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Article

Drug Standardization, HPTLC Finger Printing, Toxicity Research Studies of ASU Herbaceous Plant Fruit Seeds Part Samples of *Ipomoea nil* (Linn.) Roth

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Abstract: This study aims to evaluate the drug standardization research, physico-chemical, toxicity research studies of the fruit seeds part of plant of I N. drug standardization research, physico-chemical, toxicity research studies of ASU (*Ayurveda, Siddha, Unani*) herbal products remains a big challenging task. There needs to be more than the advance investigation research studies and screening parameters to validation, authenticate and differentiate adulterants in *Ipomoea nil* (Linn.) Roth medicinal plant is one of the herb used to treat various health wellness and therapeutic illness of public mankind. Plant samples of I N. powder were carried out using standard methods. The Quality, safety and toxicity effects of the tested drug samples were also investigated. estimated and investigated research studies data's of I N. have shown that all the parameters were within the AYUSH/WHO permissible limits. The tested drug samples showed significant Quality, safety and toxicity studies against certain pathogens organisms and promising anti-pathogenic activity. In the investigated studies of DSR, HPTLC finger printing investigation, QC, Toxicity research findings revealed that the revalidated test drug samples was free from adulterations. This investigated herb research data confirmed to drug standardization and therapeutically may treat that the drug is safe for internal use and cures in Antioxidant, Anti-Inflammatory, Antimicrobial, Antispasmodic, Anticancer, Anti-tumor, Antiproliferative Activities, Multidrug-Resistance Efflux-Inhibiting Activity, bronchodilatory along with Antiasthmatic potential, Diuretic, anthelminthic and deobstruant and are prescribed for dropsy and constipation, Antihypertensive and cardio-protective effects, Investigated drug had classically, traditional and alternative ASU medicine I N. used as a Cold, Dropsy, Gout, Joint pain, Vitiligo, Itching, Asthma and Anthelmintic.

Keywords: *Ipomoea nil* (Linn.) Roth. DSR; HPTLC finger printing; QC and QA; Toxicity; Pharmacological Quality; safety and toxicity studies

1. Introduction

The World Health Organization (WHO) reports that herbal medicines are able to fulfill the health requirements of approximately 80% of the global population, particularly those residing in rural regions of developing nations. The quality assurance and quality control of herbal crude drugs and formulated products are important in justifying their acceptability in modern system of medicine. Hence it is required to conduct the research on drugs standardization and product validation to provide effective, curable and safe drugs to the needy mass suffering from various ailments [1–10]. I N. (Convolvulaceae) is known as ivy morning glory; seed usage is common, and flowers are produced from June to October. It is indigenous to Punjab and distribution is in Pakistan in addition to India. Folkloric reputation as anti-inflammatory, blood purifier, anthelmintic, astringent, laxatives, anti-emetic, carminatives and is considered useful to treat a series of ailments like abdominal diseases, asthma, hypertension, disease of liver and joints, drying the phlegm [3,11,12]. Investigated I N herbaceous plant, Ivy leaf morning-glory, an annual climber is locally known as "Kaladana". Commonly found in gardens and wild fields, areas along railway tract and roadsides. Cambodia, China, Columbia, England, Japan, Korea, Mexico, Nigeria and Philippines [3,13]. The top role of these mediators is central in asthmatic problems, as labelled in miscellaneous

studies beforehand [3,11]. Epithelial cells, pseudo stratified cells, dendritic cells, mast cells and mucus secreting cells are present in the airway's lumen. Vagus sub-epithelial cells, in addition to receptors, discharge acetylcholine on bronchial muscles. Parasympathetic action cause airways to narrow, so, when this is going to antagonize, generate dilatation of airways so anti-muscarinics are used in asthma, COPD. and other respiratory infections [3,11]. Different medicines, e.g., short and/long acting beta 2 agonists, and corticosteroids have been engaged as therapeutic agents as desirable treatment plus prevention for these sorts of disorders to treat broncho-constriction, asthma and lung dam age [3,11]. However, tenacious treatments, besides their immediate termination outcomes as side effects, may demonstrate as being lethal for the social life cycle and express necessity to sightsee traditional medicinal flora to discover new reliable along with life-saving medicinal elements [3,11]. erectile dysfunction and for lubrication obligations. Application of seeds paste is used for cosmetic purposes i.e., dry skin, freckles, etc. In addition, it is also hepato protective, anti-diabetes, anti-interleukin-8 (IL-8), and anti-inflammatory [3,11]. Phyto analysis exposed the occurrence of alkaloids—(chanoclavine-18, penniclavine, elymoclavine)-methyl-oxybutyric acid, pharbitinic acid, phytoecdysone, tiglic acid, plytosteratin, lysergol [14], ecdysteriods, hederasterones, stigmasterol 3-O-D-glucoside, 20-hydroxyecdysone, sitosterol-3-O-D-glucoside, ethylcaffeate, anthocyanin,i.e., cyanidin, cyanidin 3-sophoroside and-d glucopyranoside [3,11]. The utmost contemporary investigated phytochemicals are hederaceterpenol, hedera terpenoside, triterpenoid plus stigmast-5-en-3-O--D-glucopyranoside [3]. The aim of the current research effort on the aqueous-methanolic crude extract of I N. seeds was commenced to observe its antioxidant, bronchodilator, anti-asthmatic and enzyme inhibition action with mechanistic approaches [11]. The seeds are diuretics (antihypertensive), anthelmintic, aphrodisiacs, blood purifiers, carminative, laxative and useful for headache, abdominal inflammation, bronchitis, hearts diseases and high blood pressure. Seeds are also prescribed to induce menstruation and cause abortion in high doses. Seeds are also useful in gout, scabies and leucoderma. Whereas, few phytochemical compounds including chanoclavine I, elymoclavine, lysergol, penniclavine and isopenniclavine have also been reported previously, antifungal and antibacterial, analgesic and CNS stimulant, Flavonoids, saponins, tannins, mucilage and proteins. Phytochemical studies have revealed the presence of different phytochemical classes including alkaloids, reducing sugars, terpenoids. Hepato-protective. and treatment of skin diseases [3,12,14–17]. Its quality, safety, and efficacy are affected due to adulterants and contamination of herbal products; therefore, its purity, safety, potency, and efficacy are major problems associated with the quality of ingredients. The regulatory bodies will have to ensure that medications given to consumers are of good quality with assurance. The regulatory authority should implement good manufacturing practices at the manufacturing operation unit and develop a quality control unit for raw materials and finished products as per the Pharmacopoeia [3,7,18]. The World Health Organization (WHO) Assembly and Ayurvedic Pharmacopeia Commission have expressed the need to use modern technology and appropriate standards like HPLC, HPTLC, and Spectroscopy to ensure the quality of Ayurvedic medicines and their products [19–21]. The study plant I N. fruit seeds parts active phytochemical constituents shown as Secondary metabolites, such as Alkaloids, Triterpenes, Flavonoids, Norisoprenoids, Glycosides, Tannins, Coumarins, Carbohydrates, Phenols, Saponins, Phlobatannins, and Steroids, Steroids/triterpenes, alkaloids, glycosides, flavonoids, resins, reducing sugars, tannins, proteins, fibers, lipids, carbohydrates, minerals (potassium, calcium, phosphorus and magnesium), amino acids, fatty acids (palmitic acid, linoleic acid, linolenic acid, and oleic acid) and seed oils [3,14,22]. Plant extract was found to contain alkaloids, saponins, anthraquinones, sterols, tannins, coumarins, flavonoids and terpenes [3,11]. Alkaloids, coumarins, anthraquinones, saponins, tannins, flavonoids, polyphenolics compounds and terpenes as potential foremost elements of the plant [3,11]. Steroids/triterpenes, alkaloids, glycosides, flavonoids, resins, reducing sugars, tannins, proteins, fibers, lipids, carbohydrates, minerals (potassium, calcium, phosphorus and magnesium), amino acids, fatty acids (palmitic acid, linoleic acid, linolenic acid, and oleic acid) and seed oils [3,14,15,17] and their therapeutics medicinal potential and pharmacological activities shown as Antioxidant, Anti-Inflammatory, Antinociceptive, Antimicrobial, Collagenase Inhibitory, Antispasmodic, Anticancer, Antitumor, Antiproliferative Activities, Multidrug-Resistance Efflux-Inhibiting Activity [3,14,22]. Antioxidant, bronchodilatory along with Anti-asthmatic potential [3,11,14,23]. Diuretic, anthelminthic and deobstruant and are prescribed for dropsy and constipation, and to promote menstruation and cause abortion [3,14,15,17]. Anti- hypertensive and cardio-protective effects [3,13,15]. I N. Investigated herbasious medicinal plant studies Graphical Illustration, Confirmation and identification shown in Figure 1, respectively.

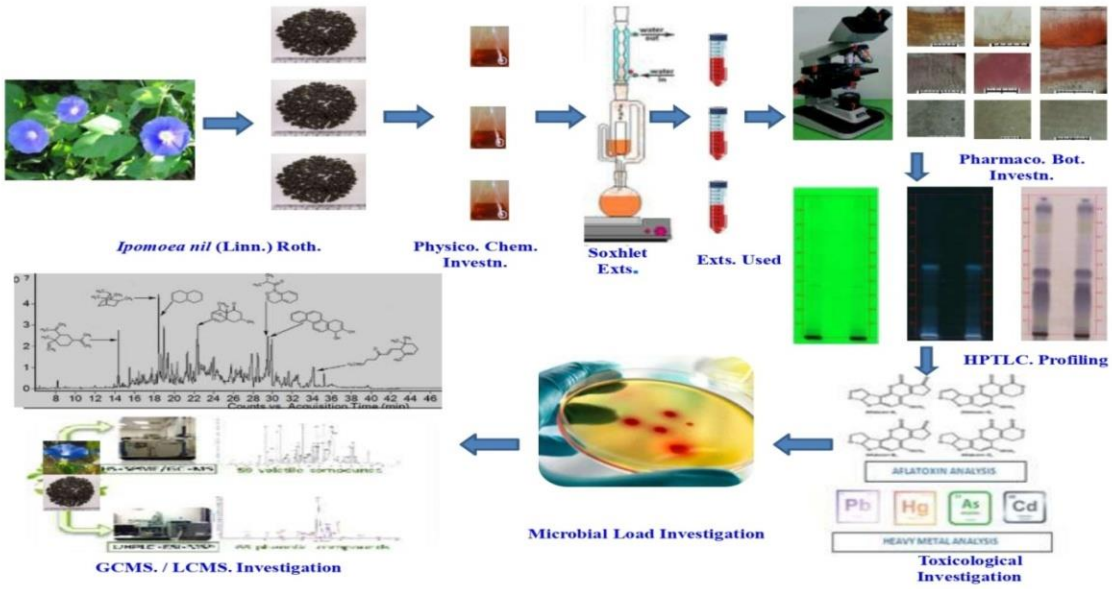


Figure 1. Graphical Illustration.

Geographical, biodiversity and Natural Occurrence: The investigated herbaceous medicinal plant Fruit seed part of I N. was shown and worldwide occurred and found in Alabama, Angola, Bangladesh, Bermuda, Burkina, California, Cambodia, Cameroon, Cape Provinces, Central African Republic, Chad, China North-Central, China South-Central, China Southeast, Christmas I., Comoros, Congo, Equatorial Guinea, Eritrea, Ethiopia, Florida, Ghana, Greece, Guinea, Gulf of Guinea Is., Hainan, India, Inner Mongolia, Ivory Coast, Jawa, Korea, KwaZulu-Natal, Laos, Lesser Sunda Is., Louisiana, Madagascar, Malaya, Maluku, Maryland, Mauritius, Myanmar, Namibia, Nansei-shoto, Nepal, New Caledonia, New Guinea, Nigeria, North Carolina, Northern Territory, Oman, Pakistan, Philippines, Queensland, Rodrigues, Réunion, Saudi Arabia, Senegal, Seychelles, Sierra Leone, Socotra, South China Sea, Sri Lanka, Sudan, Sulawesi, Sumatera, Tanzania, Texas, Thailand, Tibet, Uganda, Vietnam, Western Australia, Yemen, Zambia, Zaïre, Zimbabwe, East Himalaya and West Himalaya of Indian, Southern region of India and Pakistan, Asian region climatically and biodiversity presence appeared of herbaceous medicinal plant I N. having investigated bioactive phytochemacal constituents with immense pharmacological action properties, medicinal and therapeutic potential [3,11,12,14,15,17].

2. Material and Method

Taxonomical and Pharmacognostical Studies: The fruits seeds samples of the plant I N. were procured from the local market of Central NCR Region, New Delhi, India, Southern Region, Tamil Nadu State, India, Northern Region of Uttarakhand State, Indiaand authenticated by Botany and Pharmacognosy Laboratory Section, Researcher Scientific Staff of Regional Research Institute of Unani Medicine, Royapuram, TN. State, Drug Standardization Research Institute, Ghaziabad, UP. and PCIM&H, Ghaziabad UP.

2.1. Collection and Authentication of Ipomoea nil Samples

Fresh *Ipomoea nil* (L.) Roth samples were collected from [Delhi NCR, Chennai and Haridwar commercial source] under controlled conditions. The plant material was authenticated by a qualified taxonomist at [RRIUM Chennai], following standard botanical identification keys and comparison with herbarium specimens. A voucher specimen (No: RRIUM(C)13718) was deposited in the herbarium for future reference. The authentication was based on macroscopic and organoleptic characteristics, including leaf morphology, venation patterns, and seed characteristics.

Standard Methods applied for Detection and investigation of Physicochemical, HPTLC Fingerprinting, Toxicology parameters used and applied Advance sophisticated Instruments and operating parameters, detections and investigations of Heavy Metals-Pb, As, Cd, Hg by Atomic Absorption Spectrometer (AAS-GF), Aflatoxins-B1, B2 and G1, G2 were estimated by Kobra cell techniques. Pesticide residues and Microbial Load contamination-TBC/TFC detect in cfu/gm. *Escherichia coli*, *Salmonella typhai* spp. *Staphylococcus aurous*author pathogenic, detection and estimation in Heavy Metals-Pb, Hg, Cd and As, Aflatoxins B1, B2 and G1, G2 andPesticide Residues-Organo chlorine, pesticides, Organo phosphorus pesticides, Pyrethroids etc, in ppm levels concentrations

as per WHO/AOAC/AYUSH-API/UPI Pharmacopeial permissible limits and standard methods basis [1–10,12,14,19,24–38].

2.2. Physicochemical Parameter Analysis Studies

2.2.1. Moisture Content

The Loss on Drying (LOD), (*w*/*w*)%, method was used to determine moisture content by heating the sample at 105 °C until a constant weight was achieved, ensuring stability and preventing microbial contamination.

2.2.2. Ash Value Analysis

Ash content was evaluated to detect inorganic impurities and ensure sample purity:

- Total Ash, (*w*/*w*)%—Indicates overall mineral content by incinerating the sample at 550 °C.
- Acid-Insoluble Ash, (*w*/*w*)%—Assesses contamination from siliceous matter such as sand or soil by treating the total ash with hydrochloric acid.

2.2.3. Extractive Values, (*w*/*v*)%

Extractive values were determined to quantify the amount of active phytoconstituents soluble in specific solvents:

- Alcohol-Soluble Extractive—Indicates the presence of polar and moderately nonpolar compounds.
- Water-Soluble Extractive—Represents the proportion of water-soluble constituents.

2.2.4. pH Analysis

pH Determination

The pH of aqueous extracts was measured to assess the acidity or alkalinity of the plant material, which can influence stability and bioavailability.

- Method: pH was determined using a digital pH meter calibrated with standard buffer solutions (pH 4.0, 7.0, and 9.2).
- Procedure: A 1% *w*/*v* aqueous extract of *Ipomoea nil* was prepared and measured at room temperature (25 °C ± 2 °C).
- Regulatory Range: As per pharmacopeial standards, ensuring consistency and quality of herbal formulations.

3. HPTLC Fingerprinting Analysis

3.1. Sample Preparation

Dried *Ipomoea nil* seeds were powdered and subjected to Soxhlet extraction using 100% ethanol as the solvent. The extract was concentrated under reduced pressure, filtered, and stored at 4 °C for analysis.

3.2. Chromatographic Conditions

HPTLC analysis was performed using a CAMAG TLC system with a Linomat 5 sample applicator, TLC scanner, and Win CATS software.

- Stationary Phase: Pre-coated silica gel 60 F254 plates.
- Mobile Phase: Optimized solvent system (Toluene: Ethyl Acetate: Formic acid) in 9:1:0.5 ratio.
- Sample Application: 10 µL of extract was applied in 6 mm bands at a distance of 10 mm from the plate edges.
- Development Chamber: Twin-trough glass chamber, pre-saturated with mobile phase vapor for 20 min.
- Development Distance: 80 mm.
- Detection:
  - UV Light: 254 nm (shortwave) and 366 nm (longwave).
  - Post-Derivatization: Anisaldehyde–Sulfuric Acid reagent was used for visualizing chemical components.
- Retention Factor (R^f) Values: Used for fingerprinting and comparison with reference standards.
- Densitometric Scanning: Performed at 366 nm to quantify specific bioactive markers.

4. Toxicity and Quality Control Measurement Studies

To ensure the safety and purity of *Ipomoea nil*, comprehensive quality control parameters were analyzed, including heavy metal analysis, pesticide residue screening, aflatoxin contamination assessment, and microbial load evaluation.

4.1. Heavy Metal Analysis

The presence of toxic heavy metals (Lead [Pb], Arsenic [As], Cadmium [Cd], and Mercury [Hg]) was assessed using Atomic Absorption Spectroscopy (AAS).

- Permissible Limits: As per WHO standards.

4.2. Pesticide Residue Analysis

Pesticide contamination was analyzed using Gas Chromatography-Mass Spectrometry (GC-MS/MS).

- Screening for all the 34 Pesticides as mentioned in UPI and API like Organophosphates, Organochlorines, Pyrethroids, and Carbamates.
- Detection Limits: As per WHO guidelines.

4.3. Aflatoxin Contamination

Aflatoxins (B1, B2, G1, and G2) were analyzed by employing Kobra cell techniques.

- Regulatory Limits: Aflatoxin B1 < 5 ppb; Total Aflatoxins < 10 ppb (as per WHO standards).

4.4. Microbial Load Assessment

Microbial contamination was assessed as per Pharmacopeial Microbial Limits using standard culture methods.

- Total Bacterial Count (TBC) and Total Fungal Count (TFC) were determined in colony-forming units per gram (CFU/g).
- Pathogenic microorganisms were screened to ensure microbiological safety, including:
  - *Escherichia coli*, *Salmonella typhi* spp., *Staphylococcus aureus*
- Acceptable Limits: As per WHO, Unani Pharmacopoeia India and Ayurvedic Pharmacopoeia India guidelines.

5. Toxicological Investigation Study

Standard Methods applied for Detection and investigation of HPTLC Fingerprinting, Toxicology parameters used and applied of Advance sophisticated Instruments and operating parameters detections and investigations of Heavy Metals—Pb, As, Cd, Hg in ppm. levels by Thermo Fisher M Series, 650902 V1.27 model Atomic Absorption Spectrometer with Graphite Furnace (AAS-GF), Aflatoxins—B1, B2 and G1, G2 in ppm. levels were estimated by Kobra cell techniques using Agilent HPLC or CAMAG or Anchrom HPTLC instrument, Revolution Front (R^f) values detect in cm. range, Detector—UV-Visible detector, Detector temperature: 25 °C, Sample injector volume range 5.0 µL to 20 µL range, Pesticide residues in mg/kg and bioactive components were analyzed using Gas Chromatography Mass Spectra (GC-MS) (Instrument-Thermo Scientific, Model TSQ9000, Waltham, MA, USA), detector-mass selective detector or Triple Quadrupole mass analyzer detector, column specification-TG-5MS/Run time - usually for the investigated compounds vary according to the method of GC and temperature programming whereas roughly all the relevant bio active phyto-chemical constituents usually appear in an around 0–50 min in the GC column i.e., the Retention time, Run time usually varies with the method and GC temperature program. Mass value range 0.0 to 650 amu, Sample injector volume range 1.0 µL to 5.0 µL. Microbial Load contamination-TBC/TFC detect in cfu/gm. *Escherichia coli*, *Salmonella typhai* Spp. *Staphylococcus aurous* author pathogenic, detection and estimation in Heavy Metals-Pb, Hg, Cd and As, Aflatoxins B1, B2 and G1, G2 and Pesticide Residues- Organo chlorine, pesticides, Organo phosphorus pesticides, Pyrethroids etc., in ppm levels concentrations as per WHO/AOAC/AYUSH-API/UPI Standard methods basis [1–10,12,14,19,24–38].

6. Result and Discussion

6.1. Identification of Key Marker Compounds

Comparative analysis with established reference standards facilitated the tentative identification of key marker compounds in *Ipomoea nil*. The chemical nature of these constituents was inferred based on R^f values, spectral characteristics, and prior literature reports. Shown in Table 1. The presence of flavonoids, alkaloids, phenolic acids, and triterpenoids suggests the potential therapeutic efficacy of *Ipomoea nil* in traditional medicine, supporting its pharmacological applications in *Ayurveda, Siddha, and Unani* (ASU) medicine.

Table 1. key marker compounds in *Ipomoea nil*.

R^f Value's	Putative Compound		Pharmacological Relevance
0.32	Flavonoid glycoside	-	Antioxidant, anti-inflammatory
0.58	Phenolic acid	-	Antimicrobial, hepatoprotective
0.75	Alkaloid derivative	-	Neuroprotective, hypotensive
0.89	Glycoside	-	Cardioprotective, adaptogenic
0.65	Triterpenoid	-	Immunomodulatory, wound healing
0.78	Steroidal compound	-	Hormonal modulation, analgesic

6.2. Comparative Analysis with Existing Literature

The fingerprinting results were compared with previously reported HPTLC profiles of *Ipomoea nil* and related Convolvulaceae species to establish consistency in phytochemical markers and therapeutic applications.

- Flavonoids (R^f - 0.32, 0.75): Consistent with prior studies reporting quercetin and kaempferol derivatives in *Ipomoea* spp., known for antioxidant and anti-inflammatory activities [11,39].
- Steroidal compounds (R^f - 0.78): Align with ethno botanical studies indicating hormonal and anti-inflammatory effects in related *Ipomoea* species [11,15].
- Phenolic acids (R^f - 0.58, 0.89): Previously linked to hepatoprotective properties in the Convolvulaceae family [3,11,31]

The presence of characteristic marker compounds in this study reinforces prior findings on the phytochemical richness and medicinal potential of *Ipomoea nil*, supporting its therapeutic use in traditional medicine.

6.3. Implications for Standardization & Quality Control

The study contributes significantly to the quality assessment, authentication, and standardization of *Ipomoea nil* in herbal medicine.

6.4. Establishment of a Reference HPTLC Fingerprint

- The obtained HPTLC profile serves as a unique chemical fingerprint, ensuring authenticity and minimizing adulteration risks.
- The data can be utilized in pharmacopeial monographs for regulatory acceptance and standardization of *Ipomoea nil* extracts.

7. Quality Control Implementation

To ensure consistency, safety and toxicity in ASU., herbal formulations, quality control parameters were systematically evaluated.

Heavy Metal Analysis

- The levels of toxic metals (Lead [Pb], Arsenic [As], Cadmium [Cd], and Mercury [Hg]) were assessed using AAS, confirming compliance with WHO and USP limits.
- Ensures that *Ipomoea nil* extracts meet regulatory safety thresholds for heavy metal contamination.

Pesticide Residue Screening

- GC-MS analysis was employed to screen for pesticide residues, including organochlorines, organophosphates, pyrethroids, and carbamates.
- No detectable levels of harmful pesticide residues were found, supporting the safety of the plant material for therapeutic use.

Aflatoxin Contamination Assessment

- Kobra Test Method was used to detect aflatoxins (B1, B2, G1, G2).
- The levels were found to be within acceptable limits (<10 ppb), as per WHO guidelines.

Microbial Load and Pathogenic Contamination

Microbial safety was assessed using standard pharmacopeial methods, with results confirming the acceptability of *Ipomoea nil* for medicinal use:

7.1. Contribution to Herbal Standardization and Therapeutic Application

This study reinforces the scientific standardization of *Ipomoea nil* by providing a reliable HPTLC fingerprint and comprehensive quality control data, bridging the gap between traditional knowledge and modern pharmacognosy.

Key Contributions:

(1) Fingerprint Consistency: Ensures batch-to-batch reproducibility in herbal products. Marker Compound Standardization: Facilitates accurate quality assessment of commercial extracts.
Regulatory Compliance: Supports the inclusion of *Ipomoea nil* in pharmacopeial monographs (WHO, API, AYUSH).
(2) Therapeutic Validation: Aligns with phytochemical and pharmacological evidence, supporting traditional Ayurvedic and Unani medicine applications.
(3) Physicochemical Analysis
(4) The physicochemical parameters were analyzed to ensure consistency and purity. The results are summarized below:
(5) HPTLC Fingerprinting [12,37,38].
(6) The ethanolic extract of I N was analyzed using HPTLC to identify characteristic phytoconstituents. The results are presented in terms of R^f values under different detection methods [12,35–38].

7.2. Quality Control Measures

Microbial Load Analysis: Microbial contamination was evaluated using standard methods prescribed by WHO/AOAC/AYUSH/API/UPI. The results confirm that I N meets regulatory standards:

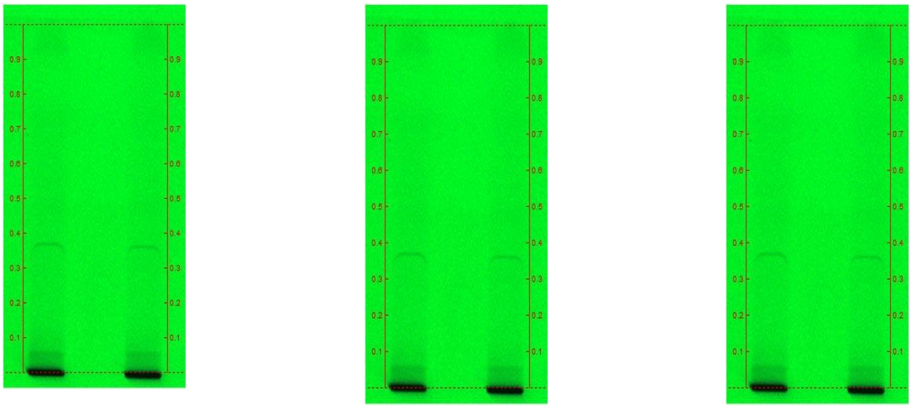
Heavy Metal Analysis: The concentration of heavy metals was evaluated using Atomic Absorption Spectroscopy (AAS-GF). The results indicate that all values fall within permissible limits:

Aflatoxin Contamination: The presence of aflatoxins was assessed using HPTLC, with no detectable levels observed.

Pesticide Residue Analysis: The analysis of pesticide residues by GC-MS confirmed no detectable residues in any sample.

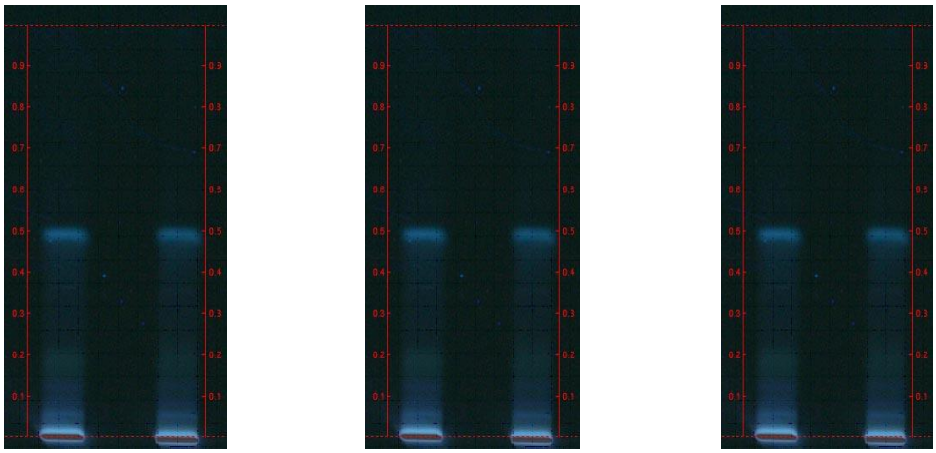
8. HPTLC Investigated Profiling

Extract 2 g of sample with 20 mL of alcohol separately and reflux on a water bath for 30 min. Filter and concentrate to 5 mL and carry out the thin layer chromatography. Applied Ethanol extract on precoated aluminium TLC plate of silica gel 60 F_{254} used HPTLC automatic sample applicator. Developed the plate in Toluene-Ethyl acetate-Formic Acid (9:1:0.5) solvent system. Allowed the plate to dried in air and examine under UV (254 nm). It shown 4 major spots at R^f - 0.93 (Green), 0.73 (Green), 0.46 (Green) and 0.36 (Dark green). Under UV (366 nm), it shown 1 major spot at R^f - 0.49 (Blue), and dipped the plate in 1% Vanillin—Sulphuric acid reagent followed by heated at 105 °C for 5 min and examined under visible light. Observed shown 6 major Spots at R_f 0.90 (Dark grey), 0.76 (Yellow), 0.43 (Dark grey), 0.36 (Dark grey), 0.28 (Dark grey) and 0.10 (Yellow). Shown in investigated Figures 2–4, their densitogram shown in Figures 5 and 6 respectively, R^f - values shown in Table 2 respectively. Resulted the all investigated Physicochemical, HPTLC Finger printing profiling data's, Toxicology research parameters found in the study medicinal plant shown complies and not found of any hazardous or highly toxic contamination in the investigated drug. It had fit for internal used. Investigated results shown in Tables 3–7 respectively [1–12,14,19,24–39].



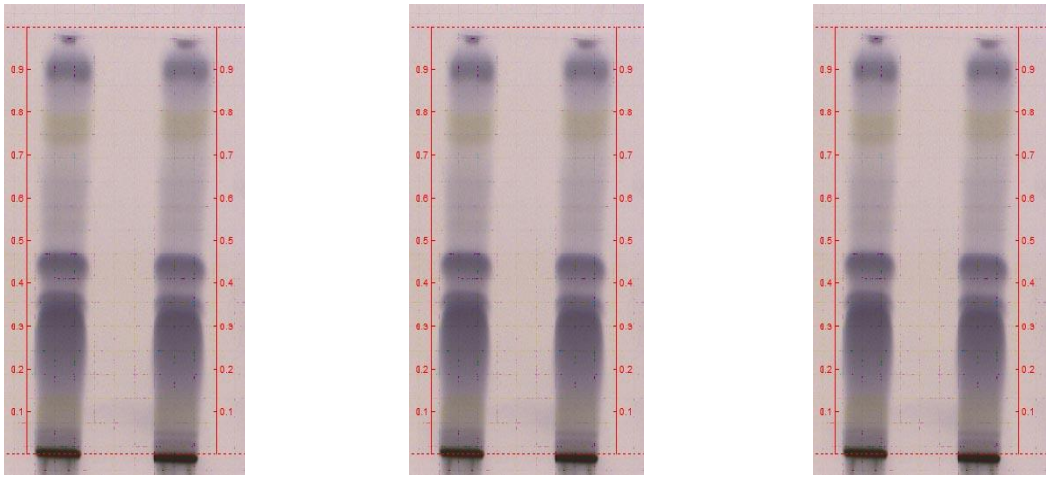
(**a**) (**b**) (**c**)

Figure 2. Thin layer chromatography (alcohol extract). (**a**) I N-1, UV-254 nm. (**b**) I N-2, UV-254 nm. (**c**) I N-3, UV-254 nm.



(**a**) (**b**) (**c**)

Figure 3. Thin layer chromatography (alcohol extract). (**a**) I N-1, UV-366 nm. (**b**) I N-2, UV-366 nm. (**c**) I N-3, UV-366 nm.



