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Composition of *Amesiodendron chinense* (Merr.) Hu Seed Oil and Assessment of Its Nrf2/ARE Induction Activity in AREc32 Cells

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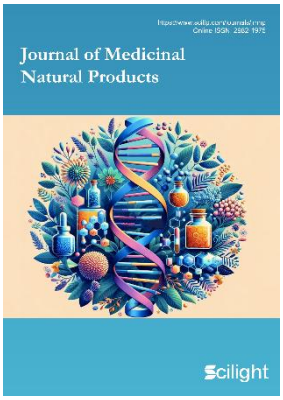

The *Journal of Medicinal Natural Products* (JMNP) aims to provide a dedicated platform for the dissemination of high-quality research related to the extraction, isolation, characterization, bioactivities, toxicities, biosynthesis, synthesis, and application of natural products with medicinal properties. Only research that makes new and impactful scholarly contributions to the area of medicinal natural products will be considered for publication. The journal encourages submissions that provide insightful contributions to the field, particularly those that address current challenges and innovations in the discovery and application of medicinal natural products. It is published quarterly online by Scilight Press.

Aims:

- To publish high-quality, peer-reviewed research on the chemistry, biochemistry, and pharmacology of natural products.
- To advance the understanding of the role of natural products in medicine, including their mechanisms of action, efficacy, and safety.
- To foster interdisciplinary collaboration among scientists in the fields of chemistry, biology, pharmacology, and medicine.

Scope:

Agriculture: Cultivation, breeding, and sustainability practices for medicinal plants.

Regulatory Science: Compliance with regulatory requirements for the development and approval of natural product-based therapeutics.

Analytical Techniques: Development and application of methods for the detection and quantification of natural products.

Biochemistry: Studies on the biosynthesis and metabolic pathways of medicinal natural compounds.

Biotechnology: Use of genetic engineering and fermentation technology to produce medicinal natural products.

Chemistry: Isolation, structural elucidation, synthesis, and chemical modification of bioactive natural products.

Ethnopharmacology: Documentation and validation of traditional medicines and practices.

Formulation and Delivery: Development of pharmaceutical formulations based on medicinal natural products and their delivery, including pharmacokinetics studies.

Metabolomics: New approaches to metabolomics of medicinal natural products.

Pharmacology: Preclinical and clinical studies on the therapeutic potential and pharmacokinetics of natural products.

In silico studies covering any of the above areas may be considered only if the finding is validated by relevant *in vitro* or *in vivo* results.

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Editorial

Journal of Medicinal Natural Products: A New Journal with a New Perspective

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Journal of Medicinal Natural Products has been launched to address various aspects of innovative research exclusively focusing on medicinal natural products. Natural products are secondary metabolites biosynthesized by various living organisms, e.g., plants and microbes. However, not all natural products have known medicinal values. Therefore, medicinal natural products refer to only those natural products that have known or clinically proven medicinal values [1]. Over the years, natural products have played a key role in the discovery of modern drugs like aspirin, cephalosporins, penicillins, quinine, taxol, vinblastine, vincristine and many more; the major contributions have been in three major disease areas: cancer, microbial infections, and malaria. The other disease areas, where the contributions from medicinal natural products are well documented include cardiovascular and neurological disorders, liver and kidney diseases, diabetes, psychosis, and obesity. Cancer chemoprevention is another emerging area, where medicinal natural products have started to play a significant role.

The practice of using crude forms of natural materials in the treatment of human ailments goes back to thousands of years and has formed several well-known traditional medicinal systems across the globe, e.g., Ayurveda and Traditional Chinese Medicine (TCM) [1,2]. The discovery of the antimalarial drug artemisinin and the award of the Noble prize in Physiology/Medicine to Youyou Tu in 2015, have brought natural products back to limelight. There is no doubt that natural products, because of their unique chemical diversity, will continue to contribute to new drug discovery in the coming years.

With the remarkable advancement in separation science (particularly, various hyphenated techniques [3]), structural characterization methods and assay technologies, natural products drug discovery process has become highly competitive with the conventional drug discovery processes. Introduction of and rapid developments in AI and computational methods have significantly enhanced the capabilities of natural products drug discovery research, Nowadays, a combination of *in silico*, *in vitro* and *in vivo* evaluation (including pre-clinical and clinical trials) has become a norm in any high-yielding and high-quality research with medicinal natural products [4].

While the aims and scope of this new journal have been well articulated in the instructions/guidelines for authors on the journal website, it can be mentioned that this journal will fundamentally capture all new developments and emerging technologies in research involving medicinal natural products. We understand that there may be several journals in natural products, but this new journal will stand out from the crowd based on its uniqueness, quality, standard and absolute focus on the medicinal aspects of natural products.

This new journal is in the process of forming a strong and active editorial board comprising members from all over the world, chosen based on their outstanding contributions in medicinal natural products research. The journal team believes that this journal will serve as a sought-after knowledgebase for a large scientific community comprising researchers from all levels and varied experiences, who have been involved in medicinal natural products research or have a keen interest in medicinal natural products and will soon become one of the leading journals in this research area. It will also cater for the readers, who are generally interested in drugs from natural origins.

