feng, Z.-T.; Xu, J.; He, G.-C.; et al. A systematic review and meta-analysis of the clinical efficacy and safety of total glucosides of peony combined with methotrexate in rheumatoid arthritis. *Clin. Rheumatol*. **2018**, *37*, 35–42.
41. Huang, Y.; Wang, H.; Chen, Z.; et al. Efficacy and safety of total glucosides of paeony combined with methotrexate and leflunomide for active rheumatoid arthritis: A meta-analysis. *Drug Des. Devel. Ther*. **2019**, *13*, 1969–1984.
42. Zhang, L.; Wei, W. Anti-inflammatory and immunoregulatory effects of paeoniflorin and total glucosides of paeony. *Pharmacol. Ther*. **2020**, *207*, 107452.
43. Čabarkapa, A.; Živković, L.; Borozan, S. Dry olive leaf extract in combination with methotrexate reduces cell damage in early rheumatoid arthritis patients—A pilot study. *Phytother. Res.* **2016**, *30*, 1615–1623.
44. Nediani, C.; Ruzzolini, J.; Romani, A.; et al. Oleuropein, a bioactive compound from *Olea europaea* L., as a potential preventive and therapeutic agent in non-communicable diseases. *Antioxidants* **2019**, *8*, 578.
45. Savairam, V.D.; Patil, N.A.; Borate, S.R.; et al. Allicin: A review of its important pharmacological activities. *Pharmacol. Res. – Mod. Chin. Med*. **2023**, *8*, 100283.

46. Zugravu, G.S.; Pintilescu, C.; Cumpat, C.-M.; et al. Silymarin supplementation in active rheumatoid arthritis: Outcomes of a pilot randomized controlled clinical study. *Medicina* **2024**, *60*, 999.
47. Wu, W.T.; Chen, Y.R., Lu, D.H.; et al. Silymarin modulates catabolic cytokine expression through Sirt1 and SOX9 in human articular chondrocytes. *J. Orthop. Surg. Res.* **2021**, *16*, 1–9.
48. Gupta, O.P.; Sing, S.; Bani, S.; et al. Anti-inflammatory and anti-arthritic activities of silymarin acting through inhibition of 5-lipoxygenase. *Phytomedicine* **2000**, *7*, 21–24.
49. Saber, M.S.; Fahim, H.I.; Ahmed, O.M.; et al. Assessment of the preventive effects of *Silybum marianum* (L.) Gaertn. seeds hydroethanolic extract and silymarin on complete Freund's adjuvant induced arthritis in Wistar rats. *Ann. Phytomed.* **2020**, *9*, 172–182.
50. Olas B. The beneficial health aspects of sea buckthorn (*Elaeagnus rhamnoides* (L.) A. Nelson) oil. *J. Ethnopharmacol*. **2018**, *213*, 183–190.
51. Zielińska, A.; Nowak I. Abundance of active ingredients in sea-buckthorn oil. *Lipids Health Dis.* **2017**, *16*, 95.
52. Solà Marsiñach, M.; Cuenca A.P. The impact of sea buckthorn oil fatty acids on human health. *Lipids Health Dis.* **2019**, *18*, 145.
53. Zugravu, G.S.; Dimitriu, D.C.; Ciobanu, C.P.; et al. Benefits of sea buckthorn fruit oil as adjuvant therapy in rheumatoid arthritis. *Farmacia* **2024**, *72*, 554–564.
54. Zugravu, G.S.; Pintilescu, C.; Cumpat, C.-M.; et al. Sea buckthorn fruit oil supplementation in active rheumatoid arthritis: A focus on composite disease activity indices and EULAR response. *Med. Surg. J. – Rev. Med. Chir. Soc. Med. Nat., Iasi* **2024**, *128*, 422–436.
55. Santa-Maria, C.; López-Enríquez, S.; Montserrat-de la Paz, S.; et al. Up-date on anti-inflammatory molecular mechanisms induced by oleic acid. *Nutrients* **2023**, *15*, 224.