(**a**) (**b**) (**c**)

Figure 4. Thin layer chromatography (alcohol extract). (**a**) I N-1, V-S Reagent. (**b**) I N-2, V-S Reagent. (**c**) I N-3, V-S Reagent. Solvent System: Toluene:Ethyl acetate (9:1).

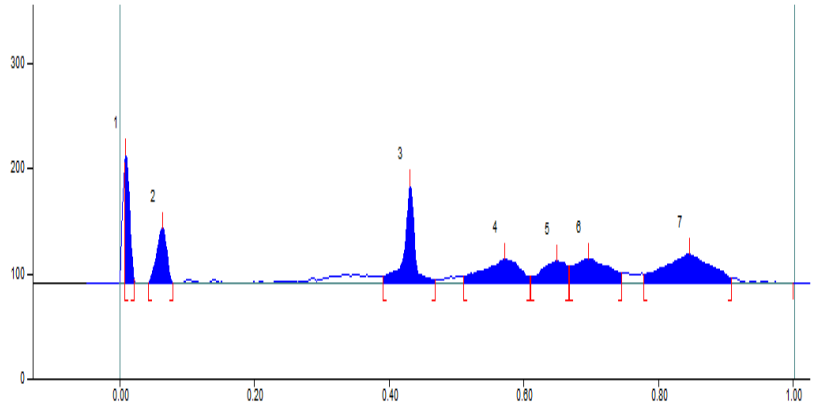


Figure 5. HPTLC finger print of Habb-ul-Neel—Alcohol extract at 254 nm.

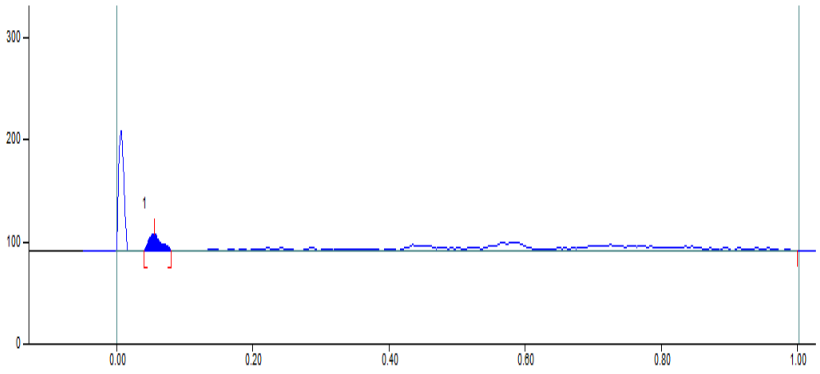


Figure 6. HPTLC finger print of Habb-ul-Neel—Alcohol extract at 366 nm.

Table 2. R^f values of Ethanolic extract: (By HPTLC).

Solvent System	R^f Values								
	UV Light at 254 nm			UV Light at 366 nm			1% *Vanillin—Sulphuric acid* Reagent		
Toluene: Ethyl acetate: Formic acid (9:1:0.5)	0.93 (Green)	0.92 (Green)	0.93 (Green)	0.49 (Blue)	0.48 (Blue)	0.49 (Blue)	0.90 (Dark grey)	0.89 (Dark grey)	0.90 (Dark grey)
	0.73 (Green)	0.72 (Green)	0.73 (Green)				0.76 (Yellow)	0.75 (Yellow)	0.76 (Yellow)
	0.46 (Green)	0.45 (Green)	0.46 (Green)				0.43 (Dark grey)	0.42 (Dark grey)	0.43 (Dark grey)
	0.36 (Dark green)	0.35 (Dark green)	0.36 (Dark green)				0.36 (Dark grey)	0.35 (Dark grey)	0.36 (Dark grey)
							0.28 (Dark grey)	0.27 (Dark grey)	0.28 (Dark grey)
							0.10 (Yellow)	0.09 (Yellow)	0.10 (Yellow)

Table 3. Physico-Chemical investigation tests.

Sr. No.	Analyzed Parameters	Results			Mean/Average Value
		I N-1	I N-2	I N-3	
1.	Colour	Brownish Black	Brownish Black	Brownish Black	Brownish Black
2.	Odour	No Characteristic	No Characteristic	No Characteristic	No Characteristic
3.	Taste	Sweetish Bitter	Sweetish Bitter	Sweetish Bitter	Sweetish Bitter
4.	Foreign matter, *w*/*w*%	ND	ND	ND	0.56%
5.	Loss in wt on drying at 105 °C	4.06%	4.14%	4.10%	3.81%
6.	Total Ash, *w*/*w*%	5.70%	5.76%	5.82%	5.51%
7.	Acid insoluble ash, *w*/*w*%	10.98%	10.96%	10.98%	4.20%
8.	Alcohol Soluble Extract, *w*/*v*%	10.98%	10.96%	10.98%	1.31%
9.	Water Soluble Extract, *w*/*v*%	26.95%	27.53%	27.34%	2.76%
10.	pH (1% Solution)	6.79	6.79	6.77	5.28
11.	pH (10% Solution)	5.94	5.94	5.92	5.37

N/D = Not Detect.

Table 4. Analysis of Microbial load (By WHO/AOAC/AYUSH/API/UPI Std. Methods).

S.No.	Parameter Analyzed	Results			WHO Limit
		I N-1	I N-2	I N-3	
1.	Total Bacterial Count	580 cfu/gm	583 cfu/gm	584 cfu/gm	10^5 cfu/gm
2.	Total Fungal Count	630 cfu/gm	636 cfu/gm	634 cfu/gm	10^3 cfu/gm
3.	*Escherichia coli*	Absent	Absent	Absent	Absent
4.	*Salmonella typhai Spp.*	Absent	Absent	Absent	Absent
5.	*Staphylococcus aurous*	Absent	Absent	Absent	Absent

Table 5. Estimation of Heavy Metals (By AAS-GF).

S.No.	Parameter Analyzed	Results			WHO Limit
		I N-1	I N-2	I N-3	
1.	Lead	3.02 ppm	3.04 ppm	3.03 ppm	10 ppm
2.	Cadmium	0.04 ppb	0.05 ppb	0.04 ppb	0.3 ppm
3.	Mercury	N/D	N/D	N/D	1.0 ppm
4.	Arsenic	0.05 ppm	0.06 ppm	0.04 ppm	3.0 ppm

N/D = Not Detect.

Table 6. Estimation of Aflatoxins (By HPTLC).

S.No.	Parameter Analyzed	Results			WHO Limit
		I N-1	I N-2	I N-3	
1.	Aflatoxin, B1	N/D	N/D	N/D	0.5 ppm
2.	Aflatoxin, B2	N/D	N/D	N/D	0.1 ppm
3.	Aflatoxin, G1	N/D	N/D	N/D	0.5 ppm
4.	Aflatoxin, G2	N/D	N/D	N/D	0.1 ppm

N/D = Not Detect.

Table 7. Estimation of Pesticide Residues (By GC-MS).

S.No.	Parameter Analyzed	Results			WHO Limit (mg/kg)
		I N-1	I N-2	I N-3	
1.	DDT (all isomers, sum of ρ, ρ'-DDT, α, ρ' DDT, ρ, ρ'-DDE and ρ, ρ'-TDE (DDD expressed as DDT)	N/D	N/D	N/D	1.0
2.	HCH (sum of all isomers)	N/D	N/D	N/D	0.3
3.	Endosulphan (all isomers)	N/D	N/D	N/D	3.0
4.	Azinphos-methyl	N/D	N/D	N/D	1.0
5.	Alachlor	N/D	N/D	N/D	0.02
6.	Aldrin (Aldrin and dieldrin combined expressed as dieldrin)	N/D	N/D	N/D	0.05
7.	Chlordane (cis & tans)	N/D	N/D	N/D	0.05
8.	Chlorfenvinphos	N/D	N/D	N/D	0.5
9.	Heptachlor (sum of heptachlor and heptachlor epoxide expressed as heptachlor)	N/D	N/D	N/D	0.05
10.	Endrin	N/D	N/D	N/D	0.05
11.	Ethion	N/D	N/D	N/D	2.0
12.	Chlorpyrifos	N/D	N/D	N/D	0.2
13.	Chlorpyrifos-methyl	N/D	N/D	N/D	0.1
14.	Parathion methyl	N/D	N/D	N/D	0.2
15.	Malathion	N/D	N/D	N/D	1.0
16.	Parathion	N/D	N/D	N/D	0.5
17.	Diazinon	N/D	N/D	N/D	0.5
18.	Dichlorvos	N/D	N/D	N/D	1.0
19.	Methidathion	N/D	N/D	N/D	0.2
20.	Phosalone	N/D	N/D	N/D	0.1
21.	Fenvalerate	N/D	N/D	N/D	1.5
22.	Cypermethrin (including other mixtures of constituent isomers sum of isomers)	N/D	N/D	N/D	1.0
23.	Fenitrothion	N/D	N/D	N/D	0.5
24.	Deltamethrin	N/D	N/D	N/D	0.5
25.	Permethrin (sum of isomers)	N/D	N/D	N/D	1.0
26.	Pirimiphos methyl	N/D	N/D	N/D	4.0

N/D = Not Detect.

Toxicological Investigated Study

I N. having investigated bioactive phytochemacal constituents with immense pharmacological action properties shown in Table 1 respectively, and toxicologically investigated of collected samples of various 3 regions from India, seeds part 3 samples of I N. medicinal plant shown, quality, safety, toxicity QC, QA research data's. Safety and Toxicity investigated research parameters revealed results were shown with in prescribed WHO/AYUSH Pharmacopeial permissible standard Limits in as Microbial Load contaminations-TBC/TFC detect in cfu/gm. *Escherichia coli*, *Salmonella typhai* spp. *Staphylococcus aurous* author pathogenic, detection and in Heavy Metals-Pb, Hg, Cd and As, Aflatoxins B1, B2 and G1, G2 and Pesticide Residues-Organo chlorine, pesticides, Organo phosphorus pesticides, Pyrethroids etc., potentially toxic elements detections in ppb. levels. Resulted the all investigated Safety, Toxicity research parameters found in the study medicinal plant investigated 3 samples shown complies and not found of any hazardous or highly toxic contamination in the investigated drug samples. It had fit for internal used. Investigated results shown in Tables 3–7 respectively. Result of Analytical and Advance Sophisticated instrumentations estimation and analysis HPTLC finger print of alcohol extract:TLC plate was developed using Toluene: Ethyl acetate: Formic acid (9:1:0.5) as mobile phase. After development allow the plate to dry in air, record the fingers print at 254 nm and 366 nm in identification of various bioactive phytochemical secondary metabolites, Quality, Safety and Toxicity research Studies [1–10,12,14,19,24–38].

9. Conclusions

The results investigated and revalidated, tested drug samples I N-1, I N-2 and I N-3 were found and confirmed that I N meets regulatory standards for quality, safety and toxicity in ASU herbal drugs. HPTLC fingerprinting revealed key phytochemical markers useful for authentication. The absence of microbial contamination, heavy metals, aflatoxins, and pesticide residues ensures its suitability for medicinal use. This study contributes to the standardization protocols of I N. enhancing its reliability in herbal medicine formulations. I N. may be treat and therapeutically used in various ASU traditional and alternative medicines confirmed and Investigated In-vitro, In-vivo research reports data's basis and shown therapeutics medicinal potent values as a Antioxidant, Anti-Inflammatory, Antinociceptive, Antimicrobial, Collagenase Inhibitory, Antispasmodic, Anticancer, Anti-tumor, Antiproliferative Activities, Multidrug-Resistance Efflux-Inhibiting Activity, bronchodilatory along with Antiasthmatic potential, Diuretic, anthelminthic and deobstruant and are prescribed for dropsy and constipation, Antihypertensive and cardio-protective effects, Investigated plant has been used classically, traditional and alternative ASU medicine as Cold, Dropsy, Gout, Joint pain, Vitiligo, Itching, Asthma and Anthelmintic disorder from since ancient time. I N. drug can be incorporated of pharmacopeial standard monograph development, DSR, QC, QA, PV aspects. However, further advance research studies on the isolation and characterisation, Structural detection upon GC-MS, LC-MS, XRD, SEM-EDX Advance sophisticated instrumental techniques of these investigated drug scan still be carried out for purposes of advance research investigation of isolated novel bioactive phytochemical, constituents, compounds, novel drug discovery, drug mechanism of action upon isolated active phytochemical constituents apply advance In-vitro or In-vivo studies trial's, thus studies on the these plant parts of I N. also be done to discover potential bioactive compounds that can be explored for discovery of novel drug development and health advantages.

10. Limitations and Future Remarks of the Study

The present study's drug standardization, physicochemical, HPTLC finger printing profiling toxicology profiles show the reconfirmation and presence of DSR, QC, QA of fruit seeds part of PG. plant. In the future, investigated data may be used to drug standardization research, pharmacopeial monographs profiling and confirm these investigated resulted data's.

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Review

Chemical Composition and Health Benefits of Grape and Grape Products

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Abstract: Grape is a highly nutritious fruit that is widely consumed around the world. It is widely cultivated around the world. We provided knowledge on the phytochemical contents, biological impacts, and economic worth of grapes. Polyphenols are main constituents of grape and grape derivated products. They exhibited antioxidant, anti-inflammatory, and antihepatotoxic properties. The most prominent effect was the cardioprotective effect. The cardioprotective effect is shown by *in vitro*, *in vivo* studies as well as human studies.

Keywords: grape; *Vitis*; Vitaceae; polyphenol

1. Introduction

The genus *Vitis* L. (grape) comprises 68 species. This genus is different from the other Vitaceae plants by having petals that remain coalescent at the top and separated at the base, falling as a capGrape, the fruits of *Vitis vinifera* L. (Vitaceae) one of the most popular fruits of the world. Grapes are grown all around the world, especially Mediterranean type temperate climates. The cultivation of grapes began 6000–8000 years ago in around the Near East [1]. The cultivated grapes are mainly *Vitis vinifera* L. ssp. v*inifera* L. (European or wine grape), *V. vinifera* L. ssp. *slyvestris* Hegi (wild grape) and their hybrids and also *Vitis labrusca* L. [2]. Grapes can be eaten fresh as table grapes or used for making wine, jam, grape juice, grape seed extract, raisins, vinegar, and grape seed oil [3]. Commercially, *Vitis* species contain valuable raw materials for the production of wine, medicine and cosmetic industry.

Grapes contain simple phenolics, flavonoids, anthocyanins, tannins, stilbenes. *V. vinifera* fruits exhibit wide range of activity including cardioprotective, antioxidant, hepaprotective, antibacterial, etc. [4].

In this review, we aim to give information on the phytochemical composition, biological effects and commercial value of grape and grape derivated products.

2. History and Economic Value

Grape is produced in the regions between 20–52° north and 20–40° degrees south latitude in the world. Grape production is also seen in high altitude regions near the equator. The Caucasus and Mesopotamia are shown as the homeland of grapes. The Epic of Gilgamesh from the Mesopotamian period confirms this situation. It is the first written work in which the subject of wine is mentioned. Geological and archaeological research conducted in the 20th century shows that the grape was grown in many parts of the world 60 million years ago. It is thought that the cultivation and domestication of the grape took place in the geographical region between the Black Sea and Iran in 6000–5000 BC [5]. Grape seeds from the press residue from approximately 10 thousand years ago have been observed today. This shows how old grape winemaking is [5,6]. In the analysis of a jar found in Godin Hill in Iran in 3500 BC, a large amount of tartaric acid was detected, indicating that it contained wine. With the development of trade in Godin Hill between 3500–2900 BC, various goods spread to a wide area in the Near East. It is thought that the intercultural transfer of wine and winemaking occurred in this way [7].

There are also Egyptian hieroglyphs dating back to 2400 BC that mention winemaking. Wine was used as a cure for many years. Many scientists, such as Hippocrates, mentioned the medicinal properties of wine during those times [6].

The history of viticulture is intertwined with Anatolian civilizations. Different shapes of the grape plant have been encountered in rock paintings and sculptures from the Hittite civilization. These findings show that grapes and wine were offered to the gods as offerings in religious ceremonies between 1800–1550 BC. The Hittites also learned viticulture from other peoples who lived in Anatolia in earlier periods [5,6]. The Hittites were the first civilization in history to use agricultural laws by today's understanding in order to protect their vineyards and gardens [6].

The grape has great economic importance worldwide [8]. According to Food and Agriculture Organization of the United Nations (FAO) 2022 data, the top 10 in grape production are China, Italy, France, Spain, USA, Turkey, India, Chile, South Africa and Argentina. While China produced 12,600,000 tons of grapes in 2022, Argentina, ranked 10th, produced 1,936,803 million tons [9]. According to FAO data in 2022, Peru ranks first in the export value comparison with 526,857 tons of grape exports worth 1,292,376,000 USD [10]. The top 10 grape producers in 2017 were China, Italy, the USA, Spain, France, Turkey, India, Chile, South Africa, and Argentina [11].

According to the Food and Agriculture Organization of the United Nations, 75.866 square kilometres of the world is devoted to grapes. Approximately 71% of the world's grape production is used for wine, 27% as fresh fruit and 2% as dried fruit [12].

In 2016, the world produced 77.4 million tons of grapes from 7.1 million hectares of vineyards. In 1966, 52 million tons of grapes were produced from 9.5 million hectares. An increase in grape production was observed from 1966 to 2016 [1].

3. Chemistry

Many factors such as grape variety, soil and climate conditions, technical and cultural processes applied, and especially the degree of ripeness affect the content of the grape [13,14].

In general, the composition of grapes includes water, sugars, organic acids, phenolic compounds (Table 1), terpenes (Table 2), nitrogenous substances, vitamins, minerals and enzymes. Grapes contain between 65–85% water, depending on the variety. Since it contains a large amount of sugar in its composition, its caloric value is quite high. In addition, it is rich in minerals (such as calcium, potassium, sodium and iron) and is also considered an important source of some vitamins (vitamins A, B1, B2, B3 and C) [13].

Table 1. Some phenolic and polyphenolic compounds of grape and grape products.

Compound	Part of Plant	Reference
Flavan-3-ols		
Catechin	Seeds Fruits Grape Skin Wine Grape pomace	[15–17]
Catechin gallate	Grape skin Seeds	[15]
Epicatechin	Fruits Grape skin	[16,17]
Epicatechin gallate	Fruits Grape skin Seeds Grape juice Wine	[15,17]
Epigallocatechin	Fruits	[17]
Procyanidin B1	Wine Seeds	[15,18]
Procyanidin B2	Grape pomace Wine Seeds	[15,16,18]
Procyanidin B3	Wine	[18]
Procyanidin B4	Wine	[18]
Procyanidin C1	Wine	[18]
Anthocyanins and Anthocyanidins		
Cyanidin	Grape pomace Grape skins Seeds Wine	[15,18]
Cyanidin-3-glucoside	Wine Grape skin	[15,19]

Table 1. *Cont.*

Compound	Part of Plant	Reference
Cyanidin-3,5-diglucoside	Seeds Grape skin	[15]
Malvidin-3-*O*-glucoside	Grape skin Seeds Wine	[15,19]
Malvidin 3,5-diglucoside	Grape skin Seeds	[15]
Delphinidin	Fruits	[17]
Delphinidin-3-*O*-glucoside	Grape skin Fruits Wine	[15,17,18]
Delphinidin 3,5-diglucoside	Seeds Grape skin	[15]
Delphinidin 3-(6″-acetyl)-glucoside	Seeds Grape skin	[15]
Delphinidin 3-(6″-caffeoil)-glucoside	Seeds Grape skin	[15]
Delphinidin 3-(6″-cumaroil)-glucoside	Seeds Grape skin	[15]
Pelargonidin	Fruits	[17]
Peonidin 3,5-diglucoside	Seeds Grape skin	[15]
Peonidin-3-*O*-glucoside	Grape skin Fruits Wine	[15,17,19]
Petunidin-3-*O*-glucoside	Seeds Grape skin Wine Fruits	[15,17,19]
Vitisin A	Wine	[20]
Vitisidin A	Wine	[20]
Vitisin B	Wine	[20]
Vitisidin B	Wine	[20]
Acetylvitisin A	Wine	[20]
Acetylvitisin B	Wine	[20]
Flavanones		
Naringenin	Grape skins Grape juice Wine	[18,21,22]
Hesperetin	Wine	[18]
Flavonols		
Rutin	Seeds Grape skin	[15]
Isorhamnetin	Seeds Grape skin	[15]
Myricetin	Seeds Grape skin Wine	[15,18]
Myricetin-3-*O*-glucuronide	Seeds Grape skin	[15]
Myricetin-3-*O*-glucoside	Seeds Grape skin	[15]
Quercetin	Seeds Fruits Wine	[15,17,19]
Quercetin-3-*O*-glucoside	Seeds Grape skin	[15]
Quercetin-3-*O*-glucuronide	Seeds Grape skin	[15]
Kaempferol	Grape skin Wine	[16,18]
Flavones		
Apigenin	Fruits Wine	[17,18]
Luteolin	Wine	[18]

Table 1. *Cont.*

Compound	Part of Plant	Reference
Stilbenoids		
Resveratrol	Wine Fruit Grape skin	[16,17,19]
Trans-Resveratrol	Grape skin Wine	[15,19]
Piceatannol	Wine	[18]

Table 2. Some terpenes of grape and grape products.

Compound	Part of Plant	Reference
Monoterpenes		
Linalool	Wine Fruit Grape skin Grape juice	[23–26]
Cis-Linalool oxide	Wine Grape juice	[23,25]
Trans-Linalool oxide	Wine	[23]
Linalool *E*-pyranic oxide Linalool *Z*-pyranic oxide	Fruit	[27]
Nerol	Wine Fruit Grape skin	[23,26,27]
Trans-8-Hydroxylinalool *Cis*-8-Hydroxylinalool	Grape skin	[26]
Nerol oxide	Fruit	[24]
Hydroxynerol	Grape skin	[26]
Geraniol	Wine Grape skin Fruit	[23,24,26]
α-Terpinene	Fruit	[24]
γ-Terpinene	Fruit	[24]
Terpinolene	Fruit	[24]
β-Citronellol	Grape juice Wine	[23,25]
Hotrienol	Wine Fruit Grape juice	[23,25,28]
α-Terpineol	Wine Grape skin Grape juice Fruit	[23,25,26,29]
Epoxylinalool-1/-2	Wine	[23]
2,6-dimetyl-3,7-octadiene-2,6-diol	Wine	[23]
Citronellol	Fruit Grape skin	[26,28]
Hydroxy-citronellol	Grape skin	[26]
Limonene	Fruit Wine	[30,31]
trans-citral (Geranial)	Fruit Wine	[31]
cis-citral (Neral)	Fruit Wine	[31]
γ-Isogeraniol	Fruit	[27]
trans-ocimenol	Fruit	[31]
cis-ocimenol	Fruit	[31]
Myrcenol	Fruit	[31]
Terpendiol I	Fruit	[27]
3-Carene	Wine	[31]

Table 2. *Cont.*

Compound	Part of Plant	Reference
Myrcenol	Fruit	[31]
Geranic acid	Fruit Wine	[31]
trans-Rose oxide *cis*-Rose oxide	Fruit Wine	[31]
Citronellyl acetate	Wine	[31]
Geranyl acetate	Wine Fruit	[31]
Neryl acetate	Wine Fruit	[31]
trans-Methyl geranoate	Fruit	[31]
Norisoprenoids		
Theaspirane A	Fruit	[30]
Theaspirane B	Fruit	[30]
β-Damascenone	Fruit Wine	[30,32]
α-ionone *β*-ionone	Fruit Wine	[31]
1,1,6-trimethyl-1,2-dihydronaphthalene	Fruit Wine	[32]
1-(2,3,6-trimethylphenyl)buta-1,3-diene	Fruit Wine	[32]
Actinidol A/B	Fruit	[28]
Vitispirane	Wine Fruit	[31]
Sesquiterpenes		
Selina-4(15),6-diene	Fruit	[30]
α-Muurolene	Fruit	[30]
γ-Muurolene	Fruit	[30]
α-Cadinene	Fruit	[30]
δ-Cadinene	Fruit	[30]
ω-Cadinene	Fruit	[30]
γ-Cadinene	Fruit	[30]
α-Calacorene	Fruit	[30]
1-epi-Cubenol	Fruit	[30]
Cubenol	Fruit	[30]
α-Copaene	Fruit	[30]
β-Copaene	Fruit	[30]
α-Ylangene	Fruit	[30]
β-Caryophyllene	Fruit	[30]
α-Guaiene	Fruit	[30]
Guaia-6,9-diene	Fruit	[30]
α-Humulene	Fruit	[30]
Zonarene	Fruit	[30]
epi-Zonarene	Fruit	[30]
β-Bourbonene	Fruit	[30]
Rotundone	Fruit Wine	[25]
Clovene	Fruit	[30]
δ-Selinene	Fruit	[30]
cis-Calamenene	Fruit	[30]
trans-Calamenene	Fruit	[30]
Germacrene D	Fruit	[31]
β-Farnesene	Fruit	[31]
α-Cadinol	Fruit	[31]
p-Cymene	Wine	[31]
Farnesol	Wine	[31]

Table 2. *Cont.*

Compound	Part of Plant	Reference
Triterpenes		
Sitosterol	Grape skin	[33]
β-Sitosterol-3-*O*-*β*- D-glucoside	Grape skin	[33]
Oleanolic acid	Grape skin	[33]

4. Bioactivity

Various parts/products of grapes were evaluated for their in vitro and in vivo activities in different test models given in below.

4.1. Antioxidant Effect

Antioxidant effect is involved in many activity mechanisms. This effect was attributed to either polyphenols or oils.

Grape, peel of grape and seed extracts scavenged different radical oxygen species higher than positive control and the latter exhibited the best effect among them in various studies [34–36]. Similarly, grape seed oil possesses a high antioxidant effect. Carignan, Sangiovese, Syrah, Muscat d'Alexandrie, Khamri, and Merlot seed oils scavenged 1,1-diphenyl-2-picrylhydrazyl (DPPH) radical higher than that of positive control, butylated hydroxytoluene (BHT). Moreover, Carignan, Sangiovese, Syrah, Muscat d'Alexandrie, Khamri, Merlot, Razagui, Razaki and Marsaoui seed oils had higher metal chelating ability than BHT [37].

The seedless black grape (pulp and skin) suppressed systemic oxidative and inflammatory stress in CCl_4intoxicated rats. It reduced radical oxygen species (ROS), nitric oxide (NO) and 2,2′-azino-bis (3-ethylbenzothiazoline)-6-sulfonic acid (ABTS) radical thiobarbituric acid reactive substances (TBARS) levels, as well as myeloperoxidase (MPO) activity as related to the CCl_4 group. Furthermore, the levels of total antioxidant capacity, reduced glutathione (GSH), as well as the activity of superoxide dismutase and glutathione peroxidase were increased associated with ROS/NF-κB (Nuclear Factor kappa-B) signalling pathways [38].

In the study examining the antioxidant effects of wines obtained from different *V. vinifera* varieties, the wine with the highest total phenol (1968.27 ± 1.50 mg/L), hydroxy-cinnamoyl tartaric acid (352.52 ± 0.66 mg/L) and flavonol (209.04 ± 1.05 mg/L) contents were examined. The highest DPPH radical scavenging effect of wine (EC50 = 49.24 ± 0.26mL/g) was observed [39].

In a study examining the effects of red wine polyphenols on heart health, rats fed a fructose-rich diet were given an aqueous solution of red wine polyphenolic extract (10mL/kg) and a solution containing the same amount of red wine polyphenolic extract and 10% ethanol. Superoxide anion production increased in the aorta and heart tissues of rats due to fructose consumption. As a result, a significant decrease was observed in superoxide radical levels in the group consuming aqueous solution of red wine polyphenolic extract and the group consuming a solution that contained the same amount of red wine polyphenolic extract and 10% ethanol compared to the control groups (10% (*v*/*v*) ethanol-water consuming group and water-only consuming group) [40].

The DPPH, ABTS, CUPRAC (Cupric Reducing Antioxidant Capacity) and FRAP radical scavenging activities of extracts obtained from wine (alcohol-free wine, alcohol-free wine aqueous extract and alcohol-free wine ethylacetate extract) and grape skin decoction, oil obtained from the seed and grape skin soxhlet extract were examined and wine extracts were found to be higher than other extracts [41].

4.2. Anticancer Effect

Saponifiable-fraction of black and green grapes seeds exhibited cytotoxicity by inducing apoptosis and reducing inflammation more than 5-fluorouracil in MCF-7 cells. Treatment with each extract 150 mg/kg for 7 days reduced tumor size in peritoneal Ehrlich ascites carcinoma inoculated mice. The treatment reduced hepatic injury due to carcinoma in comparison 5-fluorouracil. Moreover, the authors indicated that the anticancer effect may related to fatty acids. The extracts exhibited cytotoxic and apoptotic effects associated with induction of oxidative stress in cancer cells. They also alleviated cell migration by reducing the level of CD44+ cells in cancer cells and inhibiting of Matrix metalloproteinases protein-9 (MMP-9) and cathepsin B activities [42]. The non-saponifiable-fraction of grape seeds exhibited cytotoxicity and induced apoptosis in Huh7 hepatocellular carcinoma cells related to oxidant status and inflammation [43].

The seedless black grape extracts exhibited cytotoxicity and apoptotic effects in HepG2 and Huh7 hepatocellular carcinoma cells. These extracts also reduced tumor size in vivo [44]. Grape seed proanthocyanidin extract reversed drug resistance through down-regulation of the expression of MPR1 (multidrug resistance-

associated protein 1), MDR1 (Multidrug resistance protein 1) and LRP (Lung resistance-related protein) by inhibiting the PI3K/Akt pathway in adriamycin resistant acute myeloid leukemia cells (HL-60) [45]. Red wine inhibits proliferation of A549 lung cancer cells and blocks clonogenic survival at low concentrations (0.02%). This effect is associated with inhibition of basal and epidermal growth factor (EGF) -stimulated Akt (Protein kinase B) and Erk (Extracellular signal-regulated kinase) signals and enhancement of total and phosphorylated levels of p53. White wine mediates similar effects at higher concentrations (0.5–2%). Anti-proliferative effects of wine were not mediated by the associated contents of ethanol or the polyphenol resveratrol and were independent of glucose transport into cancer cells [46]. Ethanol at low concentrations (12.5 mM to 25 mM) has been shown to increase the proliferation of breast cancer (MCF-7) and esophageal cancer (KYSE-510) cell lines. Specifically, red wine concentrations ranging from 6.25 mM to 100 mM resulted in growth inhibition, with complete inhibition of MCF-7 cell growth observed at a concentration of 25 mM. Furthermore, the effects of red wine were also studied on human lung carcinoma cells (A-549) and human colon cancer cell lines, including SW-480 and RKO. Growth inhibition was noted for all these cell lines in a dose-dependent manner. Research examined both perennial and low-year wines, revealing that perennial wines were more effective at inhibiting cancer cell growth. An increase in the transcription of RNA polymerase III-dependent genes is associated with cell proliferation, cell transformation, and tumorigenesis. It was found that ethanol enhances the transcription of these genes, whereas red wine significantly reduces their transcription [47]. However, these wine concentrations are extremely high and do not appear to be biologically achievable.