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Article

Composition of *Amesiodendron chinense* (Merr.) Hu Seed Oil and Assessment of Its Nrf2/ARE Induction Activity in AREc32 Cells

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Abstract: Background: *Amesiodendron chinense* (Merr.) Hu (family: Sapindaceae) is a Thai medicinal plant. The seed oil of this species has been used by folk healers and local people in southern Thailand for the treatment of wounds, skin disorders and common hair problems. This study aimed at the GC-MS-based determination of the chemical composition of the seed oil of this plant, and evaluation of its Nrf2/ARE induction activity in AREc32 cells (modified human breast cancer cell line MCF-7) using the MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] and luciferase reporter gene assays. Results: GC-MS analysis identified 9-(*E*)-octadecenoic acid (84.82%) as the main component of this seed oil. TLC-based qualitative DPPH (2,2-diphenyl-1-picrylhydrazyl) assay revealed the DPPH radical-scavenging activity of the seed oil and its chromatographic fractions. A low-level DPPH-scavenging activity was observed in the quantitative assay, but no IC_{50} value could be determined even with the highest tested concentration (10 mg/mL). Neither the oil nor its chromatographic fractions showed any significant Nrf2/ARE induction in AREc32 cells. The seed oil was noncytotoxic against the AREc32 cells. Conclusions: *A. chinense* seed oil and its fractions had a low level of free-radical scavenging property but no significant Nrf2/ARE induction activity in AREc32 cells. However, as the oil did not show any cytotoxicity at test concentrations in the MTT assay, this oil might potentially be safe to use in cosmetic formulations or as a vehicle for the dermal delivery of drug molecules.

Keywords: *Amesiodendron chinense*; Sapindaceae; seed oil; antioxidant; cytotoxicity; cancer chemoprevention; GC-MS; Nrf2/ARE induction

1. Background

Thailand is one of the most biodiversity-rich countries in Southeast Asia, which contains *ca.* 15,000 plant species, representing 8% of the world's plant species [1]. Importantly, Thai ancestors had accumulated precious experience of health care in fighting against diseases before the Sukhothai period or before 1238 CE. As a result, the unique traditional medicine system, known as “Thai Traditional Medicine” (TTM), based on Thai medicinal plants, was developed [2,3]. Although medicinal plants form the foundation of the TTM, many of these plants have never been evaluated for their phytochemical composition and bioactivities, and for most of them, their therapeutic potential is yet to be discovered.

Amesiodendron chinense (Merr.) Hu is one of the medicinal plants of the Sapindaceae family [4]. This species is a primary rainforest tree growing up to 25 m tall (Figure 1). Its Thai name is “Khun”, and found in primary rainforests, native to valleys, hill forests and limestone areas, 300–1000 m altitude and growing on well-drained soil [4,5]. It is distributed from South China through Indochina to Peninsular Malaysia and Sumatra. In Thailand, it is an endemic tree species only found in Phatthalung and Trang province, the southern part [4–6]. *A. chinense*

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was categorized as 'near threatened' by the International Union for Conservation of Nature (IUCN) due to over-exploitation of valuable timber in certain regions and a restricted geographical distribution of this plant [7]. However, the use of seeds to produce oil is not considered a threat to this species.

Traditional healers and local people in southern Thailand externally apply *A. chinense* seed oil to treat chronic wounds such as diabetic foot and pressure ulcers, itching, skin allergies and inflammation caused by insect bites, skin diseases such as ringworm, scabies, and athlete's foot. Moreover, it is applied as a massage oil for relieving muscle pain and a hair tonic for giving shiny-black hair and antidandruff treatment [6,8,9]. This oil has also been used as an excipient in carious cream formulations [9].

a **b** **c**

Figure 1. Tree (**a**), fruits (**b**) and seeds (**c**) of *A. chinense.*

Previous phytochemical and pharmacological studies of this species identified phenolic compounds including flavonoids amesiflavones A–C, and lignans (+)-aptosimon (+)-isolariciresinol (−)-cleomiscosin A and (−)-cleomiscosin C from the leaves. Amesiflavones A and B, (+)-aptosimon, (−)-cleomiscosins A and C, and (+)-isolariciresinol showed cytotoxic activity against human cancer cell lines including KB (a subline of the ubiquitous KERATIN-forming tumour *cell line* HeLa), SK-LU-1 (lung cancer), MCF-7 (breast cancer), HepG-2 (liver cancer), and SW-480 [4,10]. Further phenolic compounds, astragalin, (−)-catechin, (−)-*epi*-catechin, chrysoeriol, kaempferide 3-*O*-β-D-glucopyranoside and quercetin 3-*O*-β-D-glucopyranoside were isolated from the flowers [11]. Moreover, *A. chinense* seed oil was shown to contain six fatty acids including, arachidic acid, eicosenoic acid, linoleic acid, oleic acid, palmitic acid, and stearic acid [6].