56. Passos, M.E.P.; Alves, H.H.O.; Momesso, C.M.; et al. Differential effects of palmitoleic acid on human lymphocyte proliferation and function. *Lipids Health Dis*. **2016**, *15*, 217.
57. Talbot, N.A.; Wheeler-Jones, C.P.; Cleasby, M.E. Palmitoleic acid prevents palmitic acid-induced macrophage activation and consequent p38 MAPK-mediated skeletal muscle insulin resistance. *Mol. Cell. Endocrinol*. **2014**, *393*, 129–142.
58. Devaraj, S.; Jialal, I. α-Tocopherol decreases interleukin-1β release from activated human monocytes by inhibition of 5-lipoxygenase. *Arterioscler. Thromb. Vasc. Biol*. **1999**, *19*, 1125–1133.
59. Bai, S.K.; Lee, S.J.; Na, H.J.; et al. β-Carotene inhibits inflammatory gene expression in lipopolysaccharide-stimulated macrophages by suppressing redox-based NF-κB activation. *Exp. Mol. Med.* **2005**, *37*, 323–334.
60. Kawata, A.; Murakami, Y.; Suzuki, S.; et al. Anti-inflammatory activity of β-carotene, lycopene and tri-n-butylborane, a scavenger of reactive oxygen species. *In Vivo* **2018**, *32*, 255–264.
61. Liao, P.C.; Lai, M.H.; Hsu, K.P.; et al. Identification of β-sitosterol as in vitro anti-inflammatory constituent in *Moringa oleifera*. *J. Agric. Food Chem.* **2018**, *66*, 10748–10759.
62. Liu, R.; Hao, D.; Xu, W.; et al. β-Sitosterol modulates macrophage polarization and attenuates rheumatoid inflammation in mice. *Pharm. Biol*. **2019**, *57*, 161–168.
63. Camillo, L.; Grossini, E.; Farruggio, S.; et al. Alpha-tocopherol protects human dermal fibroblasts by modulating nitric oxide release, mitochondrial function, redox status, and inflammation. *Skin Pharmacol. Physiol.* **2022**, *35*, 1–12.
64. Ghavipour, M.; Sotoudeh, G.; Tavakoli, E.; et al. Pomegranate extract alleviates disease activity and some biomarkers of inflammation and oxidative stress in Rheumatoid Arthritis patients. *Eur. J. Clin. Nutr*. **2017**, *71*, 92–96.
65. Golmei, P.; Kasna, S.; Roy, K.P.; et al. Review on pharmacological advancement of ellagic acid. *J. Pharmacol. Pharmacother*. **2024**, *15*, 93–104.
66. Yeoh, N.; Burton, J.P.; Suppiah, P.; et al. The role of the microbiome in rheumatic diseases. *Curr. Rheumatol. Rep*. **2013**, *15*, 314.
67. Thimóteo, N.S.B.; Iryioda, T.M.V.; Alfieri, D.F.; et al. Cranberry juice decreases disease activity in women with rheumatoid arthritis. *Nutrition* **2019**, *60*, 112–117.
68. Mur, E.; Hartig, F.; Eibl, G.; et al. Randomized double blind trial of an extract from the pentacyclic alkaloid-chemotype of *Uncaria tomentosa* for the treatment of rheumatoid arthritis. *J. Rheumatol*. **2002**, *29*, 678–681.
69. Willich, S.N.; Rossnagel, K.; Roll, S.; et al. Rose hip herbal remedy in patients with rheumatoid arthritis – a randomised controlled trial. *Phytomedicine* **2010**, *17*, 87–93.
70. Winther, K.; Apel, K.; Thamsborg, G. A powder made from seeds and shells of a rose-hip subspecies (*Rosa canina*) reduces symptoms of knee and hip osteoarthritis: A randomized, double-blind, placebo-controlled clinical trial. *Scand. J. Rheumatol*. **2005**, *34*, 302–308.