4.3. Anti-Inflammatory Effect

The ethanolic extract of *V. vinifera* (Muscat variety) seeds was found to downregulate inflammatory markers such as TNF-α (Tumor necrosis factor-α), NF-κB, p65, IKK-β, IL-1β (Interleukin-1β), and IL-6 (Interleukin-6) in the livers of rats with Type II diabetes [48]. Similarly, the extract from seedless fruits of *V. vinifera* reduced pro-inflammatory markers, including TNF-α, IL-1β, and IL-8, and also decreased levels of NF-κB, iNOS (nitric oxide synthase), and COX-2 (cyclooxygenase-2) in rats [38]. Effect of a methanol/ethanol (8:2) extract of grape pomace investigated in inflammatory bowel disease model in Caco-2 cells. It reduced the intestinal expression and release of IL-6, monocyte chemoattractant protein MCP-1 (Monocyte Chemoattractant Protein-1), and MMP-9 and MMP-2. Furthermore, it downregulated the gene expression of several pro-inflammatory markers, including IL-1β, TNF-α, macrophage colony-stimulating factor, C-X-C motif ligand (CXCL)-10, intercellular adhesion molecule (ICAM)-1, vascular cell adhesion molecule (VCAM)-1, and COX-2 mediated by the inhibition of NF-κB activity and reduced intracellular ROS levels [49]. Additionally, the ethanolic extract of grape pomace demonstrated a dose-dependent reduction in carrageenan-induced paw edema. Notably, a dosage of 40 mg/kg of the extract exhibited an anti-inflammatory effect comparable to the positive control, indomethacin, by reducing the pro-inflammatory cytokines IL-6 and IL-1β, as well as the COX-2 and myeloperoxidase enzymes [50].

Black grape seed hydroalcoholic extract (50, 100, and 200 mg/kg) and black grape seed oil (2, 4, and 8 mL/kg) were administered orally to rats, two hours before the induction of colitis and continued for an additional four days. This treatment resulted in a dose-dependent reduction in colon weight, ulcer index, and total colitis index when compared to the control group. These findings suggest that both black grape seed oil and the hydroalcoholic extract have protective and preventive effects in an acute model of experimental ulcerative colitis [51].

It has been observed that Cabernet Franc, Cabernet Sauvignon and Sauvignon Blanc wine types showed anti-inflammatory activity by reducing the levels of nitric oxide, TNF-α and IL-1β, the amount of which increases with lipopolysaccharide, in RAW 264.7 cells [52]. In another study, wine extracts (alcohol-free wine, alcohol-free wine aqueous extract and alcohol-free wine ethylacetate extract) inhibited the lipoxygenase enzyme in a concentration-dependent manner and showed 30–50% and 100% inhibition at the tested concentrations (10 and 100 μg/mL), respectively [41]. Solvent-solvent partitioning was performed on alcohol-free wines obtained through various separation methods from different types of wines, including white wines such as Robola of Kefalonia, Tsaousi, Kakotrigis, Muscat of Kefalonia, and White Thiako, as well as red wines like Petrokoritho, Vertzami, Avgoustiatis, Red Thiako, and Mavrodaphne of Kefalonia. Several subfractions were obtained from these wines. The antioxidant, anti-inflammatory, and antiplatelet activities of the subfractions were subsequently examined. All extracts demonstrated antioxidant, anti-inflammatory, and antiplatelet properties; however, the extract with the highest phenolic content exhibited the most significant activity. For assessing antioxidant activity, the DPPH radical scavenging effect and the ability to inhibit lipoxygenase activity were measured. Antiplatelet activity was evaluated by examining the inhibition of platelet-activating factor (PAF), Adenosine diphosphate (ADP), tartrate-resistant acid phosphatase (TRAP), collagen, and arachidonic acid. For the anti-inflammatory activity, it was noted that the extracts significantly reduced the increased secretion of TNF-α and IL-1β, which result from the

inflammatory response induced in peripheral blood mononuclear cells from healthy volunteers. This study indicated that both white and red wines contain biologically active microconstituents that help combat oxidative stress, inflammation, and thrombosis. Therefore, a wine's protective effect is not related to its color, but rather to its specific microconstituent profile [53]. Wine extract rich in polyphenols (extract obtained with the C18 cartridge of Port Barrel Reserve wine) reduced the increase in lipopolysaccharide-induced ROS levels in human colon CDD-18Co fibroblast cells. NF-κB, IL-6 and TNF-α gene expression, which increased with the application of lipopolysaccharide, decreased with the application of wine extract. Cell adhesion molecules have been observed to be upregulated in inflammatory bowel diseases. By downregulating these molecules, the development of inflammatory bowel disease can be prevented. For this purpose, ICAM-1, VCAM-1 and PECAM-1 (Platelet endothelial cell adhesion molecule-1) gene expression increased with the application of lipopolysaccharide. Pretreatment with wine extract resulted in a decrease in the expression increase of these genes [54].

4.4. Antihepatotoxic Effect

The ethanolic extract of red grape seeds has been shown to attenuate paracetamol-induced hepatotoxicity in rats. After administering the extract for six weeks, there was a reduction in serum cholesterol, triglycerides, low-density lipoprotein (LDL-C), and very low-density lipoprotein (VLDL-C), along with a significant increase in levels of high-density lipoprotein (HDL-C). The extract provided notable hepatoprotection by decreasing the activities of liver enzymes, improving kidney parameters, and reducing lipid peroxidation. Additionally, the extract enhanced the activity levels of endogenous antioxidants, including GSH, SOD, and CAT, bringing them close to normal levels [55].

Grape seed oil also possesses protective effects on CCl_4 induced acute liver injury in γ-irradiated rats by antioxidant, anti-inflammatory and anti-apoptotic activities. The induced activities of SOD, CAT, GSH-Px (glutathione peroxidase), GST (glutathione transferase). It reduced alleviated ALT (alanine aminotransferase), AST (aspartate aminotransferase), IL-6 and TNF-α levels associated withdown-regulation of the CYP2E1 (Cytochrome P450 2E1), iNOS, Caspase-3 and NF-κB expression, up-regulation of the trace elements concentration levels and activation of SIRT1 (silent information regulator protein-1) gene expression are responsible for the improvement of the antioxidant and anti-inflammatory status in the hepatic tissues. [56]

4.5. Antidiabetic Activity

The administration of a water extract from *V. vinifera* (Muscat variety) seeds over 28 days demonstrated an antidiabetic effect in rats with streptozotocin-induced diabetes. This treatment resulted in a reduction of fasting blood glucose, glycated haemoglobin (HbA1c), lipid profile, and serum insulin levels, bringing them closer to normal levels. Additionally, it caused less pancreatic damage. The extract-treated rats showed higher levels of insulin, GLUT-2, SOD, CAT, and glutathione peroxidase, while levels of TNF-α, IκB, and caspase-3 were lower in their pancreas [57]. A dried grape skin was extracted with water. The concentrated water extract was applied to a cationic ion-exchange resin column and eluted using ethanol, a mixture of ethanol and water, and finally water. The water portion was discarded, while the other fractions, which were rich in polyphenols, were administered to rats over a period of 19 days. This treatment demonstrated hypoglycemic effects in normal mice and antihyperglycemic effects in alloxan-induced diabetic mice. The authors suggested that these effects occur independently of increased insulin release but are related to enhanced insulin sensitivity, which is associated with increased Akt phosphorylation and higher levels of insulin receptors and GLUT-4 in skeletal muscle [58].

4.6. Antimicrobial Effect

Antimicrobial effects of seeds of grape reported in various studies. A methanolic extract of grape seeds inhibited biofilm formation of methicillin-resistant *Staphylococcus aureus and Staphylococcus haemolyticus* [59]. Moreover, seed extracts of some endemic Turkish grape varieties including Hasandede, Emir and Kalecik karası exhibited antimicrobial effects against *Escherichia coli*, *Klebsiella pneumoniae*, *Mycobacterium smegmatis*, *Proteus vulgaris*, *Pseudomonas aeruginosa*, *Pseudomonas fluorescens*, *Salmonella enteritidis*, *Salmonella typhimurium*, *Staphylococcus aureus* ve *Yersinia enterocolitica*. The effect was attributed to flavan-3-ols in the extract [60]. Furthermore, seed oil of Tamjanika, a Balkan native grape variety of *V. vinifera* exhibited antifungal activity against *Trichophyton mentagrophytes*, *Trichophyton rubrum*, *Trichophyton verrucosum*, *Microsporum gypseum* and *Microsporum canis* [61].

4.7. Miscellenous Effects

The ethanolic extract of *V. vinifera* seeds has been shown to promote wound healing in an excision wound model using albino rats. After the wounds occurred, a treatment of 2 mg of the extract per wound or the positive control, Mebo®®, was applied twice daily for 14 days. The results indicated that the extract improved wound closure rates, increased levels of TGF-β (Transforming Growth Factor-β) and VEGF, and decreased levels of TNF-α and IL-1β when compared to the Mebo®®-treated group [36].

Primitivo and Negroamaro polyphenolic extracts, inhibited monocyte adhesion to stimulated endothelial cells at 1 μg/mL. The extracts down-regulated the expression of adhesion molecules, including ICAM-1, VCAM-1, E-Selectin, as well as MCP-1 and M-CSF (macrophage colony-stimulating factor), at mRNA and protein levels. In addition, these treatments reduced NF-κB, AP-1 (activator protein-1) activation and intracellular ROS levels in lipopolysaccharide-stimulated HUVEC cells. Thus, they exhibited anti-atherosclerotic effect [62].

Pretreatment with grape seed oil at a dosage of 4 mL/kg/day was given for 14 days. After this period, ischemia was induced by isoproterenol The grape seed oil was found to reduce ventricular conduction, mitigate the cardiotoxic effects of isoproterenol in the ventricular myocardium, and lower the levels of pro-inflammatory cytokines. Additionally, the oil prevented the increase in heart rate and the reduction in RR interval caused by isoproterenol, which may contribute to its cardioprotective effects in acute myocardial ischemia [63].

4.8. Clinical Trials

A meta-analysis of 37 randomized controlled studies found a significant increase in total antioxidant capacity from the use of grape products. However, there was no significant effect on SOD and oxygen radical absorbance capacity (ORAC). While higher doses of grape products lead to increased SOD and ORAC levels, prolonged use specifically raised ORAC levels. Overall, the application of grape products in healthy volunteers significantly influenced total antioxidant capacity, SOD, and ORAC levels [64].

In a meta-analysis including 24 randomized controlled studies, the use of grape products (grape extract, grape juice, grape powder, grape seed extract, grape seed oil, raisins, and whole grapes) significantly reduced C-reactive protein (CRP) levels. However, it was observed that they had no significant effects on serum TNF-α, IL-6, total antioxidant capacity and malondialdehyde. Subgroup analysis showed that grape juice and grape seed extract had a significant CRP-lowering effect in randomized controlled studies conducted on participants who were administered high doses of grape products and fell into the normal or obese body mass index categories. In subgroup analyzes for TNF-α levels, a significant lowering effect of grape products on TNF-α levels was observed when grape seed extract was used, when high doses of grape products were used, or when administered to overweight and obese subjects [65].

A meta-analysis of 29 randomized controlled trials examined the effects of grape and grape product supplements on glycemic responses. The study found that these supplements significantly reduced the homeostatic model assessment of insulin resistance. However, there was no impact on fasting insulin levels or hemoglobin A1C percentages. Interestingly, the use of grape and grape product supplements, particularly grape juice, resulted in an increase in fasting blood sugar compared to the control group. The authors suggested that this effect might be related to the sugar content in grape juice [66].

In a meta-analysis including 48 randomized controlled studies, it was observed that consumption of grape products reduced total cholesterol, low-density lipoprotein and triglyceride concentrations. However, the use of grape products had no effect on high-density lipoprotein. Supplementation of grape products has been observed to reduce triglyceride levels in patients with hyperlipidemia, diabetes, and metabolic syndrome. At low doses and intervention periods of less than 8 weeks, triglyceride-lowering effects were observed for grape seed extract and whole grape extract forms, but not for raisins and grape juice. Supplements in the forms of whole grape extract, grape juice, and grape seed extract significantly reduced low-density lipoprotein levels, especially in hyperlipidemic patients [67].

In another meta-analysis examining 30 randomized controlled studies, it was observed that grape products significantly reduced systolic blood pressure compared to the control group. According to subgroup analyses, consumption of raisins and grape powder caused a decrease in systolic blood pressure, while such an effect was not observed with grape juice consumption. An increase in VCAM-1 has been observed. No significant effects were observed on diastolic blood pressure, endothelial function, heart rate, pulse rate and soluble intercellular adhesion molecule-1 (sICAM-1) as a result of consumption of grape products. However, the authors rated the reliability of the majority of included studies (25 out of 30) as low or moderate [68].

An open, prospective, cross-over, randomised, and controlled cross-sectional clinical trial showed that moderate red wine consumption decreases serum oxidation parameters and reduces the propensity of LDL to

undergo lipid peroxidation, whereas both red wine it increases HDL-cholesterol. However, drinking red wine lowers the production of endothelial adhesion molecules and monocytes, which, when they interact with endothelial receptors, enable monocytes to penetrate the endothelium wall and postpone the early stages of atherosclerosis. On the other hand, red wine consumption reduces the expression of monocyte and endothelial adhesion molecules, which after interaction with endothelial receptors allows the monocytes to pass through the endothelial wall, delaying the early processes of atherosclerosis [69]. A meta-analysis that included 48 animal studies and 37 human studies evaluated the impact of red wine polyphenols, which consist of a complex and varied array of molecules, including flavonoids like (+)-catechin, quercetin, anthocyanins, and stilbenes, on vascular health. The human studies found that the consumption of red wine resulted in a reduction in systolic blood pressure by an average of −2.6 mmHg. However, there were no significant effects observed on diastolic blood pressure or overall vascular function. When pure resveratrol was administered alone, a greater reduction in systolic blood pressure was recorded, averaging −3.7 mmHg. Nonetheless, these beneficial effects are not as pronounced as those seen in animal models [70].

A meta-analysis of ninety-one randomized controlled trials revealed that drinking red wine improved lipid profiles, intestinal microbiota, thrombosis, immune function and inflammation, and antioxidant status, while having no effect on body weight or glucose metabolism. While there were no changes in blood pressure, weight gain, or blood sugar levels, five of the seven studies that were examined showed positive benefits of wine consumption in terms of indicators of oxidative stress, inflammation, and nephropathy as well as a little decrease in the risk of cardiovascular disease. According to the evaluated cohort studies, moderate consumption of red wine may offer protection against atrial fibrillation [71].

Several studies were shown that light and moderate daily wine consumption decreased whereas high consumption increased colorectal, breast and prostate cancer development risk [72–74].

In a meta-analysis examining 9 studies, it was observed that products derived from grapes did not significantly change serum AST and ALT concentrations, but a significant decrease in serum alkaline phosphatase (ALP) levels was observed. Subgroup analyzes are meaningful only in studies where the effect on ALP was also evaluated in healthy participants. ALT concentration of grape products showed a significant decrease in the subgroup of healthy volunteers [75].

5. Conclusions and Prospective

Grapes and their various products have been consumed for many years. They possess a range of physiological effects due to the presence of compound groups such as flavonoids, tannins, anthocyanins, and phenolic acids. While these compounds are primarily known for their antioxidant properties, they also exhibit a variety of beneficial effects, including anticancer, antidiabetic, hepatoprotective, and anti-inflammatory properties, as well as promoting wound healing. These effects can be observed in the application of different parts of the grape or extracts derived from them. Recent studies have also focused on the biological effects of by-products (grape pomace) obtained in the production of wine from grapes. Especially cardioprotective effects of not only wine but also other grape products proved by many studies including clinical trials. Wine, an important product obtained from grapes, has attracted the attention of researchers. In particular, the cardiovascular protective effect of wine was suggested by the French Paradox, and over time, various studies have been conducted on this effect of wine. Although wine contains important phenolic compound groups, its effects on wine consumption and health are a matter of debate among researchers due to the alcohol it contains. It seems that seed extracts or seed oils are safer options.

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Abbreviations

ABTS	2,2-azino-bis(3-etilbenzotiazolin)-6-sulfonic acid
ADP	Adenosine diphosphate
Akt	Protein kinase B
ALP	Alkaline phosphatase

ALT	Alanine aminotransferase
AST	Aspartate aminotransferase
AP-1	Activator protein-1
CAT	Catalase
COX-2	Cyclooxygenase-2
CRP	C-reactive protein
CUPRAC	CUPric Reducing Antioxidant Capacity
CXCL-10	C-X-C motif ligand-10
CYP2E1	Cytochrome P450 2E1
DPPH	1,1-diphenyl-2-picrylhydrazyl
Erk	Extracellular signal-regulated kinase
EGF	Epidermal Growth Factor
FAO	Food and Agriculture Organization of the United Nations
FRAP	Ferric reducing antioxidant power
GLUT-2	Glucose transporter-2
GLUT-4	Glucose transporter-4
GSH	Glutathione
GSH-Px	Glutathione peroxidase
GST	Glutathione transferase
HDL-C	High-density lipoprotein
ICAM-1	Intercellular adhesion molecule-1
IL-1β	Interleukin-1β
IL-6	Interleukin-6
IL-8	Interleukin-8
iNOS	Nitric oxide synthase
LDL-C	Low-density lipoprotein
LRP	Lung resistance-related protein
MCP-1	Monocyte Chemoattractant Protein-1
M-CSF	Macrophage colony-stimulating factor
MPO	Myeloperoxidase
MMP	Matrix metalloproteinases protein
MDR1	Multidrug resistance protein 1
MPR1	Multidrug resistance-associated protein 1,
NF-kB	Nuclear Factor kappa B
NO	Nitric oxide
ORAC	Oxygen radical absorbance capacity
PAF	Platelet-activating factor
PECAM-1	Platelet endothelial cell adhesion molecule
PI3K	Phosphatidylinositol 3-kinase
RNA	Ribonucleic acid
ROS	Radical oxygen species
sICAM-1	Soluble intercellular adhesion molecule-1
SIRT-1	Silent information regulator protein-1
SOD	Superoxide dismutase
TBARS	Thiobarbituric acid reactive substances
TGF-β	Transforming Growth Factor-β
TNF-α	Tumor Necrosis Factor-α
TRAP	Tartrate-resistant acid phosphatase
VCAM-1	Vascular cell adhesion molecule-1
VLDL-C	Very low-density lipoprotein

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The Potential Protective Effect of the Standardized *Ginkgo biloba* Leaves Extract EGb761 against Contrast-Induced Acute Kidney Toxicity in Rats via Mitigating Renal Tissue Redox Imbalance, Inflammation, Cell Apoptosis and Mitochondrial Damage

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Abstract: In clinical medical practice, the applications of diagnostic and interventional procedures requiring iodinated contrast media (CM) administration have recently markedly increased. However, the intrinsic CM toxicity may lead to contrast-induced acute kidney injury (CI-AKI), particularly in patients with renal disease or diabetes. As successful therapy of CI-AKI is rather limited, effective strategies to prevent CI-AKI have become an insistent demand. The aim of this study was to evaluate the potential protective effects of the standardized extract of Ginkgo biloba leaves EGb761 against the pathophysiology of CI-AKI in a rat model. In this study, CI-AKI in rats was evaluated histopathologically and biochemically by measuring serum biomarkers of kidney function and tissue markers of oxidative stress, inflammation, tubular cell apoptosis and mitochondrial injury. Our results showed that CM administration led to several kidney morphological changes with alterations in serum and renal tissue parameters indicative of acute renal toxicity. These changes were moved to normality upon EGb761 treatment before CM exposure via integrated suppression of CM-induced renal tissue redox imbalance, inflammatory response, cell apoptosis activation and tubular cell mitochondrial damage. These findings demonstrated the nephroprotective effectiveness of EGb761 in alleviating CI-AKI pathophysiology through multiple effects. In conclusion, our study suggests a new therapeutic strategy for attenuating CI-AKI via administering EGb761 before CM use and may serve as an experimental basis for further studies to elucidate the promising clinical impact of EGb761 as a nephroprotective agent in patients at the risk of developing CI-AKI.

Keywords: EGb761; CI-AKI; oxidative stress; inflammation; apoptosis; mitochondrial damage

1. Introduction

Because of its role as the primary eliminator of hydrophilic drugs and metabolites besides its relatively large blood flow, the kidney is particularly prone to develop various forms of injury because of the accumulation of excreted drugs and/or their metabolites in renal tubular cells during the processes of tubular reabsorption and secretion [1]. Iodinated contrast-enhanced X-ray, computed tomography, or angiography imaging examinations are done frequently, in clinical practice, for diagnostic or interventional purposes [2]. To achieve these goals, iodine-based contrast media (CM) are administered intravenously or intra-arterially to increase tissue conspicuity and to improve diagnostic and therapeutic accuracy and ability. Despite these benefits, one potential adverse effect that occurs with intravascularly administered CM is contrast-induced acute kidney injury (CI-AKI) with rapid deterioration of renal function within 48 to 72 h after CM administration resulting in serious complications such as acute renal failure and pulmonary edema [3]. CI-AKI pathogenesis has been linked to several pathophysiological mechanisms including: (1) reactive oxygen species (ROS) overproduction in the renal tissue leading to oxidative stress and inflammation with subsequent cell injury [4], (2) direct renal tubular cell injury leading to mitochondrial damage and cell apoptosis [5], and (3) altered renal hemodynamics leading to renal medullary ischemia and hypoxia with subsequent ischemic kidney injury [6]. The latter mechanism was confirmed by the studies reported by Agmon et al., 1994 [7] and Lee et al., 2006 [8] who found that nitric oxide and

prostaglandins protected the outer medulla in rat kidneys from CM-induced nephrotoxicity via increasing regional blood flow. These authors also reported that pretreatment of rats and mice with the inhibitors of nitric oxide and prostaglandin synthesis increased radiocontrast nephrotoxicity as in these experimental models, like healthy humans, CM administration does not induce acute renal injury in animals not bearing any risk factors [6].

Due to the steady increase in the use of radiological procedures that utilize CM for better medical imaging, CI-AKI has become the third most common cause of hospital-acquired AKI after impaired kidney perfusion and medication with nephrotoxic drugs [9]. Since the effective way to treat CI-AKI remains an unmet medical necessity, it is imperative to find an effective strategy to ameliorate or prevent the development of CI-AKI after CM intravascular administration [10]. In the past few years, this issue attracted considerable scientific interest and became an existing topic of several experimental studies and clinical trials to evaluate the nephroprotective efficacy of some therapeutic procedures and agents including intravenous sodium bicarbonate, oral N-acetylcysteine, statins, ascorbic acid and calcium-channel blockers. There is a big controversy surrounding the use of most of these strategies to prevent CI-AKI as many studies have been published since the last three decades with conflicting results [11–13].

EGb761 is a standardized extract of a well-defined mixture of active constituents extracted from *Ginkgo biloba* leaves [14]. This extract contains two main groups of active compounds, flavonoid glycosides (24%) and terpene lactones (6%), which act synergistically on various targets to exert numerous pharmacological effects including antioxidant, anti-inflammatory, anti-apoptotic, and vasodilatory effects, and protection against mitochondrial dysfunction [14,15]. Clinically, EGb761 has been reported to have ameliorative effects in several disorders including cardiovascular diseases and neurodegenerative conditions [16–18]. Furthermore, EGb761 showed beneficial effects in some studies on nephrotoxicity induced by some drugs, e.g., adriamycin and cisplatin [19,20]. To the best of our knowledge, the impact of EGb761 has not been evaluated on CI-AKI despite being an interesting condition with several mechanisms involved in its pathogenesis. Therefore, the present study was designed to evaluate the effects of EGb761 on renal tissue oxidative stress, inflammation, apoptosis and mitochondrial damage, the events that play a crucial role in the development of kidney injury in a model of CI-AKI in rats.

2. Materials and Methods

2.1. Drugs and Chemicals

Ginkgo biloba leaves extract EGb761 powder (Medizen Pharma Co., Alexandria, Egypt) was used as a 4% suspension that was freshly prepared daily in 1% solution of carboxymethyl cellulose (CMC, El-Gomhouria Co. for Drugs, Cairo, Egypt). N(ω)-nitro-L-arginine methyl ester (L-NAME) powder (Thermo Fisher (Kandel) GmbH, Erlenbachweg, Germany) was freshly prepared as a 1% solution in saline. Indomethacin (INDO) ampoules were purchased from El-Nile Pharma Co. Cairo, Egypt. Urografin 76% (ampoules, Bayer Zydus Pharma, Kundaim, Goa, India), containing a mixture of sodium amidotrizoate and amidotrizoate meglumine, is an injectable iodinated ionic monomer, high osmolar radiographic contrast medium (CM) in an aqueous solution. This CM solution was used, in the present study, to induce AKI in rats.

2.2. Animals

This study was performed on adult male albino rats 8-to 10-week-old weighing 200–250 g. Animals were acclimatized for 2 weeks, housed in cages, two per cage, kept under standard conditions of temperature (20–22 °C), humidity (60%) and light (a 12-h light-dark cycle). The rats were maintained on a standard pellet diet and water *ad libitum.* All experimental procedures complied with the ethical Guidelines of the Animal Care Committee of the Medical Research Institute, Alexandria University.

2.3. CI-AKI induction and Animal Grouping

CI-AKI model in rats was conducted as previously described with minor modifications [12]. Briefly, a predisposing effect on CI-AKI induction was provided by dehydration for 24 h, followed by treatment with 10 mg/kg L-NAME, as a nitric oxide synthase inhibitor, and 10 mg/kg INDO, as a prostaglandin synthase inhibitor [12]. After that, CI-AKI was induced using a 12.5 mL/kg injection of CM. The doses of L-NAME, INDO and CM were administered intraperitoneally with 30-min time intervals. Following the completion of these injections, water restriction was continued for a further 6 h to make the kidneys more susceptible to CM nephrotoxicity. Thereafter, water was allowed for 18 h till the end of the study (24 h after CI-AKI induction). Four groups of rats (5–7 rats per group) were included in this study:

- Control group received the vehicles only throughout the study.
- EGb group received EGb761 at a dose of 100 mg/kg orally once daily [21] for 5 days.
- CM group received vehicles for 5 days. On the 4th day of the study, the rats were exposed to dehydration for 24 h. L-NAME, INDO, and CM were given after dehydration as mentioned above.
- EGb + CM group received EGb761 at a dose of 100 mg/kg orally once daily for 5 days. On the 4th day, the rats were exposed to dehydration for 24 h followed by giving L-NAME, INDO, and CM as mentioned in the CM group.

Twenty-four hours after CI-AKI induction, all rats were scarified, and blood samples were collected. Both kidneys were rapidly isolated, rinsed with ice-cold saline, blotted dry, and weighed. Blood samples were left to clot for 30 min at room temperature. Serum was separated and kept at −20 °C for later measuring of serum parameters. The right kidney was used for the histopathologic examination. The left kidney was immediately washed in ice-cold phosphate-buffered saline (0.1 M at pH 7.4), cut into small pieces, homogenized, and centrifuged at 10,000 g for 10 min at 4 °C. The supernatant was separated and stored at −80 °C until assayed.

2.4. Serum Biochemical Assays

Serum creatinine and urea concentrations were determined calorimetrically using commercially available diagnostic kits. Serum neutrophil gelatinase-associated lipocalin (NGAL) levels were determined using a rat NGAL ELISA kit (Chongqing Biospes Co., Chongqing, China).

2.5. Kidney Tissue Biochemical Assays

2.5.1. Oxidative Stress and Antioxidant Markers

Tissue malondialdehyde (MDA) content was determined using the method of Draper and Hadly [22]. The enzymatic method described by Griffith, OW [23] was used to measure glutathione and glutathione disulfide (GSSG) contents and then to calculate the content of reduced glutathione (GSH). For superoxide dismutase (SOD) assay, the activity of the enzyme was the determined by the pyrogallol method of Marklund and Marklund [24].

2.5.2. Proinflammatory Cytokines

Tissue levels of interleukin-1β (IL-1β), IL-6 and tumor necrosis factor-alpha (TNF-α) were determined using rat ELISA kits (My BioSource Co., San Diego, CA, USA) according to the instructions of the manufacturer.

2.5.3. Caspase-3 Activity

Tissue caspase-3 activity was determined using a rat caspase-3 assay kit (Elabscience Co., Houston, TX, USA) following the manufacturer's instructions.

2.5.4. Mitochondrial DNA (mtDNA) Copy Number per Cell

A quantitative reverse transcription polymerase chain reaction (RT-qPCR) assay has been developed to estimate levels of mtDNA copy numbers in samples [25,26]. This approach measures the mtDNA copy number by determining the ratio of PCR amplicons of mitochondrial sequence to that of a single nuclear gene in experimental samples. After total genomic DNA isolation, a specific primer pair for mtDNA and a primer pair for nuclear peroxisome proliferator-activated receptor-gamma coactivator-1 alpha (PGC-1α, Table 1) were used to perform the same number of PCR cycles and calculate the relative mtDNA signal to the nuclear DNA (nDNA) signal. Total DNA was isolated from the rat kidney tissue using DNeasy mini kit (Qiagen Co., Germantown, MD, USA) according to the manufacturer's instructions (The procedure of this assay was described in Supplementary Materials).

Table 1. Primers for PGC-1α and mtDNA for real time-PCR.

Gene Name		Primer Sequence
PGC-1α	F	5′-ATGAATGCAGCGGTCTTAGC-3′
	R	5′-AACAATGGCAGGGTTTGTTC-3′
mtDNA	F	5′-ACACCAAAAGGACGAACCTG-3′
	R	5′-ATGGGGAAGAAGCCCTAGAA-3′

PGC-1α, peroxisome proliferator-activated receptor-gamma coactivator-1α; mtDNA, mitochondrial DNA; F, forward; R, reverse.

2.5.5. Total Protein Content

The protein content of kidney tissue samples was determined according to the method described by Lowry et al., using bovine serum albumin as a standard [27].

2.6. Kidney Histopathology

Representative portions of the excised right kidney were fixed in 10% neutral buffered formalin for 24 h and, thereafter, dehydrated in graded alcohol concentrations and embedded in paraffin. Four micron-thick sections were cut and stained with hematoxylin and eosin (H&E) for light microscopic examination.

2.7. Statistical Analysis

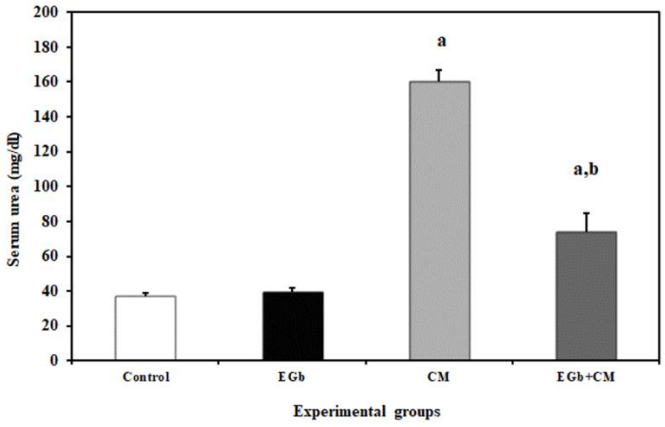
The computer package SPSS 11.5 (SPSS Inc., Chicago, IL, USA) was used for the analysis of the present study data. Shapiro-Wilk test was performed on all data sets to ensure normal distribution [28]. The results of this test are presented in a table form in Supplementary Materials. The data were expressed as mean ± SEM. For comparisons among groups, the analysis of variance (one-way ANOVA) method was applied, followed by Tukey's multiple comparison procedure. The correlation between variables was tested by computing the correlation coefficient (r, Pearson's test). Values of $p < 0.05$ were considered significant.

3. Results

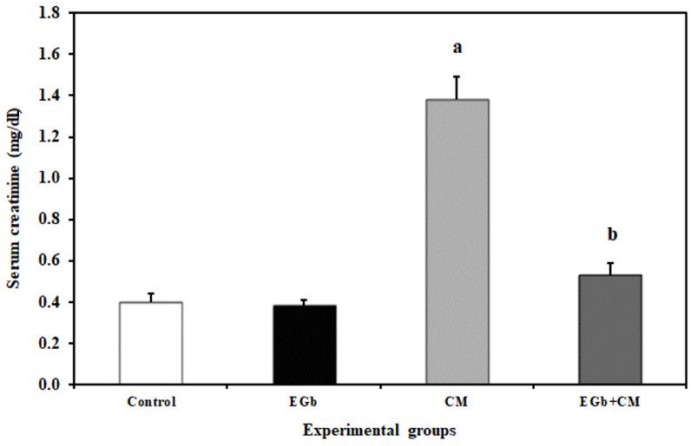
Two out of nine of rats in the CM group died in this study. No mortalities were recorded in rats of other experimental groups.