Although the seed oil of *A. chinense* has been used in folk medicine in southern Thailand with effective treatment outcomes, there are limited phytochemical and pharmacological studies reported on this species to date. No published research articles on pharmacological studies of *A. chinense* seed oil have been found in the literature. Thus, this study aimed at the GC-MS analysis of the seed oil of this plant, and evaluation of its Nrf2/ARE induction activity in AREc32 cells using the MTT and luciferase reporter gene assays**.**

2. Methods

2.1. Plant Materials

Amesiodendron chinense (Merr.) Hu seed oil was supplied by Sawat Chandang, a traditional healer in Phatthalung province, Thailand. Seed oil was extracted using traditional methods as briefly described here. The fruits were sun-dried and cracked, and the peels were removed. The seeds were ground and cooked by steaming. After that, the cooked seeds were placed in a wicker container (Figure 2a) and put in wooden equipment (Figure 2b). Then logs were inserted on both sides of the equipment to compress the seeds. Lastly, seed oil was collected, filtered, and kept in a glass bottle (Figure 2c).

a **b** **c**

Figure 2. Wicker container (**a**), wooden equipment (**b**) and seed oil (**c**) of *A. chinense*.

2.2. GC-MS Analysis

The chemical constituents of the seed oil sample were analyzed on an Agilent 6890N network GC system coupled to an Agilent 5790 mass selective detector (Agilent, Santa Clara, CA, USA). Separations were conducted using an HP-5MS capillary column (30 m × 0.25 mm i.d., 0.25 μm film thickness). The seed oil sample was diluted 10 times in *n*-hexane and then the sample (1 μL) was injected in the split mode with a split ratio of 1:50. The column oven temperature was programmed as follows: the beginning of 150 °C kept for 3 min, rising by 20 °C/min to 280 °C and maintaining for 4 min. The total run time was 14 min. Helium was used as a carrier gas at a flow rate of 1 mL/min. The mass-spectrometer was accomplished in the range of 41–500 *m*/*z* in the EI mode at 70 eV at an MS source temperature of 230 °C.

2.3. VLC Fractionation

Vacuum liquid chromatography (VLC) was carried out as described by Reid and Sarker (2012) [12] with suitable modifications. In brief, a portion of seed oil (10 mL) was mixed with normal silica gel (70–230 mesh) (Sigma Aldrich, St. Louis, MO, USA) and loaded on the top of a VLC column pre-packed with TLC-grade silica gel 60 H (Sigma Aldrich, USA). The column was washed with 200 mL of *n*-hexane and eluted with a stepwise mobile phase gradient, consisting of 2% (twice), 4%, 6%, 10%, 20%, 30%, 40% and 50% DCM in *n*-hexane and finally, 100% DCM (200 mL each) under vacuum. Ten different fractions (F1-F10) were collected separately, dried under a rotary evaporator, and stored in glass vials at 4°C until used.

2.4. TLC Analysis

Silica gel 60 coated with fluorescent indicator F_{254} on TLC aluminium foil (Sigma Aldrich, USA) was used as a stationary phase. Seed oil and 10 VLC fractions were re-dissolved (10 mg/mL) in DCM and spotted on the TLC plate. The TLC plate was developed using 5% of ethyl acetate in *n*-hexane as a mobile phase. After that, the plate was dried, and visualized under UV light at 254 and 365 nm in a UV cabinet (Analytik Jena, Upland, CA, USA). The developed plate was then sprayed with an anisaldehyde-sulphuric acid reagent followed by heating in an oven (Nabertherm, Lilienthal, Germany) at 110 °C for 10 min. Fractions with similar TLC fingerprints were combined.

2.5. DPPH Assay

Seed oil and its fractions were screened for free-radical scavenging properties using the DPPH assay following the method described by Takao et al. [13] with proper modifications [14,15]. DPPH powder (Sigma Aldrich, USA) (8 mg) was dissolved in isopropanol (100 mL) to obtain a concentration of 80 μg/mL.

2.5.1. Qualitative Assay

All tested solutions (10 mg/mL) including quercetin (Sigma Aldrich, USA), a positive control (1 mg/mL) were applied on the TLC plate and followed by the TLC development step as aforementioned procedure. The DPPH solution was sprayed onto the developed TLC plate and left the TLC plate for 30 min to allow the reactions to occur. The white spots against the purple background were indicative of the antioxidant potential of the samples.

2.5.2. Quantitative Assay

Ten-fold serial dilutions of each tested sample were prepared in isopropanol from the stock concentrations (10 mg/mL) to obtain concentrations of 1.0, 0.1, 0.01, 0.001, 0.0001 and 0.00001 mg/mL. After that, 2 mL of diluted solutions were mixed with 2 mL of DPPH and allowed to stand at room temperature for 30 min for any reaction to occur. Absorbance values were measured at 517 nm using a Jenway 7315 Advanced UV-VIS spectrophotometer (Bibby Scientific Ltd, Staffordshire, UK). The experiment was performed in triplicate. The same protocol was conducted for the positive control, quercetin (stock concentration 1 mg/mL). The percentage of DPPH scavenging activity at each concentration expressed as % inhibition was calculated by using the following formula:

$$\text{Inhibition (\%)} = (A_{control} - A_{sample}) \times 100/A_{control} \quad (1)$$

where $A_{control}$ was the absorbance value of the blank control (containing all reagents except for the tested sample) and A_{sample} was the absorbance value of the tested sample/positive control.