3.1. Influence of Treatment on Serum Biochemical Parameters of Kidney Function

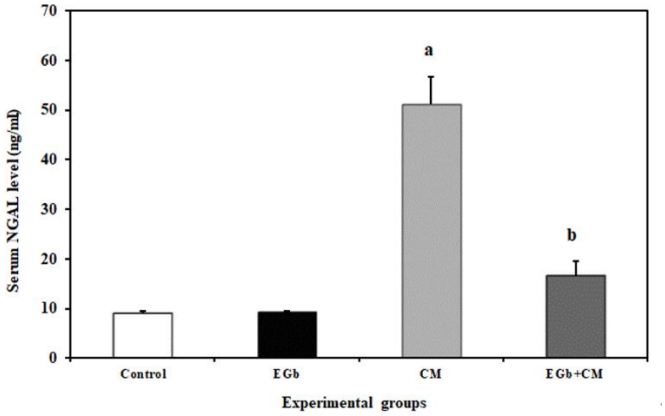
In the current study, kidney function was evaluated by measuring serum levels of urea, creatinine and NGAL. No significant changes in the levels of these parameters were observed in the EGb group, as compared to the control group. Figure 1 shows the levels of serum urea, creatinine and NGAL in all groups. In CM-treated rats, serum urea, creatinine and NGAL levels increased by 3.3, 2.5, and 4.6 folds, respectively, compared to control rats ($p < 0.05$). These notable rises in serum levels of urea, creatinine and NAGL were significantly reduced by 53.9, 61.6 and 67.3%, respectively, in rats of the EGb + CM group, as compared to the CM group.



(**A**)



(**B**)



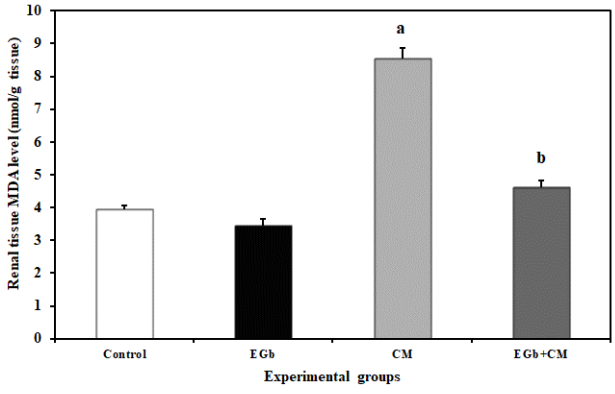
(**C**)

Figure 1. Effects of EGb761 on CM-induced changes in serum urea (**A**), creatinine (**B**) and neutrophil gelatinase-associated lipocalin (NGAL, **C**) levels. Data of all experimental groups at the end of the study are presented as mean ± SEM. EGb, *Ginkgo biloba* leaves extract; CM, contrast medium. [a] $p < 0.05$ as compared to the control group. [b] $p < 0.05$ as compared to the CM group.

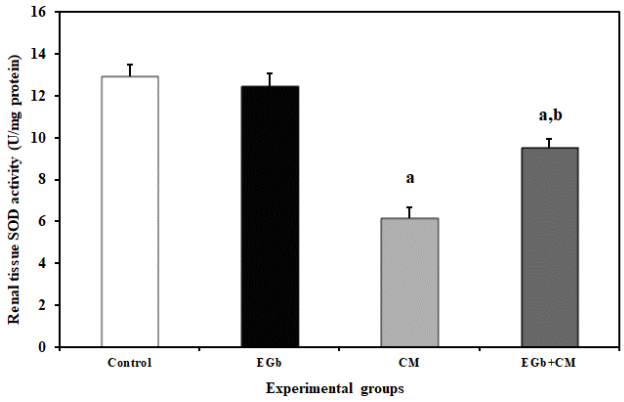
3.2. Influence of Treatment on Kidney Tissue Oxidant/Antioxidant Markers

3.2.1. Tissue Levels of MDA as a Marker of Lipid Peroxidation

As shown in Figure 2A, CM administration resulted in a significant increase by 1.2-fold in renal tissue MDA level, compared to the control level. A significant reduction of this elevated MDA level by 46% was observed in the EGb + CM group, compared to the CM group. In fact, the renal tissue MDA level in the EGb + CM group was very close to the corresponding level in control rats.



(**A**)



(**B**)

Figure 2. Ameliorative effects of EGb761 on CM-induced changes in renal tissue levels of malondialdehyde (MDA) content (**A**) and superoxide dismutase (SOD) activity (**B**). Data of all experimental groups at the end of the study are presented as mean ± SEM. EGb, *Ginkgo biloba* leaves extract; CM, contrast medium. [a] $p < 0.05$ as compared to the control group. [b] $p < 0.05$ as compared to the CM group.

3.2.2. Tissue Levels of the Antioxidant Enzyme SOD

Renal tissue SOD activity was found to be significantly reduced by 52.4% in the CM group, compared to the control group (Figure 2B). Treatment of rats with EGb761, before CM exposure, significantly elevated the CM-induced reduction in renal tissue SOD activity by 54.4% in the EGb + CM group, as compared to the CM group.

3.2.3. Tissue Changes in GSH and GSSG Levels

As shown in Table 2, the levels of the nonenzymatic antioxidant GSH and GSH/GSSG ratio, in renal tissue significantly decreased by 40.3 and 70.6%, respectively, whereas the levels of the oxidant disulfide GSSG significantly increased by 100% in the CM group, as compared to the control group. Pretreatment of CM-treated rats with EGb761 significantly elevated the CM-induced reduced renal tissue levels of both GSH content and GSH/GSSG ratios by 41.8 and 100.5%, respectively, and significantly reduced the CM-induced elevated GSSG tissue levels by 29.5% in the EGb + CM group, as compared to the CM group.

Table 2. Effects of EGb761 on CM-induced changes in renal tissue reduced glutathione (GSH) and oxidized glutathione (GSSG) contents, and GSH to GSSG ratio in rats.

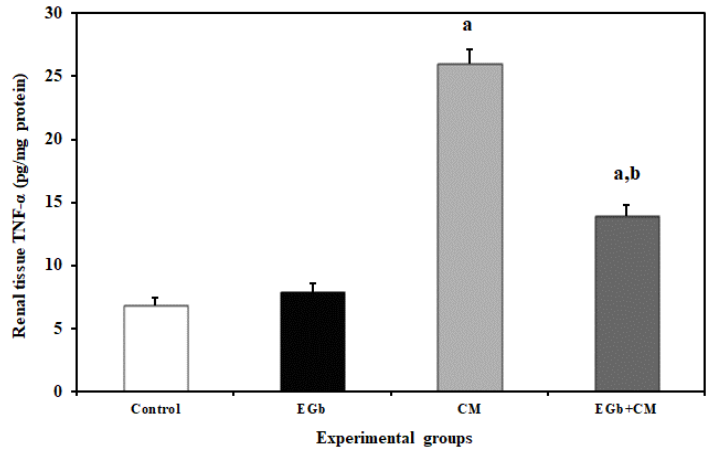
	Rat Groups	GSH (nmol/mg Protein)	GSSG (nmol/mg Protein)	GSH/GSSG Ratio
-	Control	9.94 ± 0.40	0.53 ± 0.03	18.95 ± 0.74
-	EGb	9.98 ± 0.35	0.47 ± 0.03	21.48 ± 1.35
-	CM	5.93 ± 0.26 [a]	1.06 ± 0.04 [a]	5.58 ± 0.18 [a]
-	EGb + CM	8.41 ± 0.26 [a,b]	0.75 ± 0.03 [a,b]	11.19 ± 0.30 [a,b]

Data of all experimental groups at the end of the study are presented as mean ± SEM. EGb, *Ginkgo biloba* leaves extract (EGb 761); CM, contrast medium. a: $p < 0.05$, as compared to the control group. b: $p < 0.05$, as compared to the CM group.

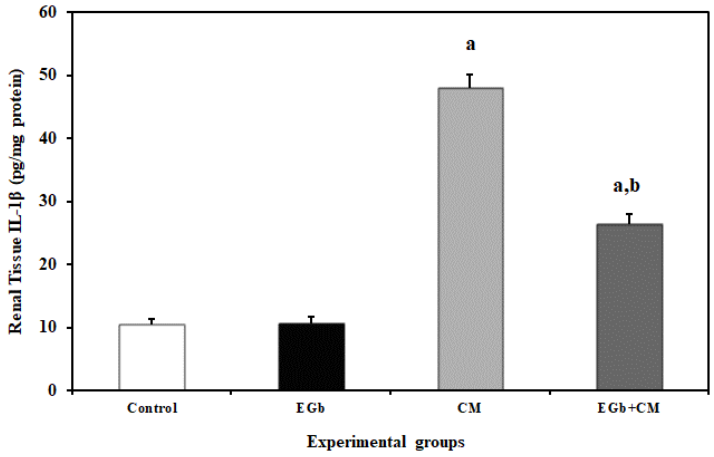
3.3. Influence of Treatment on Kidney Tissue Pro-Inflammatory Cytokines

In CM-treated rats, a significant elevation in the renal tissue TNF-α level by 2.8-fold, was observed, as compared to the control group (Figure 3A). On the other hand, pretreatment of rats with EGb761 reduced this CM-induced elevation in tissue TNF-α content by 46.6% in the EGb + CM group, as compared to the CM group ($p < 0.05$).

Also, CM administration led to a marked increase by 3.6-fold in the tissue IL-1β content compared to the control group. This elevation was found to be significantly decreased by 45.1% in the EGb + CM group, compared to the CM group (Figure 3B). In addition, CM administration resulted in a notable rise in the renal tissue IL-6 content by 2.2-fold, compared to the control group, and this CM-induced elevation in the tissue level of this parameter was significantly reduced by 42.2% in the EGb + CM group, as compared to the CM group (Figure 3C).



(**A**)



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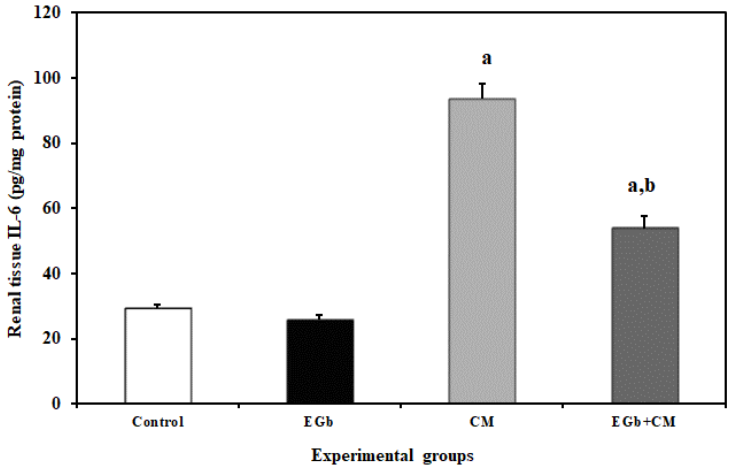


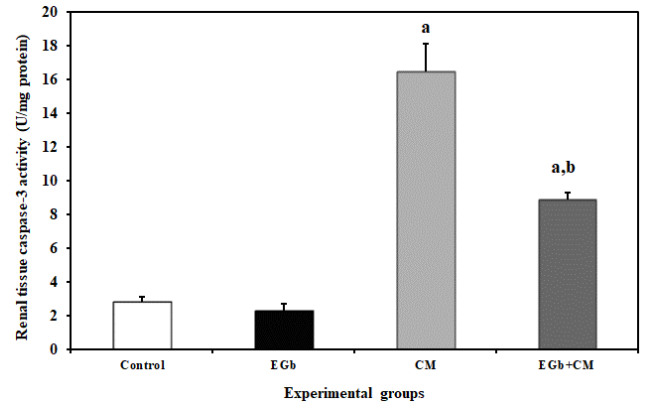
(**C**)

Figure 3. Ameliorative effects of EGb761 on CM-induced changes in renal tissue tumor necrosis factor-alpha (TNF-α, **A**), interleukin-1 beta (IL-1β, **B**) and IL-6 (**C**) levels. Data of all experimental groups at the end of the study are presented as mean ± SEM. EGb, *Ginkgo biloba* leaves extract; CM, contrast medium. [a] $p < 0.05$ as compared to the control group. [b] $p < 0.05$ as compared to the CM group.

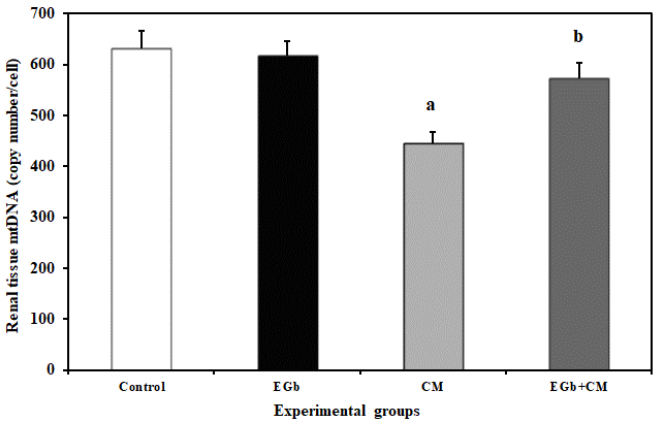
3.4. Influence of Treatment on Kidney Tissue Apoptosis and mtDNA

3.4.1. Tissue Caspase-3 Activity Changes

As shown in Figure 4A, a marked increase in tissue caspase-3 activity by 4.9-fold was noticed in CM-treated rats, compared to control rats. Pretreatments of rats with EGb761 significantly reduced the CM-induced elevations in caspase-3 activity by 46.2%, compared to the CM group (Figure 4A).



(**A**)



(**B**)

Figure 4. Ameliorative effects of EGb761 on CM-induced changes in renal tissue caspase-3 activity levels (**A**) and mitochondrial DNA (mtDNA) contents (**B**). Data of all experimental groups at the end of the study are presented as mean ± SEM. EGb, *Ginkgo biloba* leaves extract; CM, contrast medium. [a] $p < 0.05$ as compared to the control group. [b] $p < 0.05$ as compared to the CM group.

3.4.2. Tissue mtDNA Content Changes

The involvement of mitochondrial malfunction and damage in kidney cell death was evaluated, in this study, by measuring the renal tissue mtDNA content (Figure 4B). CM administration resulted in a significant decrease in renal tissue mtDNA copy number/cell by 29.4%, compared to the control group. Treatment of rats with EGb761, before CM exposure, normalized the level of mtDNA copy number/cell as this pretreatment prevented the CM-induced decline in this parameter (Figure 4B).

3.5. Tissue and Serum Data Correlation Assessment

Putting together results from all experimental groups, highly significant positive correlations were found between renal tissue levels of the apoptotic marker caspase-3 activity and both renal tissue levels of MDA, as a biomarker of lipid peroxidation, and serum levels of NGAL, as a biomarker of kidney dysfunction (Table 3). Conversely, the renal tissue levels of mtDNA content correlated negatively with the renal tissue levels of both caspase-3activity and MDA and, also, with serum levels of NGAL (Table 3).

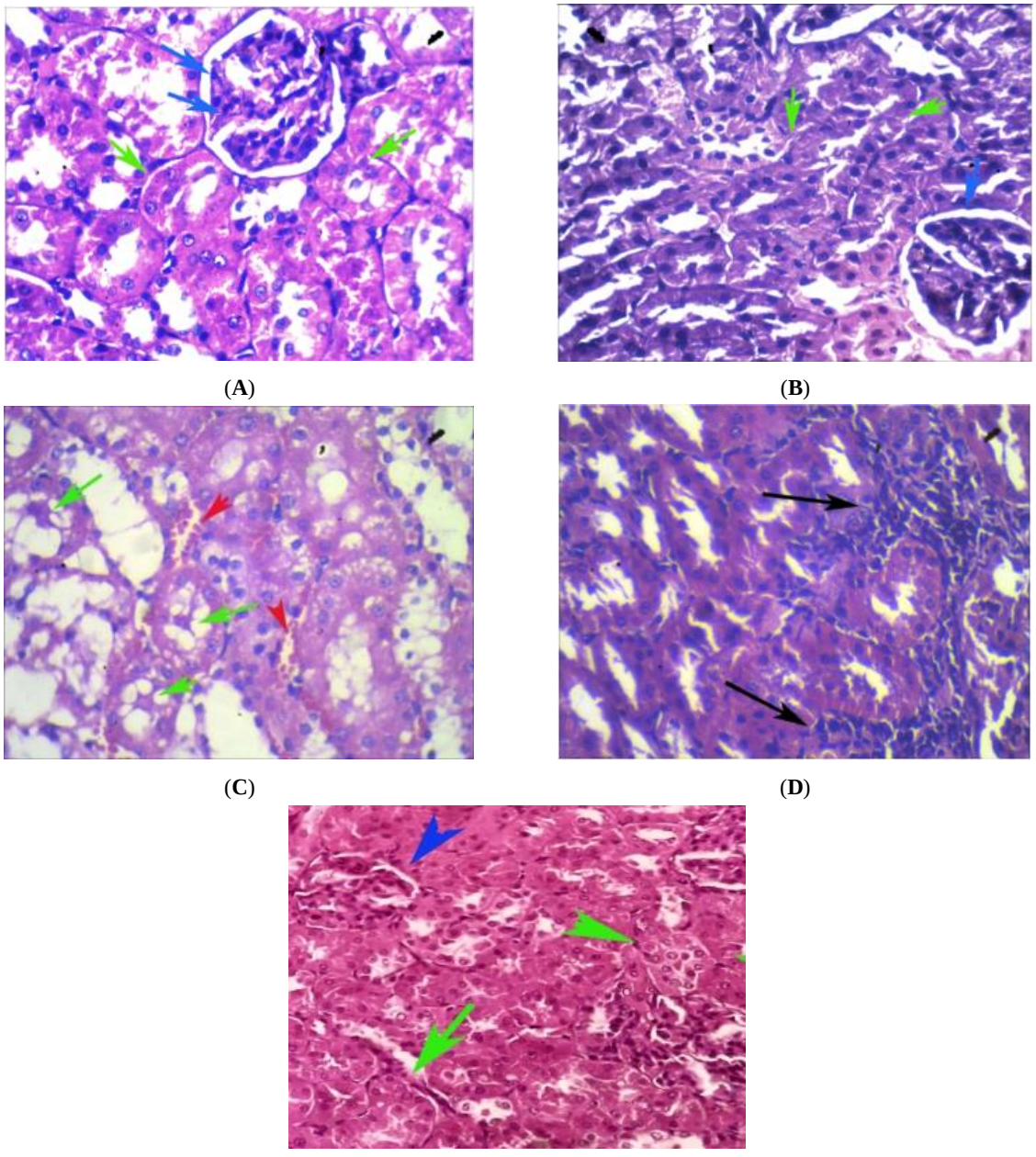
Table 3. Correlation coefficients (r values, Pearson's test) between markers of apoptosis, mitochondrial injury, oxidative stress and kidney function using the data of rats in all experimental groups at the end of the study.

Renal Tissue Markers	Serum NGAL	Renal Tissue	
		MDA	Caspase-3 Activity
Caspase-3 activity	0.707 [a]	0.827 [a]	-
mtDNA content	−0.667 [a]	−0.736 [a]	−0.637 [a]

[a] $p < 0.001$, n = 24. Caspase-3 activity, a marker of apoptosis; mtDNA content, a marker of mitochondrial injury; NGAL, neutrophil gelatinase-associated lipocalin, a marker of kidney function; MDA, malondialdehyde as a marker of oxidative stress.

3.6. Influence of Treatment on Kidney Histopathology

Photomicrographs of kidney tissue sections from rats of control and EGb groups (Figure 5 A, B, respectively) were similar and showed a normal kidney histological morphology with intact normal well-defined glomeruli and tubules. In the CM group rats, the kidney tissue sections displayed several major histopathological abnormalities including congestion of the intertubular blood vessels, interstitial mononuclear cells (mainly lymphocytes) infiltration and evidence of tubular injury in the form of vacuolar degenerative changes of renal tubular cells, tubular dilatation, and dilated tubules filled with hyaline casts (Figure 5C,D). Examination of kidney tissue sections from the EGb + CM group showed marked improvement of the CM-induced abnormalities as the glomeruli and renal tubules appeared somewhat normal (Figure 5E), having the same histological features of kidneys of control rats (Figure 5A, B).



(**A**) (**B**)

(**C**) (**D**)

(**E**)

Figure 5. Protective effects of EGb 761 on CM-induced renal histopathological changes. Photomicrographs of kidney tissue sections (H&E, ×400) from: *Control (**A**) and EGb-treated rats (**B**) showing renal cortex with intact normal glomeruli (blue arrows) and normal tubules lined with columnar epithelial cells (green arrows), *CM-treated rats (**C**,**D**) showing dilated tubules filled with hyaline casts with vacuolar degenerative changes of renal tubular cells (green arrows), congested intertubular blood capillaries (red arrows), multiple areas of atrophic cystic tubules (**C**), and heavy interstitial mononuclear cells infiltration mainly lymphocytes **(**black arrows, **D**), and *EGb + CM group (**E**) showing nearly normal kidney histoarchitecture with intact tubules lined by columnar epithelial cells (green arrows) and normal glomeruli (blue arrows).

4. Discussion

As far as we know, no previous research work studying the potential effects of EGb761 on CI-AKI has been reported. The current study was designed to investigate the potential protective effect of EGb761 against CI-AKI in rats. The results of the present study demonstrated that CM-treated rats showed the features of AKI and treatment of rats with EGb761 before CM exposure provided nephroprotection as evidenced by several major findings in the EGb + CM group. First, as compared to the nonpretreated CM group, the results showed significant decreases of the CM-induced elevated serum levels of urea, creatinine and NGAL (as indices of kidney function), and of the renal tissue levels of MDA (an index of lipid peroxidation), TNF-α, IL-1β and IL-6 (as indices of inflammation), and caspase-3 activity (an apoptotic marker). Second, significant increases of the CM-induced reduced renal tissue levels of SOD, GSH and GSH/GSSG ratio (as indices of tissue antioxidant defenses) and mtDNA copy number/cell (a biomarker of mitochondrial function). Third, the histological architecture of kidneys from the EGb + CM group rats appeared normal having features like those of control rats.

In the current study, the rats of the CM group showed marked impairment of kidney function with elevated serum creatinine and urea levels. Based only on these parameters, the assessment of renal function may not always be satisfactory as their serum levels may be affected by extra-renal factors and may not change until a significant fraction (>50%) of kidney function has already been lost [29,30]. Therefore, serum NGAL level was measured as it fulfills many criteria for a sensitive and specific biomarker for AKI as described by Bolignano et al. [31]. The CM group rats, in the current study, showed a marked rise in serum NGAL level, as compared to control rats. This finding is in agreement with the results of several studies that have shown that serum NGAL level can be used as a diagnostic biomarker for AKI as it increases proportionally to the extent of kidney damage [31–33].

There is growing evidence that CM administration may lead to imbalance between oxidative and antioxidative factors in the kidney tissue resulting in excessive accumulation of ROS, especially hydroxyl and superoxide radicals [5,34]. In our study, CM-induced oxidative stress was manifested by a reduction in renal tissue GSH content and SOD activity with a marked elevation in the tissue level of MDA. The histopathological changes observed in kidney sections from CM-treated rats confirmed the induction of AKI and provided an explanation for the observed changes in kidney function. In addition, it has been reported that increased ROS production in response to kidney injury may lead to increased synthesis and release of inflammatory mediators that can initiate and amplify inflammation and exacerbate apoptosis in renal tubular cells [4,35]. In parallel, increased ROS at the site of inflammation may cause endothelial dysfunction leading to opening of the interendothelial junctions with subsequent increased migration of inflammatory cells across the endothelial barrier into the renal tissue [34,36]. In accordance with these reports, our results showed marked elevation in renal tissue levels of proinflammatory cytokines and the apoptotic marker besides the finding of interstitial mononuclear cell infiltrations in kidney sections from CM group rats.

Noteworthily, the results of the studies on mitochondria reported by Pello et al. [37] and Vakifahmetoglu et al. [38] suggest that there is a direct link between oxidative stress-induced mitochondrial dysfunction and cell death. Mitochondrial dysfunction is a hallmark in a variety of diseases including CI-AKI as it results in inefficient cellular energy production, enhanced ROS generation and increased cellular apoptosis [37,38]. In fact, mitochondria are the main source of intracellular ROS production and, at the same time, the main target for the ROS unfavorable effects including mitochondrial damage [38]. Within the mitochondrion, mtDNA seems to be the main target for ROS-induced oxidative damage due to its lack of protective histones and its closeness to the electron transport chain, the principal site for the generation of ROS [39]. Recently, mtDNA copy number has been suggested as a promising biomarker of mitochondrial dysfunction especially in conditions associated with oxidative stress as the increased ROS production may cause damage to cellular components including mtDNA [40]. Therefore, assessment of mtDNA copy number changes could help in understanding the pathogenesis of AKI [41]. Our results indicated that renal tissue levels of mtDNA copy number, in CM group rats, were significantly lower than the corresponding values in control rats, a change that could be attributed to the CM-induced oxidative damage to mtDNA. This interpretation is supported by the negative correlation, observed in the current study, between the renal tissue levels of both mtDNA copy number and the lipid peroxidation marker MDA.

Apart from its involvement in many of physiological processes, renal cell apoptosis seems to play a pivotal role in drug-induced nephrotoxicity including CI-AKI [42]. In this regard, excessive ROS production has been suggested as an initiator of apoptotic cell death through the activation of apoptosis signaling pathways [43]. Recently, mitochondria have also gained great importance as a primary player in cellular apoptosis. It has been reported that mitochondrial damage results in the release of several proteins into the cytosol including pro-caspases and cytochrome C that activate catabolic caspases including caspase-3, a critical factor in the apoptotic execution

stage [44]. In agreement with these findings, our results showed that renal tissue caspase-3 levels correlated negatively with renal tissue mtDNA levels but positively correlated with renal tissue MDA levels.

As no available pharmaceutical agents have been proven to effectively treat CI-AKI, many researchers have evaluated a variety of synthetic drugs and natural products for the prevention of CI-AKI [9,45]. Although some of the tested agents have been proven to be effective, there is still room for improvement via targeting the pathophysiologic mechanisms involved in CI-AKI development. In recent years, herbal medicines are increasingly gaining greater acceptance from the public and medical profession due to good therapeutic efficacy, low side effects and lower cost than synthetic drugs [46]. In this regard, EGb761 is one of the most common herbal medicines that has multiple uses associated with several health claims, mostly in relation to central nervous system disorders, cardiovascular problems and pulmonary diseases [17,18,47]. In the present study, rat treatment with EGb761, before CM administration, significantly reduced the CM-induced elevation in serum urea levels and normalized elevated serum creatinine and NGAL levels as compared to control rats. This improvement in kidney function was associated with marked amelioration of the CM-induced renal tissue histopathological changes with apparently normal glomeruli and renal tubules. Moreover, our results demonstrated that rat treatment with EGb761, before CM administration, improved the endogenous renal antioxidant defense status as indicated by the observed increases in renal tissue levels of GSH, GSH/GSSG ratio and SOD activity, compared to CM group rats, leading to normalization of the elevated renal tissue MDA levels. The EGb761-induced increase in intracellular GSH content could serve to detoxify ROS by directly scavenging them as well as indirectly by acting as a co-substrate in the glutathione peroxidase-catalyzed reduction of H_2O_2 and lipid peroxides [16,48]. Also, the increased SOD activity induced by EGb761 could lead to dismutation of superoxide radicals and prevents further production of free radicals such as peroxynitrite and hydroxyl radicals [49,50].

Apart from oxidative stress, inflammation is a defensive host reaction, in response to pathogenic stimuli, that includes recruitment and proliferation of inflammatory cells in addition to the secretion of cytokines that play a crucial role in inflammation regulation [51,52]. Accordingly, inhibition of excessive production of these mediators could be a strategy to prevent the occurrence and/or progression of inflammation associated with CI-AKI. The present study showed that treatment of rats with EGb761, before CM administration, significantly reduced CM-induced elevations in renal tissue levels of the proinflammatory cytokines with marked amelioration of kidney inflammatory histopathological features, as compared to the CM group. These results agree with several studies which revealed that EGb761 exerted a marked anti-inflammatory effect in different models of acute tissue injury [21,53,54]. Several mechanisms have been suggested for the anti-inflammatory effect of EGb761 including inhibition of excessive mRNA and protein expression levels of both inducible nitric oxide synthase and cyclooxygenase-2, suppression of nuclear factor kappa-B and inhibition of secretion of pro-inflammatory cytokines [53,54]. In addition, our results demonstrated that pretreatment of rats with EGb761 displayed an antiapoptotic effect through ameliorating the CM-induced elevation of renal tissue caspase-3 activity in the EGb + CM group, as compared to the CM group. Previous studies had related the antiapoptotic effect of EGb761 to its ability to cause downregulation of tissue Bax mRNA and p53 mRNA expressions in different rat models [55,56]. Regarding its role in apoptosis, p53 expression was reported to be upregulated following mtDNA damage leading to activation of caspase-3 enzyme and initiation of mitochondrial apoptosis [55,57].

Because of the well-established role of mitochondria in cellular energy production, these organelles are especially abundant in renal proximal tubular cells and are of critical importance in kidney function as kidneys require a great amount of energy to perform their tasks [58]. As a key indicator of mitochondrial function, mtDNA copy number abnormalities have been observed during the development of AKI [41]. Mitochondrial exposure to excessive ROS might cause damage to DNA replication enzymes and thereby aggravate the reduction of the mtDNA copy number [59]. In the current study, the reduced levels of renal tissue mtDNA, observed in the CM group, were reversed by EGb761 pretreatment in the EGb + CM group. The mechanism of the EGb761 protective effect on renal tissue mitochondria may be related to both mild uncoupling of mitochondria with subsequent reduction of ROS production and direct scavenging of ROS by the flavonoids present in EGb761 [60,61].

5. Conclusions

The present study, to the best of our knowledge, is the first research work demonstrating that treatment with EGb761 before CM exposure in rats had a significant nephroprotective effect against CI-AKI development with preservation of kidney function. This beneficial effect of EGb761 could be attributed to interrelated suppressions of CM-induced increases in renal tissue oxidative stress, inflammation, apoptosis and tubular cell mitochondrial damage. Taken together, our results are potentially of clinical significance and suggest a new therapeutic strategy for attenuating CI-AKI via using EGb761, as a preventive agent, before CM administration. Thus, our findings

may serve as an experimental basis for further studies to elucidate the clinical implications of EGb761 as a nephroprotective agent in patients at the risk of developing CI-AKI.

Supplementary Materials: The supporting information can be downloaded at: https://www.sciltp.com/journals/jmnp/articles/2505000593/s1.

Author Contributions: M.M.F. conceived the idea of this work and made its design. M.A.W and H.E.A conducted the experimental work. A.H.K. carried out the histopathological work. M.M.F. analyzed and interpreted the data and wrote the manuscript. All authors have read and agreed to the published version of the manuscript.

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Institutional Review Board Statement: The protocol of this study was approved by the Animal Care and Use Committee of the Medical Research Institute, Alexandria University (Alexandria, Egypt). All experimental procedures related to the rats were carried out according to the instructions of the European Directive 2010/63/EU for animal experimentation and the ethical guidelines of the Animal Care and Use Committee, Medical Research Institute, Alexandria University (Approval No. AU 0122262211).

Data Availability Statement: All datasets generated in this study are included in this article and in its online supplementary materials.