2.6. Preparation of Cell Lines and Cell Culture

AREc32 cells (modified the human breast cancer cell line MCF-7) were used for cell culture work [16]. All cells were cultured in Dulbecco's Modified Eagles Medium (DMEM) high glucose (Biosera Europe, Paris, France) supplemented with 10% foetal bovine serum (FBS) (Thermo Fisher Scientific, USA), 5% penicillin-streptomycin suspension (Sigma Aldrich, USA) and geneticin G418 (0.8 mg/mL) (Thermo Fisher Scientific, Waltham, MA, USA) and then maintained in an incubator (Binder, Germany) at 37 °C under 5% CO_2 and 95% humidity**.** The cells were seeded into 96-well plates (Corning, USA) at a cell density of 1.2×10^4 cells in 100 µL of complete medium per well and incubated for 24 h before each experiment started. All experiments were performed in three independent experiments (5 replicates/experiment).

2.7. MTT Assay

MTT assay [17] was used to determine the cytotoxicity of the seed oil and its fractions. Cells were treated with 100 µL of the following concentrations 0.0125, 0.025, 0.05, 0.1, 0.2, 0.4, 0.8, and 1 mg/mL. Negative (untreated cells), positive (20% dimethyl sulfoxide, DMSO) and vehicle controls (0.4–1% ethanol) were also included in each experiment. After 24 h incubation, 20 µL of the MTT solution (5 mg/mL) was added to each well and incubated for 4 h. The MTT solution was discarded and 100 µL of DMSO was added in each well to dissolve the purple formazan product. The absorbance of the formazan product of viable cells was read using the microplate reader (Tecan, Männedorf, Switzerland) at 570 nm. The mean % cell viability was calculated as follows:

$$\% \text{ Cell viability} = (\text{absorbance of treated cells}/\text{absorbance of untreated cells}) \times 100 \quad (2)$$

2.8. Luciferase Assay

Luciferase assay was performed as described by Basar et al. [16]. AREc32 cells were treated with seed oil and its fractions at a non-cytotoxic concentration which was assessed by the MTT assay for 24 h. Cells were washed with 100 µL of phosphate-buffered saline (PBS), then 20 µL of 1X luciferase reporter lysis buffer (Promega, Southampton, UK) was added to each well followed by a freeze–thaw cycle (−20 °C) for 24 h to achieve complete cell lysis. The cell lysate was thawed and transferred to a white opaque 96-well plate and mixed with 100 µL of freshly prepared luciferase assay reagent. The bioluminescence was measured using the microplate reader (Tecan, Switzerland). The Luciferase activity was expressed as folds induction relative to untreated cells. Positive control of 10 µM *tert*-butylhydroquinone (*t*BHQ) was also used.

2.9. Statistical Methods

All experiments were conducted in triplicate. All results were presented as means ± standard deviation. The graphs were plotted using non-linear regression using Microsoft Excel version 2019.

3. Results

3.1. GC-MS Analysis: Composition of the Seed Oil

The seed oil derived from *A. chinense* had a yellow colour. The identified components, retention time, molecular formula, and relative peak area (%) are displayed in Table 1. The GC-MS profile is shown in Figure 3. A total of eight compounds were identified, representing 100% of the total oil. The major components of the seed oil were 9-(*E*)-octadecenoic acid (84.82%) (Figure 4) followed by di-isooctyl phthalate (8.78%), 2,3-dihydroxypropyl elaidate (1.86%) and ethyl *iso*-allocholate (1.37%).

Table 1. Chemical composition of seed oil extracted from *A. chinense*.

No	Retention Time (min)	Molecular Formula	Compounds	Relative Peak Area (%)
1	7.4	$C_{27}H_{56}$	Heptacosane	0.20
2	7.76	$C_{16}H_{32}O_2$	*n*-Hexadecanoic acid	0.47
3	8.72	$C_{18}H_{34}O_2$	9-(*E*)-Octadecenoic acid	84.82
4	10.25	$C_{21}H_{40}O_4$	2,3-Dihydroxypropyl elaidate	1.86
5	10.54	$C_{18}H_{34}O_2$	13-(*Z*)-Octadecenoic acid	0.85
6	10.62	$C_{24}H_{38}O_4$	Diisooctyl phthalate	8.78
7	10.74	$C_{18}H_{34}O_2$	Oleic acid	0.6
8	10.86	$C_{18}H_{34}O_2$	13-*(E)*-Octadecenoic acid	1.07
9	12.48	$C_{26}H_{44}O_5$	Ethyl *iso*-allocholate	1.37

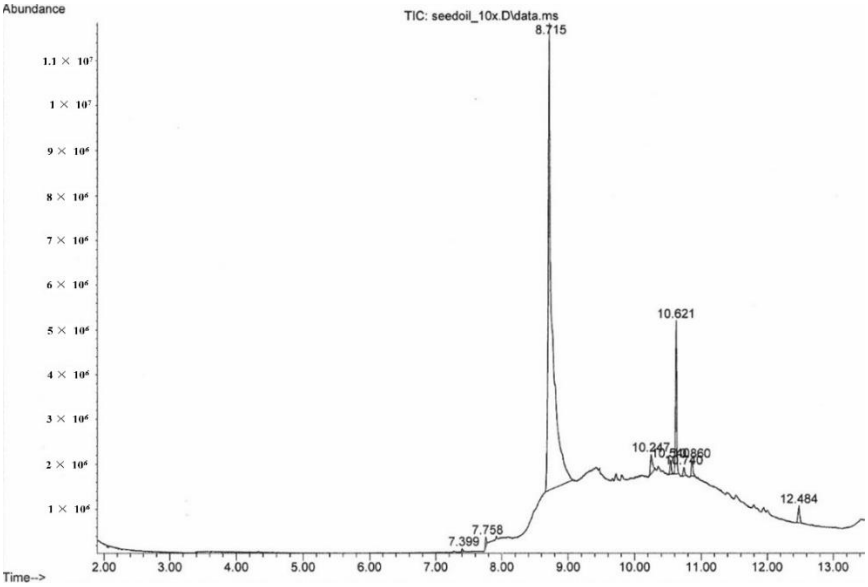


Figure 3. GC-MS chromatogram of *A. chinense* seed oil.

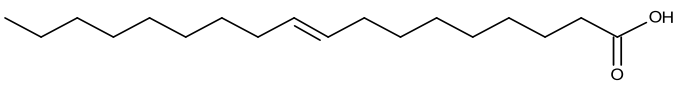
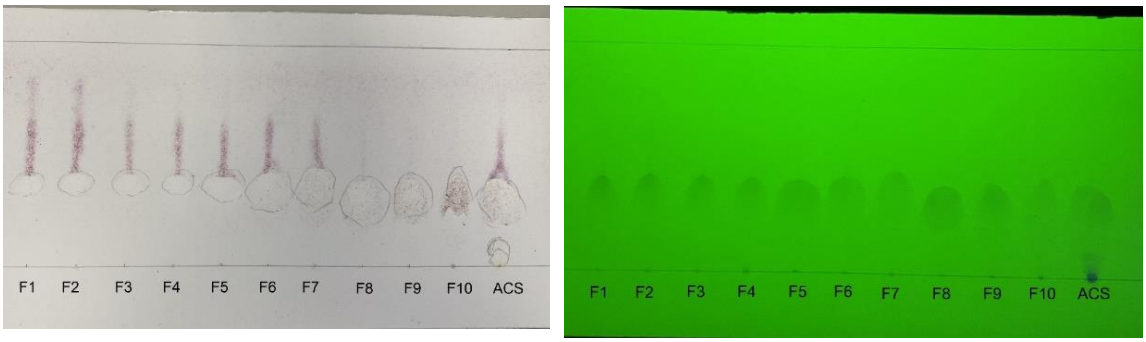


Figure 4. Chemical structure of 9-(*E*)-octadecenoic acid.

3.2. TLC Screening of the Oil and Its Chromatographic Fractions

The TLC chromatogram of *A. chinense* seed oil (ACS) and its VLC fractions (F1–F10) with different detection methods are presented in Figure 5. VLC fractions with similar TLC profiles were then combined as follows: F1 + F2, F3 + F4, F6 + F7 and F8 + F9.



A **B**

Figure 5. TLC fingerprint of *A. chinense* seed oil (ACS) and its VLC fractions with visualization under UV 254 nm (**A**) and visible light after derivatization with anisaldehyde-sulphuric acid reagent (**B**); solvent system: *n*-hexane: ethyl acetate (9.5:0.5).

3.3. DPPH-Scavenging Activity

The TLC-based qualitative DPPH assay of the *A. chinense* seed oil and its VLC fractions revealed white spots against a purple background (Figure 6), indicating the presence of compounds with free-radical scavenging activity. The seed oil itself showed a maximum of 4.51% inhibition of DPPH at its highest tested concentration of 10 mg/mL, (Table 2), and the % inhibition of the tested VLC fractions at 10 mg/mL was between 0.668% to 2.33%. At lower concentrations (0.00001–0.1 mg/mL), the seed oil and the VLC fractions F1 + 2, F3 + 4 and F5 did not show any quantifiable DPPH-scavenging activity. Among the VLC fractions, the fraction F10 showed the highest % of inhibition (2.33%) at 10 mg/mL concentration. While the 50% inhibition value (IC_{50}) for the seed oil and its VLC fractions could not be determined even at the highest tested concentration, quercetin, as a positive control, reached 50% inhibition of DPPH at a concentration between 0.01 and 0.1 mg/mL.

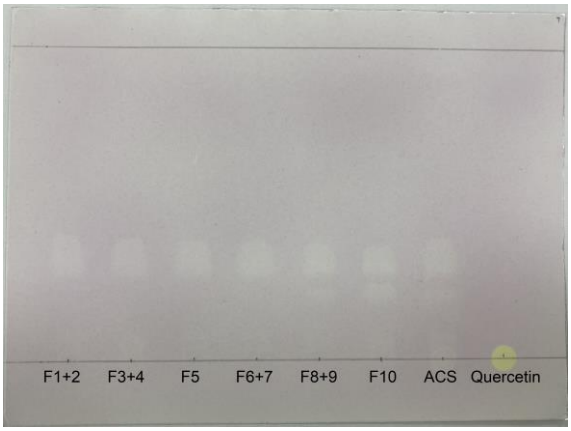


Figure 6. TLC plate of *A. chinense* seed oil (ACS) and fractions after spraying with DPPH reagent.