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

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Article

Exploring the Pharmacological Potential of *Bauhinia malabarica* Roxb.: A Comprehensive In Vitro and In Vivo Investigation

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Abstract: This study investigates the potential biological effects of organic soluble fractions from the crude methanol extract of the leaf and bark of *Bauhinia malabarica* Roxb (*B. malabarica*), which is traditionally used for various ailments. Plant materials were collected, dried, and extracted using methanol. They were then extracted into aqueous fractions, carbon tetrachloride, chloroform, and petroleum-ether. The methanolic leaf extract demonstrated excellent antioxidant activity during the DPPH assay for antioxidant testing, with an IC_{50} value of 12.15 µg/mL, which is similar in efficacy to the standard ascorbic acid (IC_{50} = 5.8 µg/mL). The cytotoxicity assay was carried out using brine shrimp nauplii. All test samples of *B. malabarica* showed significant membrane-stabilizing activity as determined by the hemolysis induced by hypotonic solutions and heat. The plant samples from leaf extract and bark extract also exhibited significant anthelmintic and analgesic activity in a dose-dependent manner. These plant extracts exerted analgesic effects that were characterized by elongation of tail immersion time and reduction in abdominal writhes. The test samples reduced the quantity of diarrhea caused by castor oil in mice in a dose-dependent manner. Additionally, the extract extended the sleep duration and delayed the onset of action compared to the control group. Based on our findings, *B. malabarica* could be a rich source of bioactive compounds, meriting further exploration for its potential in traditional medicine.

Keywords: *Bauhinia malabarica*; antioxidant; cytotoxicity; membrane stabilizing; anthelmintic; analgesic; anti-diarrheal; sedative

1. Introduction

According to a 1985 estimation by the WHO, approximately 65% of the global population primarily relied on plant-derived traditional medicines for their healthcare, as they are affordable and easily accessible to the general public. Over the past 30 years, up to 50% of approved drugs have been derived directly or indirectly from natural products. Many of the medicinal substances currently in use, such as aspirin, morphine, quinine, artemisinin and taxol were obtained from plant sources [1–3]. Moreover, the use of medicinal plants in the preparation of traditional medicine has increased during the past decade. In recent years, research on plant-based medicine has emerged as an interesting area in the scientific validation of medicinal plants that are recommended by local healers and tribal societies.

Bauhinia malabarica Roxb. (Family- Leguminosae) is a small- or moderate-sized deciduous tree which is locally known as “Kanchan”. Most *Bauhinia* species are planted for their beautiful blooms and decorative shrubs [4] In Bangladesh, the plant is widely distributed in deciduous forests of Sylhet. *B. malabarica* has many medicinal properties [5]. Stems and leaves are used as diuretics, anti-infective and anti-diarrheal in traditional Thai medicine [6]. The root extracts showed in vitro antimalarial activity [7]. An infusion of the young flowers is given in dysentery. Previously, antimalarial racemosol derivatives have been extracted from the root of *B. malabarica* [8]. Decoction of this plant root was used for liver problems. Root and stem were beneficial in the treatment of cholera [9]. Flavonols were isolated from *B. malabarica* leaves [7]. The folk people in Malabar coast of Southern India

use the stem bark extract for the treatment of jaundice [10]. There are a few ethno-pharmacological surveys on the medicinal applications of *B. malabarica* [8], however there are still few laboratory data on their bioactivity. Therefore, the current study was carried out to fill these gaps by analyzing the biological and pharmacological effects of *B. malabarica* leaf and stem bark extracts utilizing established procedures.

2. Materials and Methods

2.1. Plant Material Collection and Processing

Leaves and stem bark of *B. malabarica* were collected from Romna park area, Dhaka. After collection, the taxonomical identification of the plant was completed in Bangladesh National Herbarium, Dhaka. Next, the leaves and stem barks were carefully cleaned, air-dried, and then ground into a coarse powder for further analysis.

The powdered ingredients of the *B. malabarica* leaf and stem bark were macerated separately in 200 mL of methanol for several days, allowing the plant material to completely dissolve and release its bioactive components. After maceration, the plant material was filtered using a Whatman No. 1 filter paper, and the resulting filtrate was concentrated under decreased pressure using a rotary evaporator (Buchi Rotavapor R-3) set to a temperature of 40 °C. This evaporation process removed the methanol, yielding a semisolid mass that represented the crude methanol extract of the *B. malabarica* leaf (MEL) and stem bark (MEB).

To further isolate and separate the bioactive components of the crude extracts, the modified Kupchan method [11] was employed. This method involved solvent-solvent partitioning, where approximately 5 g of each of the crude extracts (MEL and MEB) was subjected to successive extractions using 50 mL of petroleum ether, 50 mL of carbon tetrachloride, 50 mL of chloroform, and 50 mL of water. These solvent fractions were chosen based on their differing polarities, which allowed for the separation of compounds based on their solubility in each solvent.

The 5 g of crude extract effectively achieved fractionation, as it produced clear, distinct layers of soluble fractions, which were identified as petroleum-ether, carbon tetrachloride, chloroform, and water fractions (Table 1). These fractions were dried and collected separately for further analysis. The selected solvent volumes (50 mL each) ensured adequate contact between the extract and solvent, providing efficient partitioning and separation of bioactive compounds.

After fractionation, the biological activities of each of the four soluble fractions, as well as the crude extracts of leaf and bark (Table 1), were assessed separately. Through this method, we comprehensively evaluated the different bioactive components in the crude extracts and provided insights into their individual contributions to the overall biological activity.

Table 1. Test samples of *B. malabarica* used in pharmacological investigations.

Plant Part	Sample Code	Test Sample
Leaves	MEL	Methanolic soluble fraction of leaf
	PEFL	Petroleum ether soluble fraction of leaf
	CTFL	Carbon tetrachloride soluble fraction of leaf
	CHFL	Chloroform soluble fraction of leaf
	AQFL	Aqueous soluble fraction of leaf
Stem Bark	MEB	Methanolic soluble fraction of stem bark
	PEFB	Petroleum soluble fraction of stem bark
	CTFB	Carbon tetrachloride soluble fraction of stem bark
	CHFB	Chloroform soluble fraction of stem bark
	AQFB	Aqueous soluble fraction of stem bark

2.2. In Vitro Experiments

2.2.1. Total Phenolic Content

Gallic acid was employed as the standard in the Folin-Ciocalteau's spectrophotometric method to calculate the total phenolic content [12]. Gallic acid solutions in a range of concentrations from 0.391 μg/mL to 100 μg/mL were prepared. Next, 2.5 mL of a 10-fold diluted Folin-Ciocalteau's phenol reagent was mixed with 0.5 mL of the plant extract (2 mg/mL). Subsequently, 2.0 mL of a 7.5% sodium carbonate (Na_2CO_3) solution was added, and the mixture was left to stand in the dark for 20 min. Finally, the absorbance was measured at 760 nm and the total phenolic content of the plant extracts was determined using a calibration curve.

2.2.2. DPPH Assay

DPPH (2,2-diphenyl-1-picrylhydrazyl) radical scavenging activity was measured using the Brand-Williams technique [13]. As reference standards, tert-Butyl-1-hydroxytoluene (BHT) and ascorbic acid (AA) were employed. The following formula was used to determine the percentage (%) of DPPH scavenging activity:

$$\%\ \text{Inhibition of DPPH scavenging} = \frac{\mathrm{A_{DPPH}} - \mathrm{A_{Sample}}}{\mathrm{A_{DPPH}}} \times 100\%$$

where, A = Absorbance of each group.

2.2.3. Membrane-Stabilizing Activity

Hypotonic solution- and heat-induced hemolysis experiments were performed to assess the membrane-stabilizing activity of the plant extracts [14].

2.2.4. Anthelmintic Activity

Pheretima posthuma (Annelida) was utilized to measure the in vitro anthelmintic activity of each *B. malabarica* extract at three different concentrations (20, 10, and 5 mg/mL), with albendazole and saline water serving as the standard and control, respectively.

2.2.5. Brine Shrimp Lethality Bioassay

In this experiment, brine shrimp eggs were hatched into nauplii by incubating them in seawater simulation [15]. To achieve the required concentration of the test sample, a precisely measured quantity of dimethyl sulfoxide (DMSO) was added. The nauplii were counted, placed in vials containing 5 milliliters of artificial saltwater. A micropipette was employed to add different concentrations of the samples to the vials that had already been labeled. The number of surviving nauplii was noted after a 24-h period.

2.3. In Vivo Experiments

2.3.1. Test Animals and Study Design

For the study, Swiss-albino mice of either sex, 4 to 5 weeks of age, were acquired from the pharmacy department of Jahangirnagar University in Savar, Dhaka, Bangladesh. Using a 12-h light/dark cycle for adequate ventilation, the mice were kept in standard polypropylene cages with controlled temperatures (24 ± 2 °C, 60–70% relative humidity). They were fed a regular diet, commercially available from vendors, with water provided ad libitum. The animals were allowed to acclimatize to the laboratory settings for 1 week and then divided into different experimental groups randomly. Twelve hours before and throughout the trial, the mice were fasted. All animal procedures were approved by the institutional ethical committee [16].

As part of the study design, eighteen experimental animals were chosen at random and divided into six groups (Group I, Group II, Group III, Group IV, Group V, and Group VI). Each group consisted of three mice and received a different therapy. The dosages of the test items and control materials were adjusted appropriately, and each mouse was carefully weighed before the experiment began. Animals in a group were given distinct identities in order to let them be identified during the therapy (Numbering of Mice): Mice 1 = M1; Mice 2 = M2; and Mice 3 = M3.

2.3.2. Central Analgesic Activity

The tail immersion test [17] was used to assess the central analgesic effect of the plant extracts. Morphine (2 mg/kg body weight) solution was administered subcutaneously as the standard. The *B. malabarica* extracts and the negative control (1% Tween 80 in normal saline) were administered orally at a dosage of 200 and 400 mg/kg body weight. The percentage (%) of time elongation relative to the control mice was determined using the following formula:

$$\%\ \text{Time elongation} = \frac{\mathrm{T_{Test}} - \mathrm{T_{Control}}}{\mathrm{T_{Control}}} \times 100\%$$

Here, T = mean tail deflection time in the respective group.

2.3.3. Peripheral Analgesic Activity

Acetic acid-induced writhing was utilized to test the plant extract's peripheral analgesic effect in albino mice [18]. Albino mice were administered the plant extracts at 200 and 400 mg/kg body weight, as well as the conventional reference medication, diclofenac sodium (50 mg/kg body weight), and 1% Tween 80 in normal saline as the negative control. About 40 min after the sample was delivered, the experimental animal was given 0.1 mL/10 g body weight of 1% acetic acid intraperitoneally to induce writhing.
The following formula was used to determine the percentage of writhing inhibition:

$$\%\ \text{Inhibition of writhing} = \frac{\mathrm{N_{Control}} - \mathrm{N_{Test}}}{\mathrm{N_{Control}}} \times 100\%$$

Here, N = Mean number of writhing in respective group.

2.3.4. Anti-diarrheal Activity

The castor oil-induced diarrhea model in mice was used to evaluate the anti-diarrheal properties of *B. malabarica* [19]. The test groups received 200 mg and 400 mg/kg of body weight of the soluble fractions of the crude methanol extract of *B. malabarica*. The negative control group was administered a vehicle solution (10 mL/kg body weight) that contained 1% Tween 80 in normal saline, while the positive control group was administered 50 mg/kg body weight of loperamide orally. The number of diarrheal stools produced by the mice was monitored for a maximum of four hours, and the percentage reduction in diarrhea caused by the plant extract was noted.

The percentages (%) of diarrhea decrease were calculated using the following formula:

$$\%\ \text{Reduction of diarrhea} = \frac{\mathrm{D_{Control}} - \mathrm{D_{Test}}}{\mathrm{D_{Control}}} \times 100\%$$

D = The number of cases of diarrhea in each group.

2.3.5. Sedative Activity

The phenobarbitone-induced sleeping time test was used to assess the sedative effects of *B. malabarica* leaf and bark extracts on Swiss albino mice [20]. The test groups were administered 200 mg/kg and 400 mg/kg of the leaf and bark extract orally. Diazepam (1 mg/kg, i.p.) served as the traditional positive control in this investigation. Phenobarbitone sodium (25 mg/kg body weight) was given intraperitoneally to induce sleep after a 30-min break. The onset time (in min) and the duration of sleep (from the loss to the recovery of the righting reflex) were recorded as indicators of sedative activity [21].

3. Results

The purpose of this study was to evaluate cytotoxic, antioxidant, membrane-stabilizing, anthelmintic, analgesic, anti-diarrheal, and sedative properties of *B. malabarica*.

3.1. Total Phenolic Content

A comparative analysis of the total phenol content (TPC) of all *B. malabarica* leaf extract and stem bark extracts along with various solvent fractions was carried out using the spectrophotometric method. Using the calibration curve obtained from gallic acid solutions and the regression equation ($y = 0.016x + 0.021$, $R^2 = 0.998$), the total phenol concentration in the extracts was ascertained. Table 2 shows that the total phenolic content (TPC) of *B. malabarica* leaf extracts ranged from 1.51 to 73.49 mg of GAE per gram. At 73.49 mg of GAE/g, the methanol leaf extract (MEL) had the greatest phenolic content. The petroleum ether leaf extract (PEFL) and chloroform leaf extract (CTFL) had TPC values of 28.06 and 32.38 mg of GAE/g, respectively. Out of all the extractives used for *B. malabarica* stem bark, CHFB had the highest phenolic content (10.28 mg of GAE/gm of extractives), followed by MEB (7.13 mg of GAE/gm of extractives).

This could be because these two fractions contain a considerable amount of antiradical phenolic compounds.

Table 2. Crude methanol extracts and their fractions from *B. malabarica* leaves and bark are examined for their cytotoxic effects, antioxidant qualities, and total phenolic content.

Test Sample	Antioxidant Activity		Cytotoxic Activity
	Total Phenolic Content (mg of GAE/g of Dried Extract)	DPPH Assay IC_{50} (μg/mL)	LC_{50} (μg/mL)
MEL	73.49	12.15	1.16
PEFL	28.06	45.32	2.81
CTFL	32.38	35.60	1.73
CHFL	6.57	22.81	3.52
AQFL	1.51	29.45	1.45
MEB	7.13	20.95	4.23
PEFB	2.44	38.20	28.09
CTFB	4.10	31.88	4.23
CHFB	10.28	18.22	8.97
AQFB	3.06	25.75	2.09
BHT	-	22.50	-
AA	-	5.80	-
VS	-	-	0.45

3.2. DPPH Free Radical Scavenging Activity

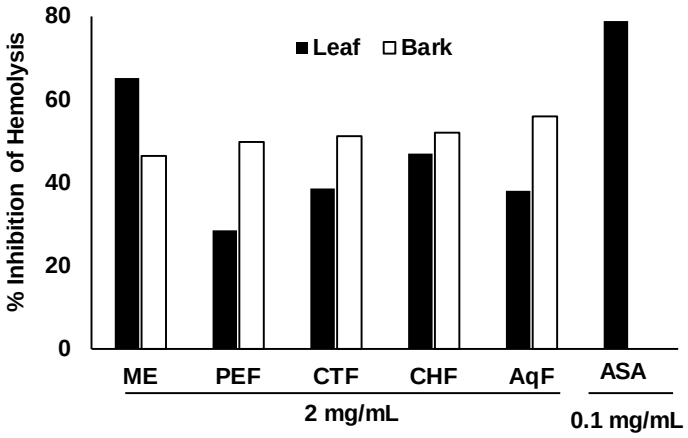
The ability of the organic components of the *B. malabarica* extract to scavenge DPPH free radicals was used to measure its antioxidant activity. With an IC_{50} value of 12.15 μg/mL, the methanolic leaf extract showed the highest free radical scavenging activity, as shown in Table 2. The chloroform fraction of the stem bark followed in next with an IC_{50} of 18.22 μg/mL. The results of the study showed that the improved antioxidant activity of the test sample is roughly close to that of conventional ascorbic acid (IC_{50} = 5.8 μg/mL). This could be because these two fractions contain a considerable amount of antiradical phenolic compounds (Table 2). This assay is based on the scavenging of DPPH free radicals [22] by antioxidants due to their hydrogen-donating capability [23].

3.3. Brine Shrimp Lethality Bioassay

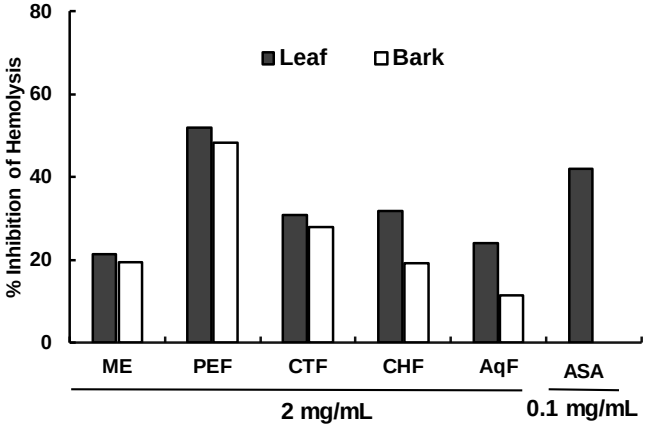
For the initial evaluation of plant extract cytotoxicity, the brine shrimp lethality test is frequently utilized. Cytotoxic compounds in this assay show significant activity, making it a useful, low-cost method for detecting anti-tumor and pesticidal compounds [24]. Table 2 presents the LC_{50} values for the plant extracts and the positive control, vincristine sulphate. All *B. malabarica* extracts displayed notable toxicity to brine shrimps, with LC_{50} values ranging from 1.16 to 28.09 μg/mL, compared to vincristine sulphate's LC_{50} of 0.45 μg/mL, suggesting the extracts as potential candidates for anticancer compounds. The methanol extract of leaf and its carbon tetrachloride and aqueous fraction showed more cytotoxic effects with LC_{50} value of 1.16, 1.73 and 1.45 μg/mL, respectively. Similarly, *B. malabarica* leaf methanol extract's aqueous fraction showed notable cytotoxicity (LC_{50} = 2.09 μg/mL) against shrimp nauplii (Table 2). Higher extract concentrations gradually boosted the plant sample's inhibitory impact. This suggests the possibility that the extract contains harmful substances, which warrants further investigation.

3.4. Membrane-Stabilizing Activity

The various soluble fractions from the methanol extract of *B. malabarica* were tested for membrane stabilizing activity using standard protocols, with the results presented in Figure 1. At a concentration of 2.0 mg/mL, the plant samples significantly reduced RBC hemolysis induced by both hypotonic solution and heat, outperforming the standard aspirin (Figure 1A, 1B). In the hypotonic solution-induced hemolysis model, the methanol extracts of both the leaf and bark showed 65.2% and 46.4% inhibition of RBC hemolysis, respectively, compared to the reference aspirin, which inhibited 78.0% of hemolysis (Figure 1A). During this experiment, most of the solvent fractions notably the pet-ether fraction (49.9%), carbon tetrachloride fraction (51.2%) and aqueous fraction (56.0%) of the *B. malabarica* bark showed prominent inhibitory activity against the hemolysis induced by hypotonic solution. In case of heat induced hemolysis, all these solvent fractions from the methanol extract of *B. malabarica* leaf showed stronger protection activity compared to the extent of inhibition observed by the solvent fractions of *B. malabarica* bark (Figure 1B).



(**A**)



(**B**)

Figure 1. The RBC membrane stabilizing properties of different organic fractions of *B. malabarica* leaf and bark extract were determined using the hypotonic solution-induced hemolytic method **(A)** and Heat-induced method (**B**).

3.5. Anthelmintic Activity

In comparison to the negative control, the methanol extract of leaves and bark of *B. malabarica* considerably shortened the paralysis and death times of *Pheretima posthuma* (Table 3). This anthelmintic activity was dose-dependent and compared to the standard albendazole at the concentration tested. Here the methanol extract of leaf, MEL at the concentration of 20, 10 and 5 mg/mL showed paralyzing time 29, 36, 48 min and death time 62, 75, 83 min, respectively. Similarly, normal albendazole (Conc. 10 mg/mL) induced paralysis at 25 min and death at 55 min, whereas the methanol extract of *B. malabarica* bark was most efficacious at a concentration of 20 mg/mL, causing paralysis and death within 31 min and 67 min, respectively.

Table 3. Anthelmintic effects of the methanol extracts from the leaves and stem bark of *B. malabarica* where n = 1.

Test Sample	Concentration (mg/mL)	Paralyzing Time (min)	Death Time (min)
Albendazole	10	25	55
MEL	20	29	62
	10	36	75
	5	48	83
MEB	20	31	67
	10	39	79
	5	50	97

3.6. Central Analgesic Activity

The central analgesic effects of standard morphine and organic soluble fractions in albino mice, as determined by the tail immersion method, are summarized in Table 4. A higher percentage of elongation indicates stronger central antinociceptive activity. The results revealed that all plant extracts at doses of 200 and 400 mg/kg body weight significantly ($p < 0.001$) and dose-dependently reduced pain sensation compared to the untreated control groups. However, morphine (2 mg/kg body weight) showed a higher analgesic effect than the plant extracts ($p < 0.001$). At 30, 60, and 90 min after the albino mice were given the plant samples, the percentage elongation times were noted. Mice given 200 and 400 mg/kg body weight dosages of the methanolic leaf extract showed substantial tail immersion elongation of 55.63 ± 0.587%, 144.30 ± 0.760%, and 167.57 ± 0.309% at 30, 60, and 90 min, respectively. In comparison, standard morphine resulted in an elongation of 447.63%. Likewise, the maximum analgesic effect of the methanolic bark extract was observed at 90 min, with a 209.34 ± 0.163% increase in reaction time after treatment with 400 mg/kg in mice (Table 4). These findings show that, in comparison to the morphine solution, the methanolic leaf and bark extracts both considerably ($p < 0.001$) postponed the start of pain sensation.

Table 4. *B. malabarica* leaf and bark extract's central analgesic effects in mice.

Test Sample	% Time Elongation (Mean ± SEM)		
	After 30 min	After 60 min	After 90 min
Morphine 2 mg/kg	106.11 ± 0.492 ***	267.15 ± 0.780 ***	447.63 ± 0.409 ***
MEL 200 mg/kg	55.63 ± 0.587 *	144.30 ± 0.760 **	167.57 ± 0.309 ***
MEL 400 mg/kg	73.20 ± 0.638 **	172.40 ± 1.060 **	193.94 ± 1.306 *
MEB 200 mg/kg	35.57 ± 0.912	130.94 ± 0.373 ***	143.57 ± 0.193 ***
MEB 400 mg/kg	41.98 ± 0.580 *	187.79 ± 0.719 **	209.34 ± 0.163 ***

Values are presented as Mean ± (standard error mean) SEM and (n=3); * $p < 0.05$, ** $p < 0.01$ and *** $p < 0.001$ were considered as significant.

3.7. Peripheral Analgesic Activity

Figure 2 depicts the effects of methanolic leaf and bark extracts of *B. malabarica* on acetic acid-induced abdominal writhing in mice. The findings show that, in comparison to the negative control group, the usual medication, diclofenac sodium (2 mg/kg body weight), and the plant extracts (at 200 and 400 mg/kg body weight) considerably ($p < 0.01$) decreased abdominal writhing. The methanolic leaf extract exhibited gradual inhibition from 0% in the control group to 58.32 ± 1.00% at 200 mg/kg and 69.51 ± 0.58% at 400 mg/kg body weight. There was a dose-dependent decrease in writhing. Similar to the standard drug diclofenac (which showed 76.73 ± 0.58% inhibition of the writhing response), the bark extract of *B. malabarica* exhibited dose-dependent anti-nociceptive effects, with 44.9 ± 0.88% and 53.06 ± 0.33% suppression of writhing at doses of 200 and 400 mg/kg body weight, respectively, in mice (Figure 2).

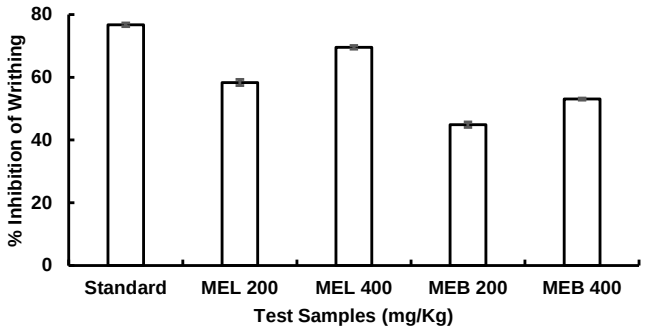


Figure 2. Response of the methanol extract of *B. malabarica* leaves and bark to peripheral analgesia. All values are represented as Mean ± SEM (standard error mean), (n = 3).

3.8. Anti-Diarrheal Activity

The leaf and bark extracts, at 200 and 400 mg/kg body weight, significantly ($p < 0.05$) decreased the overall number of diarrheal stools in the castor oil-induced diarrheal model. The leaf extract inhibited diarrhea by 42.31 ± 2.11% at 200 mg/kg and 59.23 ± 1.78% at 400 mg/kg, while the bark extract showed 53.54 ± 1.38% and 61.54 ± 1.49% inhibition at the same doses, respectively. In comparison, the standard drug loperamide achieved a 69.23 ± 1.49% inhibition (Figure 3).

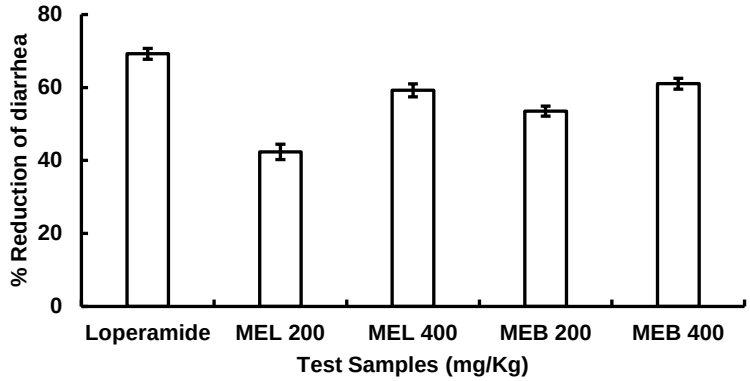


Figure 3. In the anti-diarrheal activity test, oral administration of different doses (200 and 400 mg/kg body weight) of the methanol extracts from the leaf and bark of *B. malabarica* (MEB) significantly ($p < 0.05$) reduced the number of diarrheal feces in mice (n = 3). These results were more pronounced at higher extract dosages.

3.9. CNS Antidepressant Activity

The phenobarbitone sodium-induced sleeping time test was conducted to evaluate the sedative effects of *B. malabarica*. In a dose-dependent manner, the leaf and bark extracts decreased the onset of sleep and increased its length, similar to the control group (Figure 4). The overall sleep lengths were roughly 89.7 and 99 min when the leaf extract was given at doses of 200 and 400 mg/kg body weight. The sleep start timings were 62.33 and 58.0 min, respectively. On the other hand, total sleep durations were 117.3 and 128.3 min, respectively, when the bark extract was administered at 200 and 400 mg/kg (Figure 4). These findings imply that extracts decreased locomotor activity, as demonstrated by the mice's longer sleep durations. The sedative effects may be attributed to interactions with benzodiazepine-like compounds, potentially enhancing GABAergic inhibition in the CNS [25].

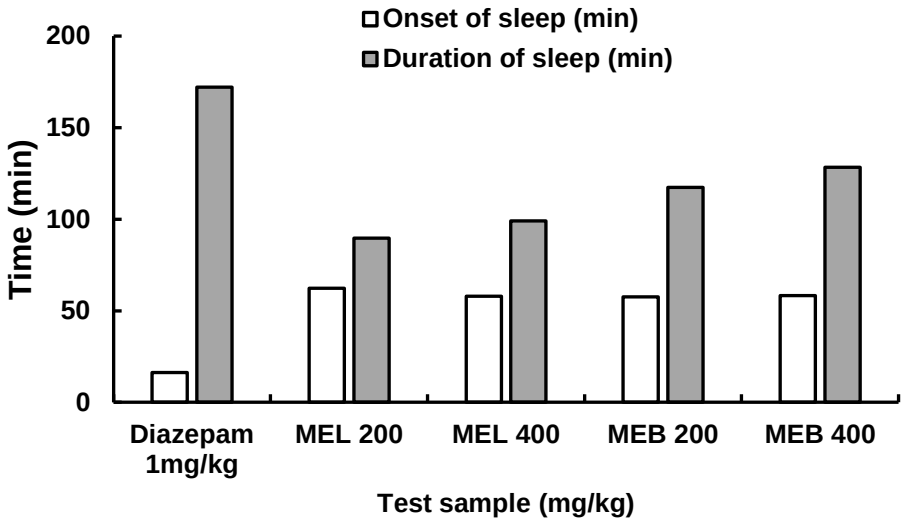


Figure 4. Sedative effect of leaf and bark extract of *B. malabarica* (n = 3).

4. Discussion

In this study, we explored the bioactive potential of *B. malabarica* through a series of in vitro and in vivo assays to assess its antioxidant, anti-inflammatory, analgesic, anti-diarrheal, anthelmintic, and sedative properties. The results of these experiments demonstrated that *B. malabarica* exhibits a range of bioactivities, making it an effective traditional medicine.

The DPPH free radical scavenging method was employed to evaluate the antioxidant activity of *B. malabarica*. It is well-known that free radicals play a key role in the pathogenesis of various diseases, including diabetes, atherosclerosis, and aging. Antioxidants counteract free radical damage, protecting cellular biomolecules like lipids, proteins, and nucleic acids. Our results demonstrated that the methanolic extract of *B. malabarica* leaves exhibited significant free radical scavenging activity, suggesting its potential as an effective antioxidant

(Table 2). These findings confirm that the plant can combat oxidative stress, which participates in diverse chronic diseases [26,27].

In addition to its antioxidant effects, *B. malabarica* also showed promising antitumorigenic potential. The methanolic leaf extract exhibited better results compared to vincristine sulfate, a known chemotherapy agent, in the current bioactivity studies. This suggests that the plant may possess components capable of modulating tumorigenic processes. Many phytochemical sources can promote the development of antitumor agents by inducing apoptosis in cancer cells [28], further supporting the potential therapeutic applications of *B. malabarica* in cancer prevention or treatment.

The RBC membrane stabilization test assessed the anti-inflammatory potential of the plant by evaluating its ability to protect red blood cell membranes from hemolysis induced by heat or hypotonic solutions [29]. Phytochemicals such as flavonoids, tannins, and other phenolic compounds, present in both the leaf and bark extracts of *B. malabarica*, likely contribute to the membrane-stabilizing activity observed in these assays. The leaf extract exhibited stronger protective effects compared to the bark, as shown in Figure 1B. This suggests that the anti-inflammatory properties of the plant may be due to its ability to stabilize cellular membranes and prevent damage from inflammatory stressors.

To determine the plant's dose-dependent anthelmintic activity, we evaluated paralysis and death times of worms exposed to the extracts. These results support the traditional use of *B. malabarica* as an anthelmintic agent. These laboratory findings reinforce the need for alternative anthelmintic treatments, especially in regions where parasites are developing resistance to synthetic drugs [30,31]. The ability of the plant to rapidly induce paralysis and death in helminths highlights its potential for further development into an affordable and effective anthelmintic therapy.

The analgesic effects of *B. malabarica* were evaluated using both the tail immersion test (for central analgesic effects) and the acetic acid-induced writhing test (for peripheral analgesic effects). Both tests demonstrated that the soluble fractions from the leaf and stem bark extracts significantly reduced pain in Swiss albino mice at doses of 200 and 400 mg/kg. These findings suggest *that B. malabarica* may possess both central and peripheral analgesic properties. The extracts may modulate pain via interacting with the central nervous system and peripheral chemoreceptors, as evidenced by their ability to reduce abdominal constrictions induced by acetic acid [32–35].