Table 2. Quantitative DPPH assay of the seed oil and its VLC fractions.

Conc. (mg/mL)	% Inhibition of DPPH							
	Quercetin	Seed Oil	VLC fractions					
			F1 + 2	F3 + 4	F5	F6 + 7	F8 + 9	F10
0.00001	-	-	-	-	-	0.165	-	0.359
0.0001	0.499	-	-	-	-	0.824	-	0.277
0.001	5.668	-	-	-	-	0.494	0.273	0.554
0.01	33.305	-	-	-	-	0.301	0.276	0.416
0.1	71.289	-	-	-	-	0.357	0.277	0.693
1	93.300	1.002	0.002	0.028	-	0.165	0.853	0.915
10	NP	4.514	0.668	1.951	-	0.770	1.323	2.328

- = No inhibition; NP = Not performed.

3.4. Cytotoxic Activity: MTT Assay

The seed oil and its fractions at the concentration ranging from 0.0125–1 mg/mL were assessed for cytotoxicity against AREc32 cells using the MTT assay to identify a non-cytotoxic concentration of each treatment. Ethanol was used at 0.4–1% *v*/*v* as the vehicle control which did not show toxicity. The non-cytotoxic concentrations chosen for the luciferase assay were 0.4, 1.0, 0.1, 0.4, 0.2, 0.4 and 0.8 mg/mL, respectively, for the seed oil, F1 + 2, F3 + 4, F5, F6 + 7, F8 + 9 and F10.

3.5. Chemopreventive Activity: Luciferase Assay

Non-toxic concentrations of seed oil and fractions as shown above were assessed for their chemopreventive property using the Luciferase assay by measuring induction of Nrf2 activation in AREc32 cells. As shown in Figure 7, *tert*-butylhydroquinone (tBHQ), a well-known Nrf2 activator at 10 μM was used as a positive control, produced a 23.2-fold activation of Nrf2 activity compared to the negative control. Seed oil and its fractions did not show any significant Nrf2/ARE induction (less than 2-fold compared to control).

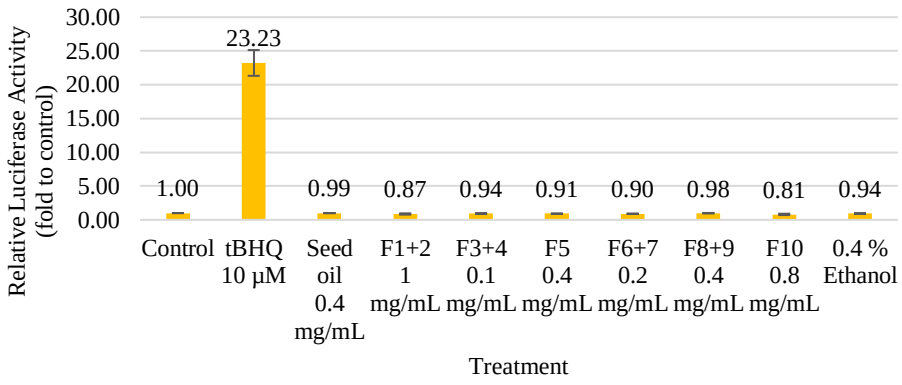


Figure 7. Luciferase activity of seed oil and its fractions on AREc32 cells.

4. Discussion

The seed oil of *A. chinense* showed the presence of long-chain alkanes and fatty acids, which is quite typical of such a fixed oil [18]. 9-(*E*)-Octadecenoic acid (84.82%), also known as elaidic acid [19], was the main component of this oil. Therefore, it is reasonable to say that the physicochemical properties of this oil are mainly because of this compound. This result is, to some extent, in agreement with an earlier report [6], where 9-(*E*)-octadecenoic acid was reported as the main component (57.14%) of *A. chinense* seed oil, but in much less proportion. However, the previous study found other fatty acids including octadecanoic acid, 9,12-octadecadienoic acid, eicosenoic acid and 11-eicosenoic acid, which were not found in the current study. This variation in the proportion of 9-(*E*)-octadecenoic acid present in the oil and the composition of the oil could be linked to the differences in the oil sample preparation for the GC-MS analysis, i.e., in the form of fatty acid methyl ester, instead of the unaltered oil itself or because of variations in geographical origin, drying method, oil extraction method and several other factors related to processing. 9-(*E*)-Octadecenoic acid, with a *trans*-configured double bond at C-9 (Figure 4), is an oleic acid *trans* isomer. It is classified as a member of the long-chain fatty acids. Consumption of this *trans* fatty acid increases low-density lipoprotein (LDL) cholesterol and decreases high-density lipoprotein (HDL) cholesterol, which is associated with an increased risk of cardiovascular disease [20,21]. However, in Thailand, the ethnobotanical uses of the seed oil of *A. chinense* are confined to external uses only, e.g., medicinal oil for chronic wounds, itching, skin allergies and inflammation and other skin conditions, and as a massage oil and hair tonic [6,8,9]. Therefore, the presence of 9-(*E*)-octadecenoic acid as the major compound in this oil will have no consequences for cardiovascular diseases.

There are many plant seed oils that possess excellent antioxidant property [22], but the seed oil of *A. chinense* did only have a low level of DPPH-scavenging activity as found in the current study. Antioxidants are well-known to be associated with the chemopreventive effect of natural products [23]. In the current study, the seed oil and its fractions did not show any significant cancer chemopreventive activity as indicated by extremely low level of induction of Nrf2/ARE activity in AREc32 cells. The absence of any significant cancer chemopreventive activity of the oil could be explained by the absence of any considerable antioxidant activity in this oil. However, it is noteworthy, that this oil or its fractions did not show any significant cytotoxicity in the MTT assay, which could provide support for its safety in external use. In cosmetic and pharmaceutical industries, it is often important to find and use excipient or vehicle that is non-toxic to use in the products only for external use [24]. To this end, the seed oil of *A. chinense*, because of its non-toxicity to cells, could be a suitable candidate for such applications.

This is the first report on the assessment of the radical-scavenging, cytotoxic and chemopreventive activities of *A. chinense* seed oil, and GC-MS analysis revealing the composition of this fixed oil.

5. Conclusion

The present study has demonstrated that the major components of the seed oil of *A. chinense* are long-chain alkenes and fatty acids. Seed oil and its chromatographic fractions did not display any noticeable cytotoxicity against the AREc32 cells at the tested concentrations. They exhibited a low level of DPPH-scavenging activity and chemopreventive potential at tested concentrations. The absence of any significant cytotoxicity in the oil might be considered a positive outcome regarding its potential use as a vehicle in cosmetic products (e.g., an excipient in carious cream formulations) or in pharmaceutical medicinal products for external use as well as its traditional use as a massage oil.

Abbreviations:

ARE	Antioxidant Responsive Element
DPPH	2,2-Diphenyl-1-picrylhydrazyl
GC-MS	Gas Chromatography-Mass Spectrometry
MTT	3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
Nrf2	*Nuclear factor erythroid 2-related factor 2*
tBHQ	*tert*-Butylhydroquinone
TLC	Thin Layer Chromatography
VLC	Vacuum Liquid Chromatography

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Conflicts of Interest: The authors declare that they do not have any competing interests.

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Article

Quality Control of Triphala Churna

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Abstract: Objectives: Triphala Churna (TC) is a tridoshashamak Ayurvedic rasayana used for treating various diseases such as skin diseases, diabetes, and as a mild laxative. Methods: In this study, TC was prepared and quality standardized standardized using various parameters such as macroscopical evaluation, physical properties, physicochemical properties, and phytochemical analysis (HPTLC fingerprinting and quantification) methods to assess its quality and purity. Results: The qualitative and quantitative phytochemical assays via HPTLC revealed concentrations of Gallic acid as 3.62 ± 0.05%, 3.67 ± 0.04%, 1.52 ± 0.03%, and 2.96 ± 0.02% for Amlaki (*Emblica officinalis*), Haritiki (*Terminalia chebula*), Vibhitki (*Terminalia bellerica*), and TC, respectively. Conclusion: This study provides valuable insights into establishing robust quality control measures and developing reliable assays for both Triphala Churna (TC) and its individual components.

Keywords: Gallic acid; HPTLC fingerprinting; mild laxative; quality standardization; Triphala Churna

1. Introduction

Ayurvedic medicines are effectively used to treat various chronic diseases as well as modern lifestyle disorders. They are mainly composed of herbal plants, minerals, aquatic drugs, and their combinations used for the treatment of diseases. They do not induce side effects and toxicity. Therefore, the demand for herbal sources is increasing daily, and it is difficult to fulfill the demand. Excessive demand increases the chances of adulteration and substitution. Herbal raw materials and their products need identification and standard parameters, which help to develop quality medicines and minimize adulteration and substitution.

The Ayurvedic medicine TC possesses tridoshashamak properties and is mainly used to treat various diseases and for detoxification. Acharya Charak said that "daily intake of TC with honey/ghee can make a person live for one hundred years devoid of old age diseases," whereas Acharya Sushrut revealed that it is useful for treating ulcers and wounds. Modern science indicates in-vivo studies on melanoma cancer [1,2]. TC is non-habit forming, is a mild laxative, and maintains healthy digestive power; hence, it is recommended for overall health. The effectiveness of this churna is due to its chemical constituents, which are mainly present in Amalki pulp (*Embelicaofficinelis*), epicarp of Haritki (*Terminalia chebula*), and epicarp of Vibhitki (*Terminalia bellerica*). The chief chemical constituents are tannin (35%), a phenolic compound (25–38%), Gallic acid (3–7%), chebulagic acid (5%), chebulinic acid (5%), ellagic acid (2%), and a small amount of flavonoids and saponins (0.053–0.33%). Gallic acid is a common chemical constituent, so it is used to assay TC and its ingredients. Gallic acid is also used as an anti-inflammatory and for treating hypoglycemia. It has antioxidant properties and gamma-ray protection [3] and is used to treat various oral diseases [4].