The anti-diarrheal effects of *B. malabarica* were examined in mice treated with castor oil, which induces diarrhea by irritating the gut mucosa. The plant's extracts significantly reduced the incidence of diarrhea, indicating that it may be effective in managing gastrointestinal disorders. The presence of bioactive compounds such as tannins, terpenes, alkaloids, glycosides, and flavonoids likely contribute to this effect, as they are known to have astringent, anti-inflammatory, and gut-modulating properties [36].

The phenobarbitone sodium-induced sleeping time test was conducted to evaluate the sedative properties of *B. malabarica*. The leaf and bark extracts exhibited a dose-dependent effect, decreasing the onset of sleep and prolonging the duration of sleep in mice. The leaf extract at doses of 200 and 400 mg/kg body weight resulted in sleep durations of 89.7 and 99 min, respectively, while the bark extract caused sleep durations of 117.3 and 128.3 min (Figure 4). These results suggest that *B. malabarica* may possess sedative properties, possibly through interactions with the GABAergic system in the central nervous system [37]. The plant's ability to reduce locomotor activity and prolong sleep could make it a potential candidate for further investigation as a sedative or anxiolytic agent.

5. Conclusions

In conclusion, this study highlights the diverse bioactive properties of *B. malabarica*, supporting its traditional use in herbal medicine. The extracts showed notable sedative, anti-diarrheal, anthelmintic, cytotoxic, antioxidant, and membrane-stabilizing properties. Based on the biological activities, *B. malabarica* could be a valuable resource for developing therapeutic agents. These findings need to be further investigated to fully elucidate the mechanisms underlying these effects and to explore the potential applications of *B. malabarica* in modern medicine.

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Institutional Review Board Statement: All experiments were performed according to the ethical standards laid down in the Declaration of Helsinki 2013. Animals were handled and treated according to the principles of the Swiss Academy of Medical

Sciences and Swiss Academy of Sciences. Animals were euthanized according to the Guidelines for the Euthanasia of Animals: 2013 edition.

Informed Consent Statement: Not applicable.

Data Availability Statement: Not applicable.

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

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Article

In Silico Investigation of *Pajanelia longifolia* (Willd.) K. Schum Bark Extract against NSCLC Targets: Potential Involvement in Apoptotic Pathways

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Abstract: Nature provides innumerable answers to human problems, but our knowledge is restricted. The use of medicinal plants to treat health problems dates back to ancient times., It has evolved into contemporary techniques that combine traditional knowledge with modern medicine. Cancer, the biggest cause of mortality worldwide, remains difficult to treat properly. This study focusses on non-small cell lung cancer (NSCLC)., The most common type of lung cancer, accounting for 85–90% of occurrences and associated with factors such as smoking and pollution. *Pajanelia longifolia*, an Indian traditional medicinal herb, has therapeutic potential and has historically been used to cure a variety of diseases. This study examines the phytochemical elements of *P. longifolia* bark using metabolite profiling. It evaluates its anti-NSCLC activity using computational methods. The key compounds were identified using liquid chromatography-mass spectrometry (LC-MS), and molecular docking was performed against protein B-Raf and EGFR, both linked to cancer proliferation. The findings emphasise the potential of *P. longifolia* as a source of bioactive chemicals for cancer therapy. They highlight the need for additional investigation into its medicinal potential, particularly in combination with proven medicines such as irinotecan.

Keywords: *Pajanelia longifolia*; antioxidant; anticancer; irinotecan; metabolites profiling

1. Introduction

There is a belief that nature contains the solution to every problem, it is we, the living creatures, who need to discover them. Our knowledge about nature is very limited, we have managed to utilise the blessings of nature to meet our needs from daily essentials to life-saving drugs. In ancient times, when modern medical sciences were unavailable, people treated various ailments using the medicinal plants. Today, by combining traditional knowledges of medicinal plants with modern medical science, numerous life-saving drugs are curing millions lives.

Currently, cancer is the disease that worries the world. This deadly disease is the second most notable cause of mortality after cardiovascular disease [1] Cancer is characterized by uncontrolled mitosis and cell proliferation [2]. Lung cancer, colorectal cancer, prostate cancer, and stomach cancer are the leading types of cancer in male. In contrast, breast cancer, colorectal cancer, lung cancer, and cervical cancer are predominant in females [1,3]. The proper therapy for cancer is unavailable. The existing therapies include chemotherapy and radiation therapy. However, these therapies have unwanted side effects and do not promise an optimistic prognosis. Thus, it is essential to develop alternative treatment strategies against cancer. In this work, we focus on the non-small cell lung cancer (NSCLC), one of the most predominantly diagnosed cancer types. NSCLC is the most common form of lung cancer, accounting for 85–90% of cases, and is strongly associated with smoking, exposure to certain industrial substances, family history, and high air pollution. NSCLC includes adenocarcinomas (LUADs), large cell cancers, and squamous cell cancers (LUSCs), which show a reduced sensitivity to radiation and chemotherapy

[4]. The NSCLC results in severe morbidity and mortality each year, with millions of new cases and deaths worldwide. Globally, lung cancer is among most common cancers, with NSCLC making up about 85% of cases [5]. More than 2.2 million new cases of lung cancer are diagnosed annually, and the disease causes over 1.8 million deaths world-wide [5].

Pajanelia longifolia is an important medicinal plant traditionally used to cure the various complexities [6]. This plant belongs to the family Bignoniaceae and varies from small to medium evergreen type. This plant is commonly distributed in the Eastern Bengal and Western Ghats in India and other tropical countries, such as Myanmmar, Burma and Bangladesh. This plant is so alued that it is recorded in Charaka Samhita (1000 BCE) for treating diseases such as urinary disorders, arthritis, stomach disorder etc. [7,8]. In local folklore practitioners also used this plant for treatment. In Karnataka, this plant is used for obesity, in Tripura and Cachar district of Assam, India. This plant is used for liver disease, such as jaundice, stomach ulcer, etc. In southern Assam, the leaves of *P. longifolia* are used on the skin for treating the infections [6,7,9]. Different studies have revealed the presence of phenolics and flavonoid compounds in the plant. It is also mentioned that, the bark of this plant is noted for its hepatoprotective and antimicrobial activity [8,10,11]. However, a comprehensive phytochemical screening has not yet been conducted. In this study, we have carried out the metabolites profiling of the bark extract and predicted its anti-NSCLC activity through computational methods.

2. Materials and Method

2.1. Preparation of Plant Extracts

The bark of the plant was collected from Silchar, Southern Assam, in the Cachar district, Northeast India during the month of June and July. It was then washed thoroughly with water and then air-dried in shade. Once moisture-free, it was crushed into a fine powder using an electric grinder for extract preparation. A powdered of the bark sample (150 g) was used for the extraction by following the maceration process [12], using the increasing solvents polarity as petroleum ether, ethyl acetate, acetone and methanol. The filtrate was first dried using a rotary evaporator under the reduced pressure and then with a lyophilizer. The extracts were stored at 4 °C for further experiments. Consequently, the four extracts were named as petroleum ether extract (PL-PE), ethyl acetate extract (PL-EA), acetone extract (PL-AC) and methanolic extract (PL-ME).

2.2. Quantitative Phytochemical Screening

2.2.1. Total Phenolic Content (TPC) Estimation

The total phenolic content of the various extracts of the plant was determined using a modified version of the method originally described in the literature [13]. In brief, the sample was prepared at a concentration of 1 mg/mL in methanol. From this stock solution, 0.5 mL of the sample was taken, and 0.1 mL of Folin–Ciocalteu reagent along with 2.4 mL of distilled water was added. The mixture was thoroughly mixed and allowed to stand for 3 min. Subsequently, 2 mL of a 2% Na_2CO_3 solution was added, and the mixture was kept in complete darkness for 60 min. The absorbance was then measured at 750 nm, and the results were expressed as Gallic acid equivalents (GAE/mg) of the plant extract.

2.2.2. Total Flavonoid Content (TFC) Estimation

To quantify the total flavonoids present in the extracts, a slightly modified standard protocol was implemented [14]. An equal volume of plant extracts (1mg/mL) and $AlCl_3$ (2%) was mixed properly and incubated at dark for a period of 15 min. After the incubation period, the absorbance was recorded at 415 nm and the results were expressed as quercetin equivalents (QE/mg) of the plant extract.

2.3. In Vitro Antioxidant Assay

Determination of DPPH Free-Radical Scavenging Activity

Antioxidant properties of all the four extracts of the plant sample was determined using DPPH free radicals scavenging activity following the protocol described in the literature [15]. Briefly, 80 µg/mL DPPH solution is prepared in methanol and kept it in dark. Then six serial dilutions of each extract were carried out from stock solution of 1 mg/mL. An equal volume of each sample solution and the stock DPPH solution was mixed and kept it in dark for 30 min. The absorbance was taken at 517 nm after the incubation period. The DPPH solution in

methanol was used as a control and 95% methanol was used as a blank. The results were compared with standard ascorbic acid. The percent inhibition of the free radicals was calculated using the following formula:

$$\% \text{ inhibition} = [(Ac - As)/Ac] \times 100$$

where 'Ac' is the absorbance of control and 'As' is the absorbance of the sample. The IC_{50} value, which is the concentration of the test material that reduces 50% of the free-radical concentration, was calculated through sigmoidal dose-response curve.

2.4. Metabolites Profiling

A detailed metabolite profiling was performed for the plant extract to identify the active compounds. Liquid Chromatography-Mass Spectrometry (LC-MS) were employed for this analysis at the Sophisticated Analytical Instrument Facility (SAIF) at IIT Bombay. LC-MS analysis was performed using a Varian Inc. 410 Prostar Binary LC system, equipped with 500 MS IT PDA Detectors. The separation was achieved using an RRHT C18 column (2.1 mm × 100 mm, 1.8 µm). The mobile phase consisted of two solvent systems: Solvent A (water with 0.1% formic acid) and Solvent B (acetonitrile with 10% water and 0.1% formic acid). The injection volume was set at 5 µL, with a flow rate of 0.300 mL/min, and the column temperature was maintained at 40 °C.

2.5. In Silico Analysis

2.5.1. Target

Malignant cells are characterized by their rapid proliferation, driven by uncontrolled cell proliferation. In this study, the focus was placed on two specific targets, B-Raf (PDB id: 4R5Y) and EGFR (PDB id: 6LUB) responsible for cell proliferation identified through comprehensive literature research. These proteins, when mutated, play a critical role in cancer development. The 3D structures of these mutated proteins from the Protein Data Bank (www.rcsb.org/pdb; accessed on 30 August 2024) and utilized as drug targets.

2.5.2. Ligand

The identified phytocompounds from the plant extract, as determined through LCMS analysis, were used as ligands for this study. The SMILES format of these compounds was obtained from the PubChem database (https://pubchem.ncbi.nlm.nih.gov/; accessed on 23 July 2024) and generated using ACD/ChemSketch (version 2021.1.2, Advanced Chemistry Development, Inc., Toronto, ON, Canada) whichever compounds are not available in PubChem database. Since molecular docking requires the compounds in mol format, the conversion from SMILES to mol was carried out using Open Babel software v 3.1.1.

2.5.3. Molecular Docking

Molecular docking is a computational method used to estimate how well a ligand can bind to the active site of a target protein. This technique not only predicts the binding efficiency but also provides insights into the binding conformation and orientation. In this analysis, Molegro Virtual Docker (MVD) version 6.0 was used for performing the docking simulations. The receptor proteins were prepared by removing any bound inhibitors, cofactors, and water molecules before loading them into the software. The protonation states of amino acids were adjusted using the built-in protein preparation tool. MVD's cavity detection feature facilitated the identification of the active sites of the receptors, which were designated as the docking sites. After conducting energy minimization and optimizing hydrogen bonds, the software generated key metrics, such as the MolDock score, hydrogen bond score, and the geometry of ligand binding at the active site.

2.5.4. Prediction of ADME Profile and Drug-Likeness

ADMET analysis was performed using the SwissADME server (https://www.swissadme.ch/) provided by the Swiss Institute of Bioinformatics. The compounds were input in SMILES format, and the server's algorithm generated data on physicochemical properties, lipophilicity, water solubility, pharmacokinetics, medicinal chemistry, and drug-likeness characteristics.

3. Result

3.1. Crude Yield of Plant Extracts

A powdered of the bark sample (150 g) was extracted and upon drying, the yield of crude extracts obtained from their respective solvents is presented in Table 1 below.

Table 1. Crude yield of the extracts per 100 g powdered sample.

Crude Extract Yield per 100 g Powdered Sample			
PL-PE	PL-EA	PL-AC	PL-ME
1.5 g	1.77 g	2.35 g	19.4 g

3.2. Total Phenol and Flavonoid Content

The results indicate that the acetonic extract of *P. longifolia* contains the highest levels of total phenolic content (TPC) and total flavonoid content (TFC) compared to other extracts. The acetonic extract has a TPC of 109 GAE/mg and a TFC of 135 quercetin/mg (Figure 1). The TPC was calculated using the gallic acid standard curve equation and the TFC was determined using the quercetin standard curve equations. The equations were as follows:

$$y = 0.0045x + 0.4498,\ R^2 = 0.9867$$

$$y = 0.0002x + 0.117;\ R^2 = 0.9999$$

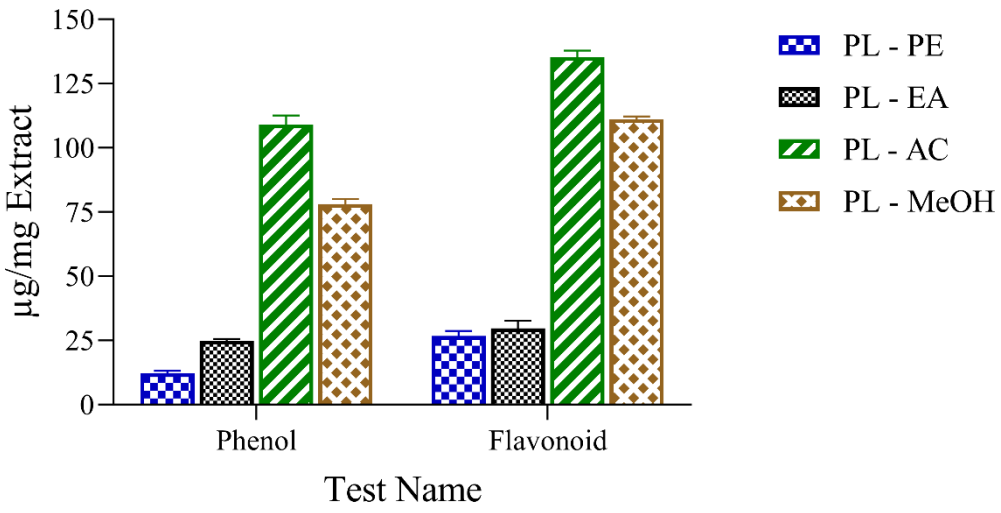

Figure 1. Comparative TPC and TFC in the different extracts of *Pajanelia longifolia*.

3.3. Antioxidant Activity

The results of the DPPH radical scavenging activity for *P. longifolia* and the standard ascorbic acid are presented in the Figure 2. The percentage inhibitory activity of free radicals, particularly the ability to inhibit by 50%, is widely used as a parameter to measure antioxidant activity. In this study, both the plant extract and standard significantly scavenged the DPPH radical with increasing concentrations. The Figure 2 showed the dose response curve of DPPH radical scavenging activity IC_{50} (µg/mL) of the acetone extract (10.54 ± 0.01 µg/mL) was found to be the lowest, while the IC_{50} for the methanolic extract (13.85 ± 0.01 µg/mL) of *P. longifolia*is ranked the second lowest among all extracts analysed. Both the acetone and methanolic extracts demonstrated better DPPH radical scavenging activity compared to the standard ascorbic acid (12.50 ± 0.01 µg/mL).

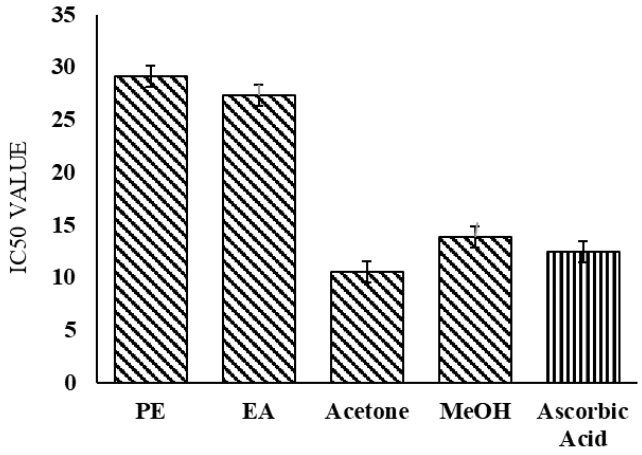

Figure 2. Graph showing the IC_{50} concentration in µg/mL of inhibiting DPPH free radicals by the different extract of P. longifolia and standard ascorbic acid.

3.4. Metabolites Profiling

Based on the results from the TPC, TFC, and DPPH free radical scavenging assays, the acetonic extract of *P. longifolia* (PL-AC) was selected for further phytochemical analysis using LC-MS techniques. These metabolomic analyses provide insights into the phytochemicals present in the plant, facilitating their identification. The compounds detected in the LC-MS chromatograms (Figure 3) of PL-AC are listed in the Table 2.

Table 2. (**A**) Compounds detected from PL-AC extract of bark extract of *P. longifolia* through LC-MS analysis (‘+’ ve ESI). (**B**) Compounds detected from PL-AC extract of bark extract of *P. longifolia* through LC-MS analysis (‘−’ ve ESI).

(A)				
Name	**Score**	**Mass**	***m/z***	**RT**
D-Lombricine	74.41	270.07	293.06	1.14
Myoinositol 1-phosphate	76.04	260.03	261.04	1.28
5-Hydroxy-3,3′,7,8-tetramethoxy-4′,5′-methylenedioxyflavone	79.52	402.09	425.08	1.99
Tiracizine	69.27	367.19	390.17	3.30
Ricinine	95.08	164.06	165.07	3.41
1,5-Dibutyl methyl hydroxycitrate	88.84	334.16	357.15	3.63
3,4,5-Trimethoxycinnamic acid	85.94	238.08	239.09	3.68
2,4,6-Trihydroxytoluene	87.03	140.05	141.05	4.02
Sulprostone	89.66	465.18	466.19	4.34
(R)-Cryptone	86.17	138.10	139.11	4.40
Funtumine	80.53	317.27	340.26	4.68
Pivmecillinam	40.91	439.21	462.20	4.73
Methylergonovine	63.92	339.19	362.18	4.97
Puromycin	48.68	471.23	494.22	5.02
Alfuzosin	81.49	389.20	390.21	5.03
Istamycin C1	85.37	431.27	432.28	5.26
Netilmicin	87.91	475.30	476.31	5.61
3-Oxo-12,18-ursadien-28-oic acid	45.04	452.33	475.32	6.42
Vernodalin	92.63	360.12	361.13	6.87
16,17-Dihydro-16a,17-dihydroxygibberellin A4 17-glucoside	96.95	528.22	551.21	7.08
Cubebin	85.51	356.13	357.13	7.13
Methyl trans-*p*-methoxycinnamate	83.95	192.08	193.09	8.69
N_1,N_5,N_{10}-Tricoumaroyl spermidine	59.11	583.26	584.27	9.38
Dihydrodeoxystreptomycin	92.07	567.29	568.30	10.18
Manumycin A	55.04	550.26	573.25	10.26
Neuraminic acid	55.79	267.09	290.08	10.50
N-(1-Deoxy-1-fructosyl)serine	56.61	267.09	290.08	10.79
Protorifamycin I	59.33	639.31	640.32	10.88
Cortolone	97.94	366.24	367.25	11.02

Eugenol	94.65	164.08	165.09	11.25
Glycine, *N*-[(3a,5b,7a)-3-hydroxy-24-oxo-7-(sulfooxy)cholan-24-yl]-	67.6	529.28	552.27	11.39
1-(b-*D*-Ribofuranosyl)-1,4-dihydronicotinamide	78.82	256.10	279.09	11.47
Sulfadimidine	73.4	278.09	279.09	11.74
7-Hydroxyflavanone beta-*D*-glucopyranoside	97.58	402.13	403.14	11.83
Prunetin	84.97	284.07	285.08	12.14
5,6,7,8,3′,4′,5′-Heptamethoxyflavone	92.22	432.14	433.15	12.29
(9*Z*,11*E*,13*E*,15*Z*)-4-Oxo-9,11,13,15-octadecatetraenoic acid	81.72	290.19	291.19	12.33
Gingerenone C	96.48	326.15	327.16	13.06
Mitoxantrone	74.04	444.20	445.21	13.38
Butyl 2-aminobenzoate	98.49	193.11	194.12	13.41
Kanamycin	81.02	484.24	507.23	15.31
Cycloate	85.5	215.13	238.12	15.35
Medroxyprogesterone	75.06	344.24	345.25	16.51
Gingerglycolipid C	96.35	680.40	703.39	16.80
Irinotecan	86.84	586.28	609.27	19.98
Oxidized dinoflagellate luciferin	90.41	602.28	625.26	20.26
Pheophorbide a	96.4	592.27	593.27	21.01

(B)

Name	**Score**	**Mass**	***m/z***	**RT**
Vanillylmandelic acid	84.16	198.05	197.05	3.07
Diethyl L-malate	93.61	190.09	235.08	3.11
MeIQ	94.25	212.11	257.10	3.66
8-*D*-Olivosyl-landomycin	59.95	468.14	467.14	4.06
Aspirin	90.11	180.04	179.04	4.36
Esculetin	81.46	178.03	177.02	4.60
Byakangelicin	63.36	334.11	379.11	4.65
Inumakilactone A glycoside	85.22	526.17	525.16	4.88
4′,7-Di-*O*-methylcatechin	81.35	318.11	363.11	5.45
Phloroacetophenone 6′-[xylosyl-(1->6)-glucoside]	83.74	490.17	489.16	5.53
Silandrin	53.14	466.13	525.14	5.54
Isoacteoside	81.27	624.21	623.20	6.51
Lindleyin	73.51	478.15	523.15	6.51
(2*S*,2′*R*,3*S*,3′*R*,4*S*)-3,4′,5,7-Tetrahydroxyflavan(2->7,4->8)-3,3′,5,5′,7-pentahydroxyflavan	62.79	560.13	559.13	6.51
Glaucolide A	76.01	464.17	509.17	6.81
Guibourtinidol-(4alpha->6)-catechin	62.09	546.15	545.15	6.82
Aloesin	79.52	394.13	393.12	6.96
Artonol B	61.55	420.12	479.14	6.98
Ethofumesate	91.67	286.09	345.10	7.04
Methyl 3,4-dihydroxy-5-prenylbenzoate 3-glucoside	78.96	398.16	443.16	7.04
Aromadendrin 4′-methyl ether 7-rhamnoside	72.89	448.14	507.15	7.28
(2S,2′‘S,3S,3′‘R,4S)-3,4′,5,7-Tetrahydroxyflavan(2->7,4->8)-3,4′,5,7-tetrahydroxyflavan	63.4	544.14	543.13	7.29
Salfredin B11	91.17	232.08	231.07	7.36
Mahuannin D	63.55	528.14	573.14	7.67
2-(3,4-Dihydroxyphenylethyl)-6-*epi*-elenaiate	74.57	378.13	377.13	7.75
Vernolide	68.25	362.14	361.13	8.07
Morusignin B	75.36	328.10	327.09	8.96
Galactopinitol A	66.45	356.13	401.13	9.30
Elephantin	77.4	374.14	373.13	10.74
9*Z*-Octadecenedioic acid	86.8	312.23	311.22	16.01

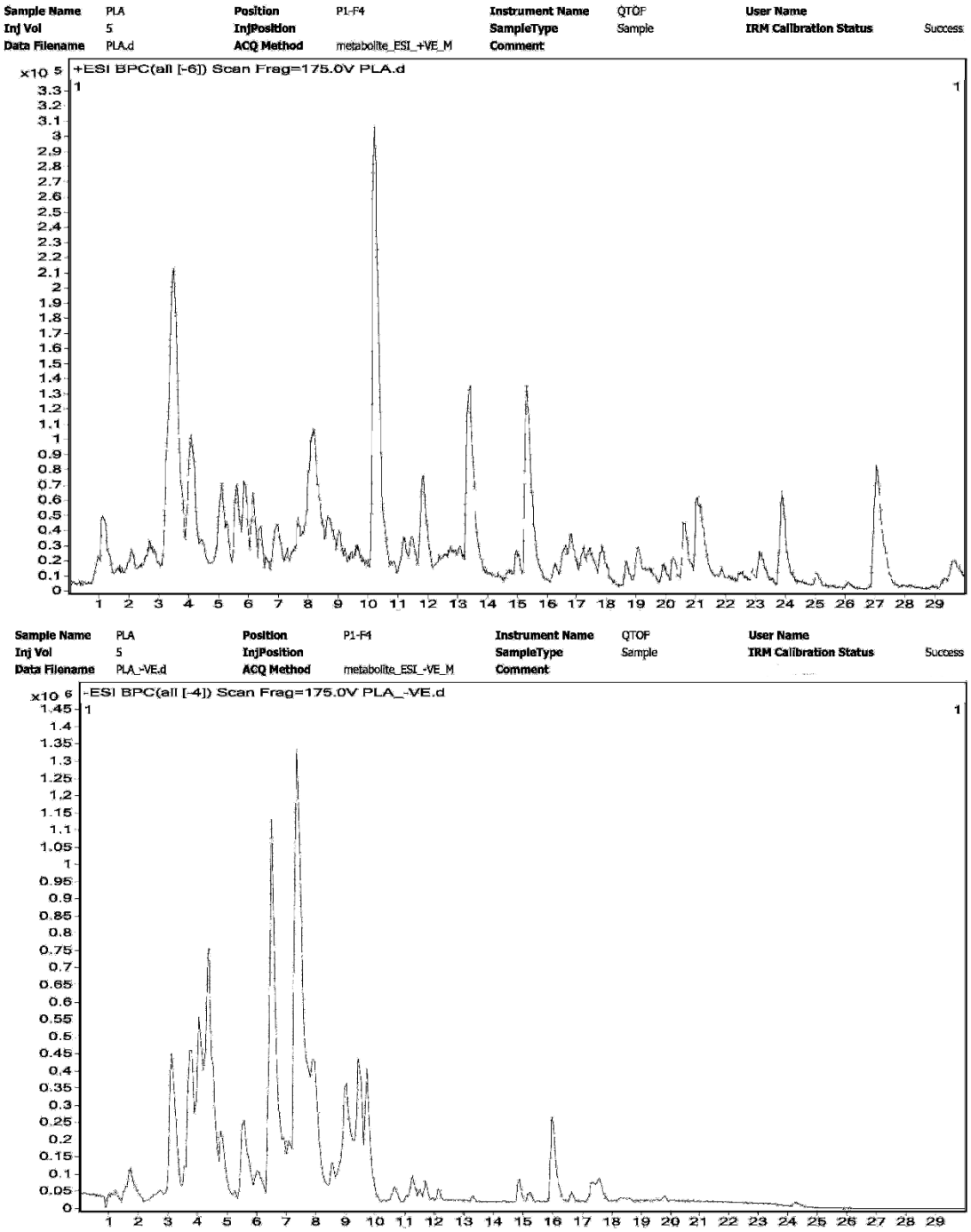

Figure 3. LCMS chromatogram of PL-AC.

3.5. Docking Scores and Inhibition of Receptors

Among the two selected targets, several identified compounds demonstrated superior binding efficacy compared to the respective positive controls. In the case of the EGFR triple mutant protein, Pheophorbide A exhibited the strongest binding, with a MolDock score of −182.13, surpassing the positive control gefitinib, which had a score of −118.65. In addition to Pheophorbide A, 18 other phytocompounds from this plant also showed stronger binding than the positive control (Table 3). Similarly, the docking results for the B-Raf V600E mutant protein revealed that the compound Manumycin A had the highest binding affinity, followed by four additional compounds, which outperformed the positive control Dabrafenib, with a MolDock score of −193.48. Both proteins are implicated in cancer cell proliferation, and binding to these targets could potentially reduce cancer cell growth and the formation of malignant tumours.

Table 3. Provides a comparison of docking scores of ligands against the targets, alongside the positive control, i.e., market-approved drugs for these targets.

Compound Name	EGFR		BRAF	
	Moldock Score	H-Bond Score	Moldock Score	H-Bond Score
Gefitinib (Positive Control of EGFR)	−118.651	−7.31214	-	-
Dabrafenib (Positive control of BRAF)	-	-	−154.12	0
Pheophorbide A	−192.13 *	−8.84	−186.93	4.19
Manumycin A	−171.62	−9.09	−193.48 *	−5.90
Irinotecan	−141.10	−4.06	−157.87	−1.91
Sulprostone	−139.38	−9.39	−155.77	−4.19
Isoacteoside	−137.83	−4.73	−159.60	−20.26
Lindleyin	−146.16	−15.70		
Elephantin	−138.44	−5.28		
Cubebin	−137.38	−3.54		
(9Z,11E,13E,15Z)-4-Oxo-9,11,13,15-octadecatetraenoic_acid	−135.28	−7.90		
Puromycin	−133.78	−5.17		
Dihydrodeoxystreptomycin	−131.71	−13.26		
Glaucolide_A	−127.96	−6.26		
Vernodalin	−126.43	−5.99		
Mahuannin_D	−125.87	−10.89		
Glycine,N-[(3a,5b,7a)-3-hydroxy-24-oxo-7-(sulfooxy)cholan-24-yl]-	−123.63	−9.58		
8-D-Olivosyl-landomycin	−121.38	−9.31		
Protorifamycin_I	−120.47	−3.32		
Methyl_3,4-dihydroxy-5-prenylbenzoate_3-glucoside	−119.94	−9.77		
Manumycin_A	−171.62	−9.09		

* Highest binding affinity.

After reviewing the data, it was observed that the five compounds, namely Manumycin A, Pheophorbide A, Isoacteoside, Irinotecan and Sulprostone (Figure 4), demonstrated the potential to inhibit the selected target proteins., This suggests their use as possible anti-NSCLC drugs. The docking poses and 2D interactions of these five compounds, along with the positive controls, are displayed in Figure 5.

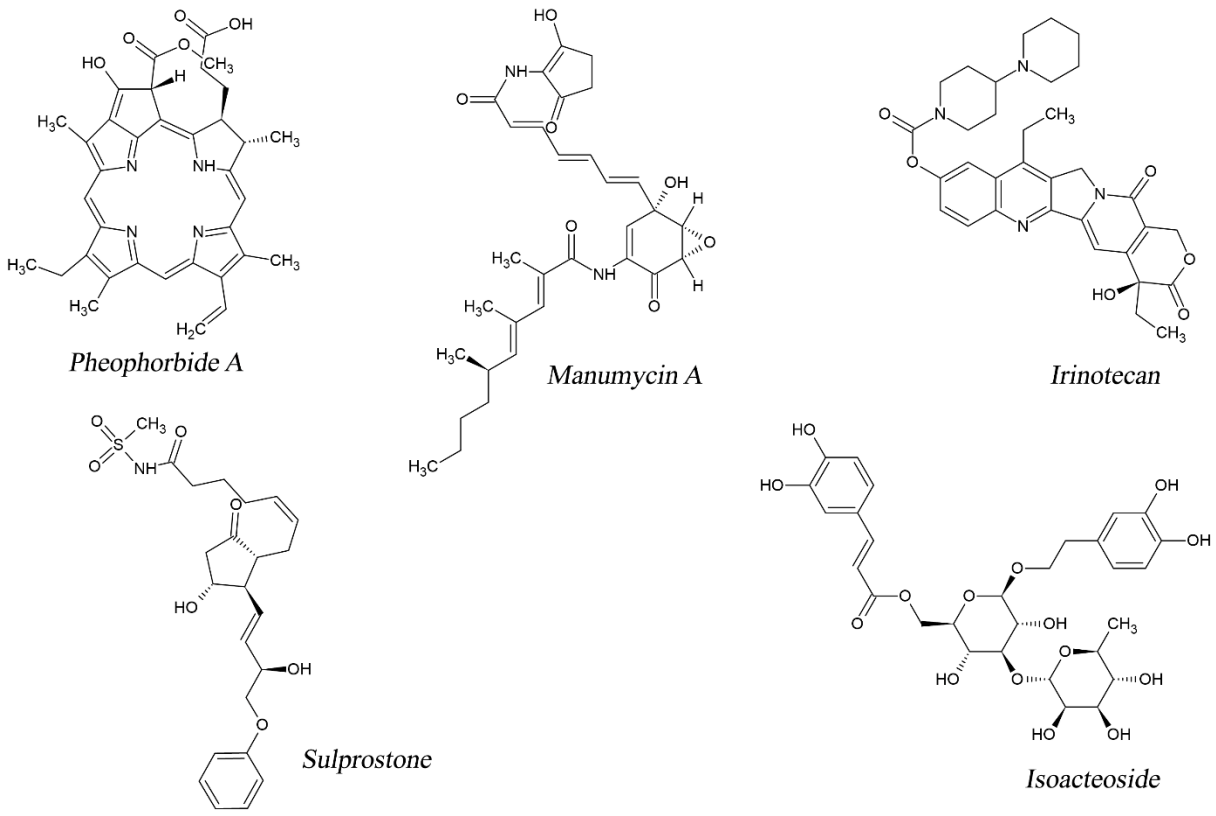

Figure 4. Chemical structure of best five compounds.

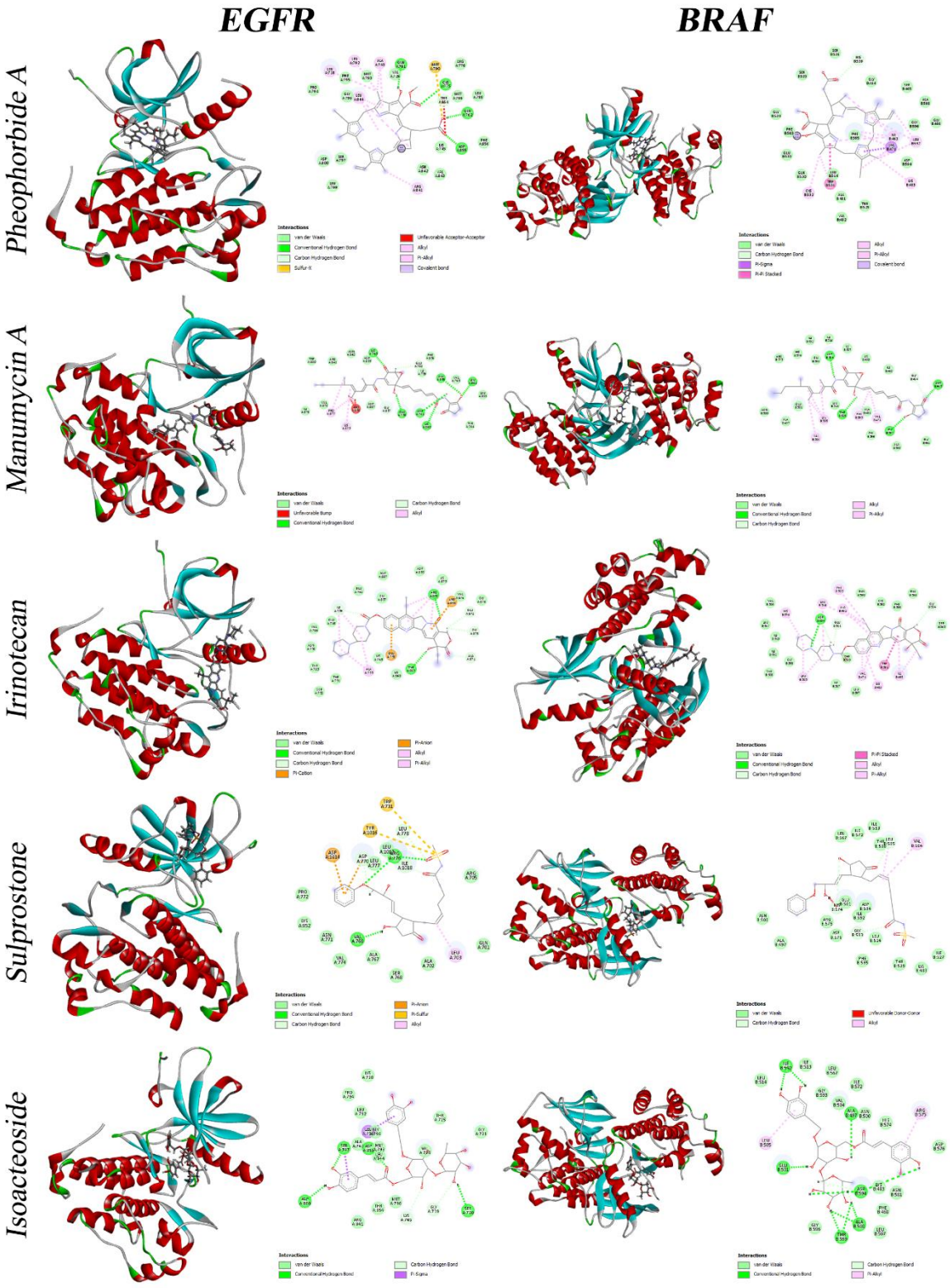

Figure 5. Docking pose of best five compounds with EGFR and BRAF protein.

3.6. ADMET Profile Analysis

Among the compounds evaluated for potential drug-likeness, irinotecan emerged as the most promising candidate based on an analysis of its physicochemical and pharmacokinetic properties. This assessment was conducted using widely accepted criteria, including Lipinski's Rule of Five, solubility, gastrointestinal (GI) absorption, bioavailability score, and safety alerts. Irinotecan displayed a moderate bioavailability score of 0.55, suggesting reasonable potential for oral bioavailability, and its GI absorption was classified as high, a desirable trait for orally administered drugs. Additionally, it demonstrated acceptable drug-like characteristics with only one

Lipinski violation, and it was free from PAINS and Brenk alerts, which indicates a lower likelihood of promiscuous binding or toxicity issues. This is particularly advantageous, as compounds with fewer alerts are less likely to cause off-target effects or adverse reactions. In contrast, other compounds such as Pheophorbide A, Manumycin A, Sulprostone, and Isoacteoside exhibited limitations, including low GI absorption, multiple rule violations, or safety alerts. Notably, Isoacteoside had a very low bioavailability score (0.17) and multiple Lipinski violations, making it an unsuitable candidate. Although irinotecan is a P-glycoprotein (Pgp) substrate, which may limit its bioavailability in certain tissues due to potential drug efflux, its overall profile, high GI absorption, moderate bioavailability score, and minimal rule violations—positions it as the most favorable compound for further investigation as a potential therapeutic agent. The pharmacokinetics and drug-likeness scores for all the compounds are detailed in Table 4 and boiled egg illustration at Figure 6

Table 4. Calculated pharmacokinetics and drug-likeness parameters of the ligands.

Molecule	Pheophorbide A	Manumycin A	Irinotecan	Sulprostone	Isoacteoside
Molecular Weight	592.68	550.64	586.68	465.56	624.59
H-bondacceptors	8	7	8	7	15
H-bond donors	3	4	1	3	9
ESOL Class	Moderately soluble	Moderately soluble	Moderately soluble	Soluble	Soluble
Ali Class	Moderately soluble	Moderately soluble	Moderately soluble	Soluble	Moderately soluble
GI absorption	Low	Low	High	Low	Low
BBB permeant	No	No	No	No	No
Pgp substrate	Yes	Yes	Yes	Yes	Yes
Lipinskiviolations	1	1	1	0	3
Ghoseviolations	3	3	3	0	4
Veberviolations	0	0	0	1	2
Egan violations	1	1	0	1	1
Muegge violations	0	0	0	0	4
Bioavailability Score	0.56	0.56	0.55	0.55	0.17
PAINS alerts	0	0	0	0	1
Brenk alerts	0	0	0	1	2

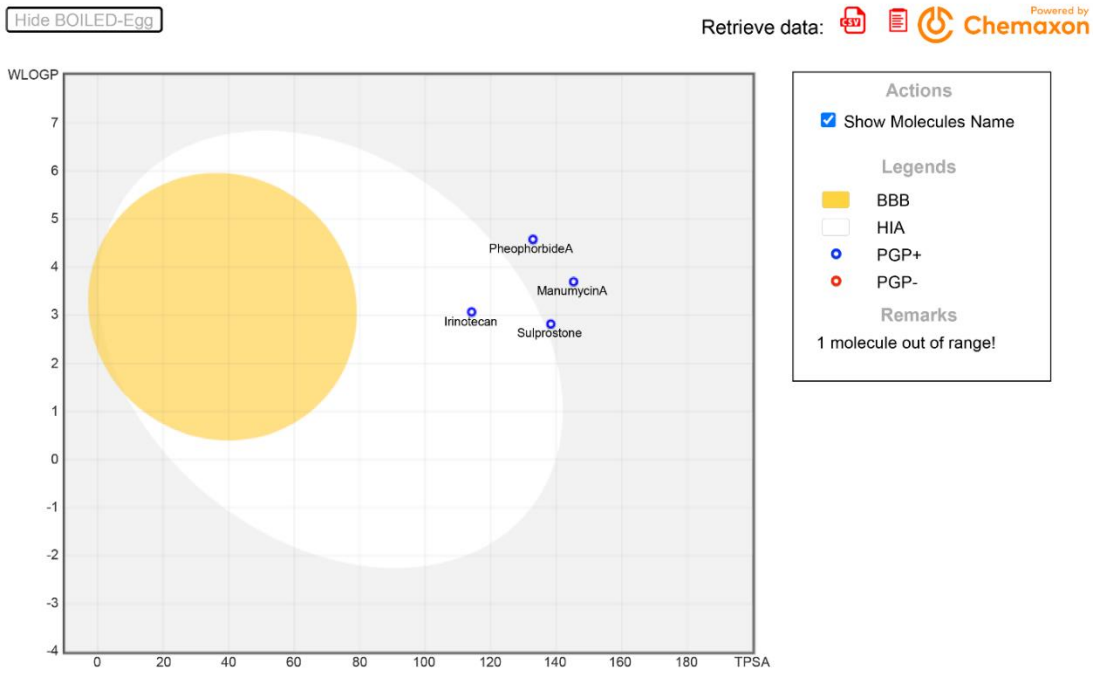

Figure 6. The BOILED-EGG MODEL is used to study gastrointestinal absorption and brain penetration. Molecules in the yolk of boiled eggs are considered capable of passing through the blood-brain barrier (BBB). Molecules in the white of a boiled egg are assumed o be passively absorbed through the gastrointestinal tract. P-glycoproteins are believed to actively remove the blue-dotted molecules from the Central nervous system (CNS).

4. Discussion

The use of natural compounds in cancer research has gained significant interest as researchers seek alternative therapies that present fewer side effects than conventional approaches. This study focusses on the possible

anticancer effects of *P. longifolia*, an ethnomedicinal plant traditionally been used to treat numerous diseases [6]. Notably, the acetone extract (PL-AC) of *P. longifolia* showed significant antioxidant activity, displaying a DPPH radical scavenging potential of 10.54 μg/mL, lower than the standard ascorbic acid (IC_{50} = 12.50 μg/mL). This data implies that *P. longifolia* contains active compounds with high free radical scavenging capacities, which gives the clue for the selection of potential extracts [15]. Further phytochemical screening using LC-MS identified various bioactive components in the PL-AC extract, including flavonoids and phenolics, which are known to have antioxidant and anticancer activities [10]. Flavonoids have been linked to the modulation of signalling pathways in cancer cells, causing apoptosis, and the inhibition tumour development [16]. The high concentration of these phytochemicals is consistent with previous studies highlighting the anticancer properties of *P. longifolia* and supports its traditional medicinal use for treating liver and skin diseases [7].

The anticancer activity of the isolated compounds was also verified by in silico molecular docking studies focusing on B-Raf and EGFR proteins as two main oncogenes associated with NSCLC. In many cancers, B-Raf and EGFR are often mutated, resulting in an overproduction of cells [4]. The favourable scores obtained in our study with MolDock indicate that compounds from *P. longifolia* may effectively interact with the active sites of these proteins, inhibiting their activity. This inhibition could be vital in controlling the growth and proliferation of NSCLC cells that are usually less responsive to conventional therapies [5].

After analysing the ADMET (Absorption, Distribution, Metabolism, Excretion, and Toxicity) profiles of selected five drugs, irinotecan was found as the most viable option for further development. This evaluation used known criteria such as Lipinski's Rule of Five, solubility, gastrointestinal (GI) absorption, bioavailability score, and safety alerts. Notably, irinotecan, a camptothecin analogue, is a strong topoisomerase I inhibitor used to treat metastatic colorectal cancer [17]. It has a moderate bioavailability score of 0.55, indicating a fair potential for oral administration, as well as high GI absorption, an important characteristic for medications intended for oral usage [4]. Furthermore, it followed Lipinski's guidelines with only one violation and was free of both PAINS and Brenk alerts, indicating a lower risk of harmful effects and promiscuous binding [18,19]. In contrast, other tested compounds, including Pheophorbide A, Manumycin A, Sulprostone, and Isoacteoside, have severe limitations such as limited GI absorption, multiple rule violations, and safety alerts. Isoacteoside was particularly problematic, with a very low bioavailability score of 0.17 and three Lipinski violations, making it an unsuitable candidate. Despite beinga P-glycoprotein (Pgp) substrate, which may reduce its bioavailability due to drug efflux mechanisms, irinotecan remains the best option due to its favorable pharmacokinetic profile. The comprehensive pharmacokinetic and drug-likeness data for all substances, highlighting their relative strengths and shortcomings, are presented in Table 4.

The research findings contribute to the growing body of research supporting the therapeutic benefit of plant-based drugs in oncology. The high incidence and mortality rates associated with lung cancer, particularly NSCLC, highlight the need for innovative therapeutic modalities that are both effective and safe. This study serves as a foundation for future research using plant-derived therapeutics on NSCLC, combining ancient knowledge with modern scientific methodologies to improve patient outcomes through reduced toxicity from treatments such as chemotherapy or radiation [20]. Further in vitro and in vivo studies are necessary to validate the findings and to investigate the pharmacokinetics and bioavailability compounds derived from *P. longifolia.*

5. Conclusions

The research findings indicate that *P. longifolia* possesses significant anticancer capabilities, particularly against non-small cell lung cancer (NSCLC). Through extensive phytochemical investigation and metabolite profiling, a diverse array of active compounds in the bark extracts has been identified, contributing to their medicinal potential. The high antioxidant activity of the extracts suggests a mechanism by which these substances protect against oxidative stress, a condition frequently linked to cancer development. Moreover, the results highlight the importance of irinotecan, a well-known chemotherapeutic drug, implying that its efficacy may be amplified by the synergistic effects of bioactive components obtained from *P. longifolia*. This study emphasises the potential of traditional medicinal plants as a source of new therapeutic molecules. Further research is necessary to explore the unique mechanisms of action and clinical applications of these compounds, as this could lead to the development of more effective and targeted cancer treatments. This research findings contribute to the expanding body of evidence supporting the use of natural ingredients in modern oncology, opening up the way for future study and drug develop.

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validation, reviewing. SK: writing and editing—original draft preparation. All authors have read and agreed to the published version of the manuscript.

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Article

Potential Antioxidant, Cytotoxic & Antimicrobial Activity of Edible Plant *Dioscorea alata* L. (Leaves & Stems)

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Abstract: Ethanolic extracts of leaves & stems of *D. alata* L. from the Dioscoreaceae family were subjected to cytotoxic, antioxidant, and antimicrobial activity. Crude extracts of *D. alata* L. showed lethality against the brine shrimp nauplii (LC_{50} = 52 µg/mL) compared to the standard vincristine sulphate (LC_{50} = 0.54 µg/mL) in the brine shrimp lethality bioassay test. In the free radical scavenging activity (DPPH assay), the crude extracts exhibited notable antioxidant activity (IC_{50} was approximately 531 µg/mL) compared to the standard ascorbic acid (IC_{50} was approximately 5.10 µg/mL). At the 250 µg/disc & 500 µg/disc concentration the crude extracts showed feeble antimicrobial activity against *S. aureus* (Diameter of zone of inhibition is 5.21 mm & 7.29 mm at 250 & 500 µg/disc concentration respectively) & *S. epidermidis* (Diameter of zone of inhibition 5.18 mm & 6.19 mm at 250 & 500 µg/disc concentration respectively) in comparison with standard drug ciprofloxacin (5 µg/disc) in disk diffusion assay test. The results suggest that the crude extracts might possess some chemical constituents responsible for cytotoxic, antioxidant & antimicrobial activities.

Keywords: *Dioscorea alata* L.; cytotoxic; antioxidant; antimicrobial; DPPH scavenging

1. Introduction

Edible herbs serve as an accessible and affordable source of natural products for nutrition and health benefits. Bangladesh has a rich flora of medicinal plants, especially edible plants used as vegetables. Dietary supplements are currently receiving recognition worldwide as being beneficial in coronary heart diseases, cancer, osteoporosis, and other chronic and degenerative diseases such as diabetes, Parkinson's, and Alzheimer's diseases [1,2]. Dietary antioxidants are now getting more attention as they are rich in polyunsaturated fatty acids, which are easily oxidized, with the formation of free radicals that are harmful if present in high amounts. Antioxidants are also produced synthetically, but an increasing demand for natural additives has shifted attention from synthetic to natural antioxidants due to minimal side effects. Again, synthetic antioxidants are less active than natural antioxidants [3]. *D. alata* L. is widely known as greater yam, purple yam, ube, Kath Alu, Banra, or Bahra, is one of approximately 600 yam species cultivated globally. This vigorous climbing plant, both annual and perennial, features purplish, winged stems, long petioled leaves of vivid green, and yellow-white flowers. Renowned as a major nutritional powerhouse, *D. alata* serves as a vital food source in many regions, providing essential energy and nutrients to diverse populations [4,5].

D. alata is native to the Indian Subcontinent, Indochina, Eastern Asia region & also found in Africa, North America, Southern America, Pacific region [6]. *D. alata* is a powerful medicinal and nutritional plant packed with bioactive compounds. Its aerial tuber contains 68.51% moisture, 5.61% starch, and 1.39% protein, while the underground tuber is richer in carbohydrates, vitamin C, and essential minerals like iron and potassium [7]. The 70% methanolic leaf extract showed potent antioxidant activity, strong ROS scavenging, and notable cytotoxicity against breast cancer cells [8]. Ethanol extract had a high total phenolic content and exhibited clear antibacterial action against *E. coli*, *S. aureus*, and *B. subtilis* [9]. This plant stands out as a potent source of nutrition and natural therapeutic agents. GC-MS showed that the major components were sesquiterpenes, namely α-gurjunene (30.31%), (−)-isoledene (13.69%), alloaromadendrene (3.28%), β-caryophyllene (3.14%), γ-gurjunene (3.14%), and spathulenol (1.11%). Since the study report of tuber of *D. alata* on the antioxidant, antidiabetic, antifungal, immunomodulatory, estrogenic activity & physicochemical characterization [4,10] has been attempted. But

systematic pharmacological screening of the crude extracts of leaves & stems of *D. alata* has not been carried out so far. Therefore, the objective of the present study was to determine the cytotoxic, antioxidant, and antimicrobial activity of ethanolic crude extracts of leaves & stems of *D. alata*.

2. Materials & Method

2.1. Plant Material

D. alata specimens were collected from Shyamnagar, Satkhira, Bangladesh, located at coordinates 22°19.8′ N, 89°6.2′ E. The plant was identified by the experts of Bangladesh National Herbarium, Mirpur, Dhaka (Accession no: DACB–37524), and a voucher specimen was also deposited there.

2.2. Drugs & Chemicals

Methanol and DMSO (dimethyl sulfoxide) were purchased from Merck, Darmstadt, Germany. Ciprofloxacin was collected from Square Pharmaceuticals Ltd., Gazipur, Bangladesh. Vinchristin sulphate was purchased from Cipla Ltd., Goa, India. DPPH was purchased from Wako Pure Chemical Industries Ltd., Chuo-ku, Japan, & Ascorbic acid was also used in this experiment.

2.3. Preparation of Ethanol Extracts

The identified plant was dried in the shade at room temperature. After complete drying, the sample was cut into small pieces and then ground into coarse powder with the help of a mechanical grinder, and the powder was stored in a suitable container. About 250 mg of powder was extracted by maceration over 20 days with 1500 mL of 98% ethanol. The extract was filtered off. The solvent was evaporated at room temperature with an electric fan to get the dried extract (approx. yield value 6.8%).

2.4. Phytochemical Screening

Phytochemical screening of the crude extracts was performed using the following chemicals & reagents: Reducing sugars with Fehling's reagent, steroids with Libermann-Burchard reagent, gums with Molish reagent & concentrated sulfuric acid, tannins with ferric chloride & potassium dichromate solution, alkaloid with Dragendorffs reagent, saponins with ability to produce suds, & flavonoids with the use of concentrated hydrochloric acid.

2.5. Cytotoxic Activity

The brine shrimp lethality bioassay is widely used in bioassays for bioactive compounds. Here simple zoological organism (*Artemia salina*) was used as a convenient monitor for the screening [11]. The eggs of the brine shrimp were collected from an aquarium shop (Khulna, Bangladesh) and hatched in artificial seawater (3.8% NaCl solution) for 48 h to mature shrimp called nauplii. The cytotoxicity assay was performed on brine shrimp nauplii using the Meyer method (Meyer, et al., 1982) [12].

The solutions of crude extract were prepared by dissolving them in DMSO (not more than 50 μL in 5 mL solution) plus sea water (3.8% NaCl in water) to attain concentrations of 5 μg/mL, 10 μg/mL, 20 μg/mL, 40 μg/mL, 80 μg/mL, 160 μg/mL & 320 μg/mL ensuring final volume up to 5 mL. Standard Vincristine sulphate was used as the positive control. The solutions of Vincristine sulphate were prepared by dissolving them in DMSO (not more than 50 μL in 5 mL solution) plus sea water (3.8% NaCl in water) to attain concentrations of 0.312 μg/mL, 0.625 μg/mL, 1.25 μg/mL, 2.5 μg/mL, 5 μg/mL ensuring final volume up to 5 mL. A vial containing 50 μL DMSO diluted to 5 mL plus sea water (3.8% NaCl in water) was used as a negative control, ensuring a final volume of 5 mL. Then, 10 matured shrimps were placed to each of the experimental vials and the negative control vial. The number of nauplii that died after 24 h was counted. Then the % of mortality was plotted against the respective concentrations used, and from the graph LC_{50} was calculated [13,14].

2.6. Antioxidant Screening

2.6.1. Qualitative Assay

Optimally diluted extract solutions were spotted on pre-coated Silica gel TLC (Thin layer chromatography) plates, and the plates were developed in solvent systems of different polarities (polar, medium polar, and non-polar) to resolve polar and non-polar components of the extract and to choose the solvent system in which extract

solutions run well. The plates were dried at room temperature and were sprayed with 0.02% DPPH in ethanol. Bleaching of DPPH by the resolved bands was observed for 10 min, and the color changes (yellow on purple background) were noted [15].

2.6.2. Quantitative Assay

The antioxidant potential of the ethanolic extract was determined based on its scavenging activity of the stable 2,2-diphenyl-1-picryl hydrazyl (DPPH) free radical. DPPH is a stable free radical containing an odd electron in its structure and is usually utilized for the detection of the radical scavenging activity in chemical analysis [15,16].

The solutions of extract were prepared by dissolving with ethanol to attain a concentration of 1 μg/mL, 2 μg/mL, 4 μg/mL, 8 μg/mL, 16 μg/mL, 32 μg/mL, 64 μg/mL, 128 μg/mL, 256 μg/mL & 512 μg/mL, respectively. Ascorbic acid was used as the positive control. The solutions of ascorbic acid were prepared by dissolving with ethanol to attain a concentration of 1 μg/mL, 2 μg/mL, 4 μg/mL, 8 μg/mL, 16 μg/mL, 32 μg/mL, 64 μg/mL, 128 μg/mL, 256 μg/mL & 512 μg/mL, respectively. Ethanol is taken as a negative control or blank solution. DPPH was weighed and dissolved in ethanol to make a 0.004% (*w*/*v*) solution. Then 3 mL of 0.004% DPPH solution was applied to each of the experimental solutions and the negative control solution. The room temperature was recorded, and the test tubes were left for 30 min in the dark to complete the reactions. DPPH was also applied to the blank test tubes at the same time, where only ethanol was taken as a blank. After 30 min, the absorbance of each test tube was determined by a UV spectrophotometer at 517 nm [16]. % of inhibition was calculated as

$$\% \text{ inhibition} = [(\text{Blank absorbance} - \text{Sample absorbance})/\text{Blank absorbance}] \times 100$$

IC_{50} was determined from the % inhibition versus concentration graph.

2.7. Antimicrobial Activity

Inhibition of microbial growth was tested by using the paper disc agar diffusion method. The disc diffusion technique is highly effective for rapidly growing microorganisms. The microorganisms were collected from the International Centre for Diarrheal Disease and Research, Bangladesh (ICDDRB). After that, these organisms were cultured at the Microbiology Lab of Pharmacy Discipline, Khulna University, Khulna-9208 [17,18]. The sample solution of the material to be tested was prepared by dissolving it in an appropriate solvent to attain a concentration of 25 μg/μL and 50 μg/μL. 10 μL of the test sample from 25 μg/μL and 50 μg/μL solutions were applied on the discs with the help of a micropipette in an aseptic condition under the laminar air flow to get concentration 250 μg and 500 μg per disc respectively and allowed to dry off the solvent in an aseptic hood. To compare the activity with standard antibiotics, Ciprofloxacin (5 μg/disc) was used. 6 sterile filter paper discs (5 mm in diameter) were taken as blank discs. 10 μL of ethanol was applied to the blank discs as the negative control. They ensured that the residual solvents' activity and the filter paper were not active themselves. The extracts of *D. alata* were tested against two Gram-positive (*Staphylococcus aureus* and *Staphylococcus epidermidis)* and four Gram-negative *(Escherichia coli, Shigella dysenteriae, Proteus vulgaris, Vibrio cholerae*) bacteria. Then, test discs and standard discs were placed in a Petri dish seeded with bacteria and then left in a refrigerator at 4 °C for 12–18 h to diffuse the material from the discs to the surrounding media in the Petri dishes [19,20]. The Petri dishes were then incubated at 37 °C overnight to allow the bacterial growth. The antibacterial activities of the extracts were then determined by measuring the respective zone of inhibition in mm.

3. Results

3.1. Phytochemical Screening

The Result of the Phytochemical screening is given in the following Table 1.

Table 1. Result of the Phytochemical screening.

Reducing Sugars	Steroids	Gums	Tannins	Alkaloids	Saponins	Flavonoids	Glycosides
+	−	+	+	+	−	+	+

+ = Presence − = Absence.

3.2. Results of Cytotoxic Activity

Following the procedure of Meyer, the lethality of the crude extracts of *D. alata* leaves and stems to brine shrimp was determined on *D. alata* (Figure 1). The LC_{50} values for *D. alata* & standard vincristine sulphate were represented in Table 2 below.

Table 2. LC_{50} values for *D. alata* & standard vincristine sulphate.

Sample	LC_{50}
Vincristine sulphate	0.54 µg/mL
D. alata L. extract	52 µg/mL

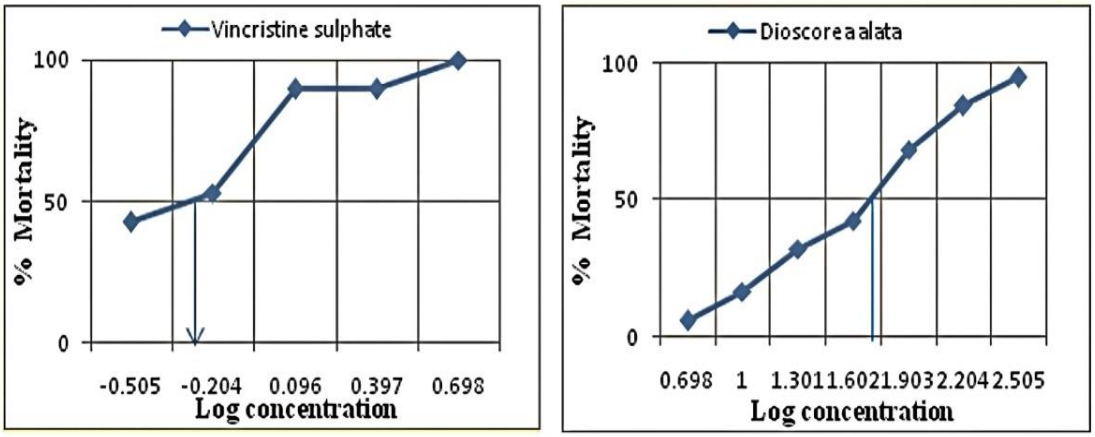

Figure 1. Graphical presentation of brine shrimp lethality bioassay and LC_{50} for Standard and D. alata.

3.3. Results of Antioxidant Activity

3.3.1. Qualitative Assay

The color changes (yellow on purple background) on the TLC plates were observed due to the bleaching of DPPH by the resolved bands (Figures S1 and S2).

3.3.2. Quantitative Assay

The antioxidant activity of the extracts was assessed by the DPPH free radical scavenging assay, as shown in Table 3 and Figure 2. These results denoted the presence of antioxidant principles in the extractives [15].

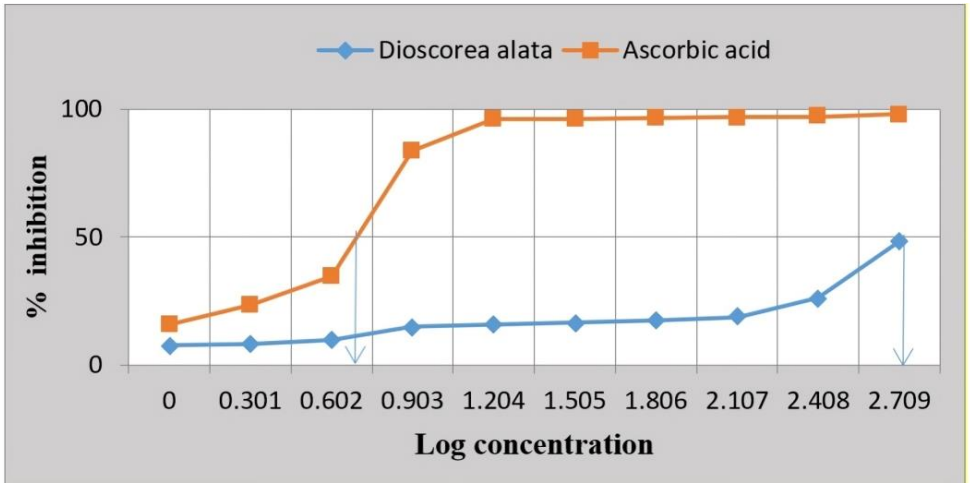

Figure 2. % Inhibition of DPPH vs log concentration graph for standard (Ascorbic acid) and D. alata L. extract.

Table 3. DPPH free radical scavenging assay of Ascorbic acid and *D. alata* L. extract.

Sample	IC_{50}
Ascorbic acid	5.10 µg/mL
D. alata L. extract	531 µg/mL

3.4. Result of Antimicrobial Activity

After proper incubation, the antibacterial activity of the test was determined by measuring the diameter of the zone of inhibition in terms of millimeters with a calibrated scale. The crude extract of *D. alata* L. reflected mild antibacterial activity against the bacterial strains *Staphylococcus aureus* and *Staphylococcus epidermidis* but showed no activity against *Shigella dysenteriae,* & *Proteus vulgaris*, *Escherichia coli* & *Vibrio cholerae* in comparison with the standard drug ciprofloxacin (5 µg/disc), which was represented in the following Table 4.

Table 4. Zone of Inhibition of plant extract and particular standard drug.

Bacterial Strains	Type of Bacterial Strains	Diameter of Zone of Inhibition in mm			
		Blank	Ciprofloxacin (5 µg/disc)	Extract (250 µg/disc)	Extract (500 µg/disc)
1. *S. aureus*	Gram(+)	-	30.59	5.21	7.29
2. *E. coli*	Gram(−)	-	24.42	-	-
3. *V. cholerae*	Gram(−)	-	25.94	-	-
4. *S. dysenteriae*	Gram(−)	-	20.64	-	-
5. *P. vulgaris*	Gram(−)	-	23.74	-	-
6. *S. epidermidis*	Gram(+)	-	25.88	5.18	6.19

Gram(−): Gram Negative Bacteria; Gram(+): Gram Positive Bacteria; (-): No inhibition.

4. Discussion

Phytochemical analysis of ethanolic extract from *D. alata* leaves and stems revealed potent bioactive compounds with promising therapeutic potential due to their diverse pharmacological actions [21]. The ethanolic extract of *D. alata* exhibited moderate cytotoxic activity and relatively low antioxidant activity (IC_{50} = 531 µg/mL) compared to ascorbic acid (IC_{50} = 5.1 µg/mL), which may be attributed to variations in flavonoid structure, such as hydroxyl group positioning, conjugation, and glycosylation, as glycosylated flavonoids (e.g., rutin) typically show reduced activity compared to aglycones [22,23]. The potent cytotoxicity of the oleo-resin is primarily driven by its sesquiterpene constituents, while the strong cytotoxic and antioxidant activities in leaf, bark, and twig extracts are directly linked to their high phenolic content. Previous study on *D. alata* reported total polyphenol content of 89.45 mg/g, with ethyl acetate fraction showing 144.1 ± 3.20 mg/g. DPPH IC_{50} was 78.32 µg/mL, and the water fraction exhibited the highest reducing power [24], suggesting that different plant parts may vary in their bioactive profiles. Another study reported that the methanol extract of *D. alata* tuber contained 0.68 g/100 g total phenolics and 1.21 g/100 g flavonoids, showing strong hydroxyl (IC_{50} = 26.12 µg/mL), superoxide (30.65 µg/mL), ABTS (25.53 µg/mL), and ethanol extract showed potent DPPH radical scavenging activity (IC_{50} = 27.16 µg/mL) [25]. Our present study shows that crude extracts of *D. alata* leaves & stems contain antioxidant & cytotoxic properties. Therefore, *D. alata* L. may contribute to the national and global economy if active compounds are isolated which is responsible for the effect mentioned in this paper. Additionally, the extract demonstrated mild antimicrobial effects. In contrast, related species such as *D. bulbifera* have shown weak activity against *S. aureus*, indicating that further research is needed to explore the antimicrobial potential of *D. alata* [26]. Given that our study utilized crude extracts, the isolation and characterization of pure compounds could enhance their bioactivity and uncover novel nutraceutical applications. Future research on *D. alata* must advance beyond crude extract analysis and prioritize rigorous fractionation to isolate and characterize the precise bioactive constituents responsible for its cytotoxic, antioxidant, and antimicrobial activities. Utilizing state-of-the-art analytical platforms such as HPLC, LC-MS, and GC-MS will enable the development of a detailed phytochemical fingerprint, essential for identifying high-impact compounds [27,28]. Bioassay-guided isolation, combined with structure-activity relationship (SAR) studies, will help elucidate the molecular features driving bioactivity. Moreover, in vivo validation and comprehensive toxicity profiling are critical to ensure therapeutic relevance and safety. These strategic approaches will not only clarify the pharmacological potential of *D. alata* but also lay the groundwork for its translation into high-value nutraceuticals or phytopharmaceuticals, ultimately fostering innovation and economic growth in the biomedical and agricultural sectors.

5. Conclusions

Based on the findings of this study, the ethanolic extracts of *D. alata* L. leaves and stems demonstrated noteworthy bioactivities, including cytotoxic, antioxidant, and antimicrobial properties. The extracts exhibited significant cytotoxic effects in the brine shrimp lethality assay, indicating the presence of bioactive compounds with potential anticancer or therapeutic implications. Although the antioxidant activity was moderate compared to standard ascorbic acid, the extracts showed promising free radical scavenging ability, suggesting their potential role in combating oxidative stress-related conditions. The antimicrobial assessments revealed limited but discernible activity against certain bacterial strains, indicating that *D. alata* may contribute to antimicrobial strategies, albeit with further optimization. Collectively, these results affirm that *D. alata* holds considerable promise as a source of natural bioactive compounds, warranting further detailed phytochemical and pharmacological investigations to fully elucidate its therapeutic potential and possible applications in healthcare.

Supplementary Materials: The following supporting information can be downloaded at: https://media.sciltp.com/articles/others/2505261446258069/JMNP-2025-000013-supplementary-online.pdf, Figure S1: TLC plate after spraying DPPH. Figure S2. TLC plates for D. alata tuber after applying 10% H_2SO_4

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Scilight

Review

Herb-Drug Interactions: A Critical Exploration in Modern Healthcare Practices

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Abstracts: Herb-drug interactions (HDIs) are a growing concern in modern healthcare, with almost 70% of individuals using herbal remedies alongside conventional pharmaceuticals. These interactions can have either beneficial or adverse consequences, and the concentration of a medicine in a certain tissue may change due to these interactions. Herb-mediated blockage and activation of protein transporters and drug-metabolizing enzymes, such as the CYP450 framework, is a common method that pharmaceuticals and herbs collaborate on. Herbovigilance, is the surveillance and assurance of the responsible use of conventional drugs, is crucial for the safety of herbal treatments. However, the frequency of adverse pharmacological reactions associated with herbal treatments has been refuted by numerous scientific studies. Herbal molecules can interact with medicines via pharmacodynamic pathways, leading to antagonistic, combined, and synergistic effects. The quality of herbal remedies is another significant risk factor, as it is difficult to precisely evaluate the safety and efficacy of herbal remedies due to problems like adulteration, misidentification, and contamination. Unpredictable therapeutic effects and an elevated risk of interaction can also result from variations in the content of herbal products, often caused by variations in production and preparation techniques. Despite the increasing awareness of the importance of monitoring HDIs, there is still a lack of standardized guidelines for assessing HDIs, leading to unreported adverse reactions and underestimated risks of using traditional and herbal remedies simultaneously.

Keywords: herb-drug interactions; modern healthcare; pharmacokinetics; herbal drugs; interactions; conventional drugs

1. Introduction

Herb-drug interactions (HDIs) are a significant issue with the increasing use of herbal treatments around the world. When a plant and a drug are taken together, certain interactions can happen and have either beneficial or adverse consequences [1,2]. All over the world, almost 70% of individuals take herbal remedies in addition to or instead of conventional pharmaceuticals. The increasing popularity of herbal remedies may be attributed to its perceived safety, affordability, and ease of use [3]. Whereas varying concentrations of active phytochemicals in herbal remedies may alter several physiological functions, including carriers and enzymatic systems. It is established that these compounds have pharmacological activity [4]. Herb-mediated blockage and activation of protein transporters as well as drug-metabolizing enzymes, that generally include the CYP450 framework, is a quite common method that pharmaceuticals and herbs collaborate [3,4]. The concentration of a medicine in a certain tissue may change because of these interactions, which could have a detrimental effect on medicine metabolism. A wide range of interaction found in accessible sources are hypothetical or dependent on unreliable information, and there are relatively few comprehensive studies assessing the interactions between prescription drugs and herbal products. But this topic must be taken into consideration because certain medications, such as warfarin, digoxin, and chemotherapy therapies, have a therapeutic index that is relatively small [5]. HDIs are a developing concern due to the increased usage of herbal medicines worldwide. Sufficient clinical data are needed

to assist with making well-informed decisions about patient safety to maximize clinical results and reduce the adverse effects of herbs while minimizing potential interactions [3].

2. Mechanisms of HDIs

The pharmacokinetic and pharmacodynamic routes may result in interactions between herbs and drugs [4,6]. When most of herbal extracts change how a medicine is absorbed, distributed, metabolized, and eliminated, this is referred to as a pharmacokinetic interaction [4,7]. Drug interactions between medications are often mediated by drug-metabolizing enzymes, particularly those belonging to the cytochrome P450 (CYP) enzyme family, or drug-carrying proteins such as P-glycoprotein (P-gp) [6,8]. Ashwagandha (*Withania somnifera*) possesses synergistic effects and increases CYP3A4 activity. It provides protection to the liver and blood cells against drug-induced harm when taken in conjunction with ritonavir. Moreover, it may intensify the sedative effects of drugs include clonazepam, diazepam, or lorazepam if administered with benzodiazepines [9]. Garlic (*Allium sativum*) activates the CYP3A4 enzyme, which can decrease drugs metabolism. Moreover, it inhibits P-glycoprotein (P-gP) and CYP2C9. Consequently, a decrease in Saquinavir metabolism may lead to adverse medication responses, a higher risk of bleeding while taking Warfarin, and a decrease in docetaxel clearance [10]. Ginkgo (*Ginkgo biloba*) suppresses the activity of the liver enzyme CYP2C9, it significantly reduces the metabolism of many drugs, such as tamoxifen, celecoxib, glipizide, tolbutamide, and piroxicam. When evaluating patient treatment alternatives, this interaction needs to be considered [11]. Grapefruit juice (*Citrus* × *paradisi*), inhibits CYP1A2, CYP3A4, and CYP2C9, three enzymes involved in drug metabolism. This inhibition may result in higher blood levels of different drugs, which could have harmful effects and be hazardous. People on drugs including saquinavir, acyclovir, lovastatin, atorvastatin, nifedipine, amiodarone, clomipramine, and carbamazepine should be aware that grapefruit juice can raise blood levels of these drugs. The safe and efficient administration of treatment regimens depend on knowledge of these interactions [12]. Kava (*Piper methysticum*), Pineapple bromelain (*Ananas comosus*), and Saw palmetto (*Serenoa repens*) all induce the CYP2C9 enzyme, that substantially reduces the efficacy of warfarin, a vital anticoagulant. While utilizing these medications together with warfarin, take caution and seek advice from a medical professional [13,14]. Liquorice (*Glycyrrhiza glabra*) includes Glycyrrhizin, an 11-keto steroid that can inhibit CYP 2C9 and 3A4 activation and mimic corticosteroid effects. Warfarin and lidocaine suppress activities, whereas corticosteroids increase actions through synergistic. The effects of spirolactone are amplified [15]. Peppermint (*Mentha piperita* L.) has a capability to suppress CYP3A4, that could enhance Felodipine's efficacy. Additional research concerning the combination use of these medications to maximize treatment outcomes is made possible by this interaction [16]. Pippali (*Piper longum*) inhibits the enzymes CYP3A4, CYP2D6, and CYP1A2. Essential drugs including digoxin, propranolol, and verapamil are substantially less efficient because of this inhibition. It is essential to take these interactions into account while prescribing or using these medicines [17]. St. John's wort (*Hypericum perforatum*) can activate CYP1A2, which improves the metabolism of several medicines. Moreover, it stimulates P-glycoprotein (P-gP) and CYP3A4. Therefore, the efficiency of cyclosporine may be reduced when used with warfarin and its anticoagulant effects, protease inhibitors, and atorvastatin. To maximize patient care and avoid possible problems such transplant graft rejection, it is essential to comprehend these relationships [18]. Tulsi (*Ocimum sanctum*), contains active ingredients such as linalool, carvacrol, and eugenol that efficiently block the CYP1A1 and CYP1B1 enzymes. This inhibition is essential because it stops the procarcinogen benzo[a]pyrene from becoming the extremely toxic diolepoxide. Since the liver's CYP1A1 and CYP1B1 are primarily responsible for the conversion process, inhibiting them is an important defense mechanism. It is crucial to recognize that extended exposure to diolepoxide may result in unfavorable dermatological effects, including as thickening, darkening, and pimple development [19,20]. Turmeric (*Curcuma longa*) substantially inhibit the enzyme activity of CYP3A4, CYP1A2, CYP2B6, CYP2C19, and CYP2C9, the efficacy of rosuvastatin, warfarin, clopidogrel, and losartan is certainly reduced [17]. Aloe vera (*Aloe barbadensis miller*) shall be administered with precaution because it may cause hypokalemia whenever utilized together with diuretics or corticosteroids. In these circumstances, potassium levels should be checked to assure safe and efficient use [21]. Amla (*Emblica officinalis*) is well-known for containing a high tannin content that can bind with iron. Although ascorbic acid breaks down at high temperatures, it is frequently advised to take raw amla powder. Amla is also known for its high calcium concentration. Raw amla powder ingestion may reduce iron levels and perhaps reduce the potency of iron tonics. It is also vital to understand that an excessive consumption of amla might lead to the formation of renal stones [22]. Coffee (*Coffea arabica*) and Tea (*Camellia sinensis*) both substantially enhance stomach acidity. The efficiency of antibiotics including erythromycin and penicillin is impacted directly by this rise, which leads to their degradation. Limiting the intake of these drinks during antibiotic treatment is essential for the best outcomes [23,24]. Flax seeds (*Linum usitatissimum*), Marshmallow (*Althea officinalis*), and Aloe vera

(*Aloe barbadensis miller*) might decrease the absorption of certain drugs in the body. The substantial mucilage content of these plants may prevent the absorption of drugs [25]. Ginger (*Zingiber officinalis*) inhibits thromboxane synthetase, that could prolong bleeding time. When using ginger with anticoagulant drugs like warfarin, care should be taken because this combination may increase the risk of severe bleeding. For using ginger in nutritional and medicinal applications, it is crucial to comprehend this possible interaction [13]. Passion flower (*Passiflora incarnata*) possesses additive effects, which substantially increase the efficacy of central nervous system (CNS) depressants [26]. Pomegranate (*Punica granatum*), Tomato (*Solanum lycopersicum*), composed of melatonin, and sweet flag (*Acorus calamus*) has significance because of their potent combined actions. These herbs are a potent combination for managing epilepsy since they absolutely amplify the anti-epileptic effects of carbamazepine when taken with it [27]. Purple coneflower (*Echinacea purpurea*) & Coneflower (*Echinacea angustifolia*) possess prominent antagonistic effects. Echinacea's immunostimulant effects can significantly decrease the effectiveness of immunosuppressive drugs [4]. Sugandhbala (*V. wallichi*) & Garden heliotrope (*Valeriana officinalis*) possess a synergistic action that substantially boosts the efficacy of central nervous system depressants [28]. Herbal phytoconstituents can interact with medicines via pharmacodynamic pathways, that include the medications' receptors or biological processes, and lead to antagonistic, combined, as well as synergistic effects [4,6]. Herbal treatments typically contain multiple active substances that may interact with one another to achieve distinct therapeutic goals. As a result, there is a higher chance of potentially major HDIs than there is with medication interactions [6]. It is challenging to forecast the quantity and therapeutic significance of these interactions due to the complicated composition of herbal extracts and the dearth of clinical data [3,7]. It is necessary to carefully monitor any possible interactions, particularly in high-risk patients who are taking drugs with limited therapeutic indices or in people who have long-term illnesses that call for several prescriptions [3].

3. HDIs and Herbovigilance

Herbal products have the potential to change the pharmacokinetics of traditional medicines, resulting in HDIs serious pharmacological complications (Figure 1). They typically occur when components found in herbal medicines have an impact on transporters or enzymes that metabolize drugs, changing the toxic effects or beneficial effects of the drug. Ginkgo biloba, for example, has been demonstrated to reduce the area under the curve (AUC) for alprazolam & midazolam, two CYP3A4 enzyme substrates, suggesting possible inhibiting effects on drug metabolism. However, the pharmacokinetics of flurbiprofen and warfarin were unaffected by the identical plant, indicating that the effects of herbal remedies can differ depending on the medication and dosage [7,29]. The heterogeneity in herbal formulation hampers the estimation and assessment of HDIs, because many consumers self-medicate these medicines without consulting medical professionals, raising the probability of adverse reactions [29,30]. To clarify these HDIs and create guidelines for secure utilization of herbal remedies when combined with traditional medicines, systematic investigations are required [30,31].

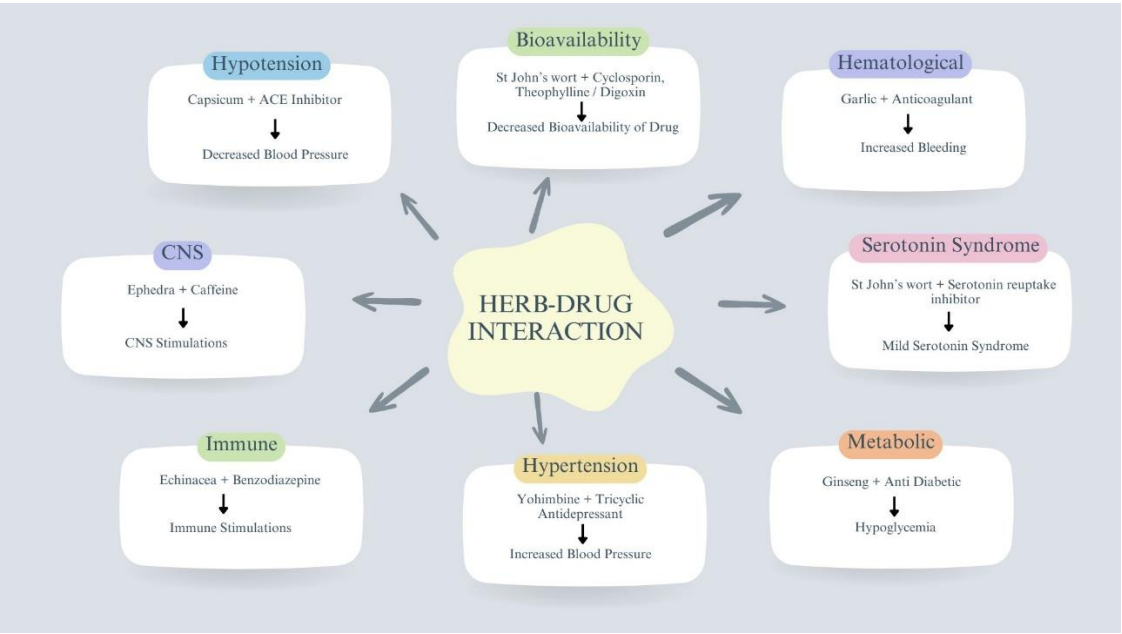

Figure 1. Common Herbs and Their Drug Interactions.

"Herbovigilance" is a surveillance and assurance of the responsible use of conventional drugs. Conventional medicines and their preparations are widely utilized to treat a wide range of ailments and health problems. But everyone believes that conventional treatments are risk-free and have zero adverse side effects. This misconception has been refuted by numerous scientific studies that show the frequency of adverse pharmacological reactions associated with herbal treatments. Pharmacovigilance, or the processes involved in the identification, evaluation, awareness, and avoidance of side effects or any other drug-associated difficulties, is crucial for the safety of herbal treatments. Although the already in use pharmacovigilance platforms were designed and executed successfully for synthetic pharmaceuticals, modifications are needed to take into consideration the distinct qualities and implications associated with natural goods [25,32].

4. Risk Factors

HDIs are a major problem in modern healthcare, especially as the utilization of herbal remedies grows alongside traditional therapies. The pharmacological qualities of both herbal remedies and traditional medicines, particular to the patient attributes, including the potency of the herbal formulations alone are some of the factors that increase the risk of adverse reactions (Table 1). A significant risk element is an individual's overall wellness. People who are experiencing comorbidity or individuals upon several drugs are more likely to acquire HDIs. Genetic variations can also affect the metabolism for both herbal and traditional drugs, compounding the effects of these interactions [33].

Herbal medications' pharmacokinetic and pharmacodynamic features are crucial when analysing HDIs. Most herbal items can inhibit the function of drug-metabolizing enzymes, specifically those in the cytochrome P450 family, resulting in changed drug concentrations in the body. For example, certain herbs can activate or block those enzymes, which results in either decreased efficacy or the increased toxicity of traditional medicines [3,29]. The potential for adverse interactions is increased when active ingredients in herbal remedies collaborate with protein carriers which help with drug absorption and excretion [29].

A further significant risk factor involves the "quality of herbal remedies." It is difficult to precisely evaluate the safety and efficacy of herbal remedies due to problems including adulteration, misidentification, and contamination, which can result in unforeseen pharmacological consequences. Unpredictable therapeutic effects & an elevated risk of interaction can also result from variations in the content of herbal products, which are frequently caused by variances in production and preparation techniques [3,33].

This scenario is complicated by the "lack of standardized guidelines" for assessing HDIs. There is still a lack of a systematic method for preventing and handling HDIs, despite the increasing awareness of the significance of keeping an eye on these interactions. Due to this lack of information, adverse reactions may go unreported and the dangers of using traditional and herbal remedies at the same time may be generally underestimated [29,33].

HDIs relate to a variety of risk parameters, including pharmacological features, product quality, patient preferences, and the requirement for standard assessment techniques. Since incorporating herbal remedies into contemporary medical regimens is becoming more and more popular, it is imperative that these factors be taken into consideration to ensure patient safety.

Table 1. Key Risk Factors for HDIs.

Risk Factor	Description
Patient Health Status	Comorbidities and polypharmacy increase the likelihood of adverse effects from HDIs [33].
Genetic Variability	Genetic differences impact drug metabolism, affecting the body's processing of both herbal and conventional drugs, and raising interaction risks [33].
Pharmacokinetic and Pharmacodynamic Properties	Many herbs influence drug-metabolizing enzymes (e.g., cytochrome P450), which can alter drug efficacy or increase toxicity [3,29].
Interaction with Protein Carriers	Active ingredients in herbs may interact with protein carriers, affecting drug absorption and excretion, and altering safety and effectiveness [29].
Quality of Herbal Products	Adulteration, contamination, and misidentification can lead to unforeseen effects, increasing the risk of adverse interactions [3,33].
Variability in Herbal Product Composition	Inconsistencies in the production and preparation of herbal products cause variations in content, leading to unpredictable therapeutic effects and interaction risks [3,33].
Lack of Standardized Guidelines	Limited systematic assessment methods for HDIs lead to underreported interactions and underestimated risks of combining herbal and traditional treatments [29,33].

5. Clinical Implications

HDIs are important clinical issues in contemporary medicine, especially given the increasing prevalence of simultaneous use of herbal remedies and prescription medications. Such interactions may cause patients' medications to become less effective, more toxic, or even have potentially fatal effects [4,34].

A major worry regarding HDIs is the diminished therapeutic effectiveness of traditional drugs. A variety of herbs can either stimulate or inhibit the cytochrome P450 group of enzymes, which is responsible for metabolizing drugs and changing the amounts throughout the human system [34,35]. Hypericum perforatum, the herb St. John's wort, is one example of a strong CYP3A4 trigger and can drastically decrease the plasma levels of medications processed by this enzyme, involving as immune-suppressive antiviral medications, and some chemotherapeutic medicines [3,34]. This combination may result in treatment failure for HIV-positive people, rejection of the organ in transplant patients, and decreased effectiveness of cancer treatments [3].

HDIs, on the other hand, can also raise the possibility of negative medication responses because of higher drug concentrations. Medicines including statins, Calcium channel blockers (CCBs), as well as benzodiazepines, are more widely distributed when taken in combination with herbs like grapefruit juice, which suppress gastrointestinal CYP3A4 [3,34]. An elevated risk of myopathy caused by statins, severe drowsiness from benzodiazepines, and potentially fatal arrhythmias from calcium channel blockers can arise from this combination [3].

The screening of HDIs risk is further complicated by the intricate and changeable composition of herbal preparations. It can be difficult to anticipate and control the interactions between various active ingredients found in many herbs, as they may interact with diverse pharmacological targets [29,35]. There is frequently a lack of regulation surrounding the safety and efficacy of herbal remedies, that raises the possibility of problems including adulteration, contamination, and inappropriate dosage [4,34].

Interactions between herbs and drugs present serious therapeutic difficulties for contemporary medicine. Elevated toxicity, decreased medication effectiveness, and possibly dangerous Adverse events (AEs) can result from such interactions. To guarantee patient safety and maximize therapeutic results, healthcare providers and patients need to be aware of the possible hazards connected with using herbal remedies and prescription medications together. They need to talk about and keep updated on such interactions.

6. Regulatory Considerations

Regulatory considerations over HDIs are increasingly significant in modern medical care because of the growing utilization of herbal remedies alongside standard drugs. The safety, efficacy, and quality of herbal medications are impacted by regulations, which vary widely across countries. For example, herbal products are governed under the Dietary Supplement Health and Education Act (DSHEA) in the USA, which classifies them as nutritious foods instead of drugs. The Food and Drug Administration (FDA) oversees it. It means that until and unless they are proven to present a significant risk, herbal remedies are treated for granted. It is the FDA's responsibilities to demonstrate this prior to a product gets withdrawn from distribution [4,29]. The shortage of recognized dosing and composition variation among herbal remedies further complicates the evaluation of their safety and the possibility of interactions. Whenever specific active ingredients in herbal remedies react with drug transporters and pharmaceutical-metabolizing enzymes, the pharmacokinetics and pharmacodynamics of combination pharmacological therapy get altered [34,36]. Healthcare providers might not possess enough access to accurate data about HDIs, that could cause patients' threats to go unidentified. For some, this inconsistency causes problems. Worldwide regulatory harmonization is required when disparities in regulatory standards may significantly impact the quality and reliability of products. The disparate ways that safety laws have been applied in different countries have left patients and medical personnel with a confusing patchwork of rules [36]. To enhance patient safety and optimize treatment outcomes, regulatory agencies should prioritize the evaluation of HDIs and provide explicit guidelines for the safe combination use of herbal medicines with conventional medications. This means promoting better communication about the application of herbal medicines between patients and medical professionals and ensuring that patients are informed of any potential risks associated with HDIs [29,34]. Regulatory frameworks are evolving to include guidelines for monitoring and managing HDIs, advocating for evidence-based approaches that ensure both safety and efficacy in the concurrent use of herbal and conventional medicines. Enhanced pharmacovigilance and research into the interactions between herbal products and pharmaceuticals are essential for safeguarding public health as the popularity of herbal remedies continues to rise [37,38].

HDIs are regulated differently by the Chinese Drug Evaluation (CDE), European Medicines Agency (EMA), as well as the Drug Management and Pharmaceutical Affairs (DMPA), each of these which reflects region-specific regulatory concerns. Herbal goods are subject to EU regulations through Guidelines 2001/83/EC & 2004/24/EC,

which mandate that conventional goods be registered simply or have full marketing approval. Herbs include St. John's Wort, that stimulates CYP450 enzymes and P-glycoprotein even may reduce plasma levels of medications including cyclosporine along with warfarin, are an emphasis of pharmacokinetic together with pharmacodynamic study used by the EMA to assess HDIs. Although HDI data is included in the monographs established through EMA's Committees upon Herbal Medicinal Products (HMPC), approximately 20% of them make inclusion of HDIs, suggesting most of the interaction require substantial clinical documentation [39,40]. The CDE in China requires comprehensive evaluations of HDIs throughout the drug manufacturing alongside regulatory process with the aim to guarantee the appropriate use of herbal remedies. It entails carrying out clinical studies and assessing pharmacological pathways. Regarding frequently utilized plants including ginseng and turmeric, DMPA guidelines mandate stringent pre-market assessments. Employing information from world-wide databases and regional instances, appliances include the Drug Interaction Probability Scale (DIPS) serve as vital for determining potential links among drugs and herbs [41]. Significant obstacles persist in the harmonization of worldwide HDI regulation requirements. Global commerce in herbal products is hampered by disparities in regulatory systems. In this regard, the U.S. FDA categorize herbal products as therapeutics or nutritional supplements depending upon their intended application, frequently avoiding the stringent pre-market reviews needed in Europe, while the EMA requires substantial proof for HDI labelling. immediate intervention is required to address this discrepancy to safeguard consumers and maintain a competitive advantage [40].

7. WHO Guidelines for HDIs

The World Health Organization (WHO) has issued recommendations for dealing with HDIs to improve the safety and efficacy of medical therapies that combine herbal treatments alongside conventional medications. These recommendations emphasize how important it is for medical practitioners to comprehend the pharmacokinetic and pharmacodynamic interactions that may occur when herbal medicines are taken with prescription drugs. Because herbal ingredients affect the enzymes and transporters that break down drugs, pharmacokinetic interactions can change how drugs are absorbed, distributed, metabolized, or excreted. On the other hand, pharmacodynamic interactions take place when herbs use comparable biological pathways to either increase or decrease the therapeutic effects of medications. WHO urges medical professionals to ask patients about all supplements they may be taking and stresses the value of informing patients about possible HDIs. To efficiently monitor side effects and interactions, the guidelines also support the incorporation of herbal remedies into current pharmacovigilance systems. WHO also urges greater research to better understand the mechanisms behind HDIs, which are still a poorly understood field of pharmacology. The ultimate objective is to improve patient outcomes in contemporary healthcare settings by giving medical practitioners all the materials they need to make educated decisions and guarantee secure simultaneous utilization of traditional and herbal medications [37,42].

8. Research and Future Directions

HDIs studies is becoming more vital in modern healthcare due to the increased usage of herbal supplements in conjunction with conventional pharmaceuticals. There is a high risk of negative interactions since users frequently self-administer these medications without telling their medical professionals. The body of current literature highlights the need for more thorough study, especially through observational studies that make use of real-world data, to comprehend the therapeutic implications of HDIs. Because of ethical considerations and the intricacy of herbal formulations, observational studies, for instance, might shed light on the prevalence and effects of HDIs in routine clinical settings, which are frequently missed in standard Randomized controlled trials (RCTs) [34,43]. Subsequent investigations ought to concentrate on formulating uniform approaches for evaluating HDIs, encompassing sophisticated computer models that forecast interactions predicated on the pharmacokinetic characteristics of the plant components. Furthermore, by utilizing cutting-edge study designs like case-control as well as cohort studies, we can improve our comprehension about these interactions and their practical consequences [43,44]. Increased interaction between scholars, medical practitioners, and government agencies is also necessary to develop thorough protocols that guarantee the secure incorporation of herbal items into treatment plans. In the end, this cooperative effort will enhance integrative medicine practices' therapeutic results and patient safety [4,29].

9. Conclusions

HDIs pose serious problems for contemporary medicine, requiring patients and healthcare professionals to exercise extra caution and awareness. The growing trend of using herbal supplements raises the possibility that they may negatively interact with prescription drugs, resulting in severe side effects or reduced therapeutic efficacy.

According to recent studies, these interactions involve both pharmacokinetic and pharmacodynamic pathways, with many herbs having the ability to modify drug metabolism and transport mechanisms. In clinical practice, there is often inadequate reporting and documenting, which contributes to the significant knowledge gap that persists despite the increasing body of information regarding interactions and their treatment consequences. Before prescribing medication, healthcare providers should have candid conversations with patients on the use of herbal supplements and the possibility of HDIs. Furthermore, more research is required to better our understanding of the mechanisms driving these interactions and to develop standard criteria. In the end, tackling the complexity of HDIs will help to improve integrative healthcare procedures and make them safer and more efficient. This will guarantee that patients can obtain the advantages of both conventional and herbal therapies without taking any risks.

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Abbreviation

Abbreviation	**Full Form**
AEs	Adverse Events
AUC	Area Under the Curve
CCBs	Calcium Channel Blockers
CDE	Chinese Drug Evaluation
CYP450	Cytochrome P450
DIPS	Drug Interaction Probability Scale
DMPA	Drug Management and Pharmaceutical Affairs
DSHEA	Dietary Supplement Health and Education Act
EMA	European Medicines Agency
FDA	Food and Drug Administration
HDIs	Herb-Drug Interactions
HMPC	Committee on Herbal Medicinal Products
P-gp	P-Glycoprotein
RCTs	Randomized Controlled Trials
WHO	World Health Organization

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