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 [5]. 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 [6,7]. Hence, in this connection, an attempt has been made to develop the

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quality control parameter of TC and its raw materials, which would be useful in the Pharmacognosy, Phytochemistry, Botany, and Herbal industry for further research activities.

2. Materials

2.1. Collection of Plant Material

The Ayurvedic formulation Triphala Churna (TC) contains Amalki pulp (AP), epicarp of Haritki (HE), and epicarp of Vibhitki (VE). It was obtained from an Ayurvedic pharmacy in Raipur, Chhattisgarh, India. The authentication was performed in the Drug Testing Laboratory AvamA nusandhan Kendra.

2.2. Method of Preparation of Triphala Churna

Triphala Churna was prepared in the laboratory using a pulverization method with slight modifications. Equal amounts of Amalki pulp, epicarp of Haritki, and Vibhitki were mixed and converted into a fine powder. The fine powder was passed through sieve no. 44 for uniform size distribution [8]. It was further used for quality control assessment.

2.3. Extracts Preparation of Plant

The powdered sample of TC (10 g) was defatted using 200 mL of petroleum ether. The defatted sample was extracted with 300 mL of distilled water using a Soxhlet apparatus until the cycle turned transparent. The aqueous extract thus obtained was dried in a water bath at 90 °C. The dried sample was weighed and transferred into different sample bottles for storage in a refrigerator at 4 °C until required for further analysis. The same procedure was applied for AP, HE, and VE samples.

3. Methods of Standardization

3.1. Macroscopic Standardization

Triphala Churna, AP, HE, and VE samples were evaluated according to sensory observations such as color, odor, and taste.

3.2. Physical Characteristics Standardization

The physical characterization of TC was determined using parameters such as particle size distribution, angle of repose, Hausner's ratio, bulk density, and Carr's index.

3.2.1. Bulk Density

The Bulk density was determined using USP guidelines. In which 10 g of TC sample was taken into a 25 mL graduated measuring cylinder, and measured the bulk volume. Bulk density was calculated by using given formula.

$$\text{Bulk density} = \frac{\text{Weight of sample taken}}{\text{Bulk volume}} \quad (1)$$

3.2.2. Tapped Density

Triphala Churna (10 g) was taken in a graduated measuring cylinder and tapped on a wooden surface from the height of 2.5 cm at the second interval and after tapping measured the tapped volume. Tapped density was calculated by using given formula.

$$\text{Tapped density} = \frac{\text{Weight of sample taken}}{\text{Tapped volume}} \quad (2)$$

3.2.3. Angle of Repose

The angle of repose was estimated using the funnel method. The height of the funnel was fixed at 6 cm from the bottom surface. Ten grams of the powdered sample of TC was poured to flow through a funnel fixed on a stand.

A heap was formed, and its height and radius were measured. The angle of repose was calculated using the given formula.

$$\text{Angle of repose}(\theta) = \tan - 1\left\{\frac{\text{Height of heap (h)}}{\text{Radius of heap (r)}}\right\} \quad (3)$$

3.2.4. Compressibility / Carr's Index

Compressibility/Carr's Index is estimated using a previously applied procedure of tapped density and bulk density. The formula applied is as follows:

$$\text{Compressibility / Carr's Index} = \frac{\text{Tappeddensity} - \text{Bulk density}}{\text{Tappeddensity}} \quad (4)$$

3.2.5. Hausner's Ratio

The ratio of tapped density to bulk density is called Hausner's Ratio. The Hausner's Ratio is calculated using the following formula:

$$\text{Hausner's Ratio} = \frac{\text{Tapped density}}{\text{Bulk density}} \times 100 \quad (5)$$

3.3. Physicochemical Standardization

The physicochemical parameters mentioned, namely foreign matter, loss on drying, total ash, acid insoluble ash, and extractive values, are used to identify and assay the formulation according to the Ayurvedic Pharmacopoeia of India.

3.3.1. Loss on Drying

The powdered sample of TC (2 g) was accurately weighed on a watch glass plate. The weighed sample was spread evenly on the plate, dried in an oven at 105 °C, cooled, and then re-weighed. The procedure was repeated until a constant weight was obtained. The weight loss of the sample was recorded and calculated as a percentage. The same procedure was applied to all samples (AP, HE, and VE) in triplicate.

3.3.2. Total Ash Value

The powder sample of TC (3 g) was accurately weighed into a silica crucible. The crucible samples were placed in a muffle furnace at 450 °C for 3–4 h until complete combustion occurred. After cooling, the crucible was re-weighed, and the percentage of total ash was calculated. The same procedure was applied to all samples (AP, HE, and VE) in triplicate.

3.3.3. Acid Insoluble Ash

The total ash from TC was obtained as described in the above method. The ash was treated with 25 mL of 6 N HCl on a water bath with continuous heating until a solution was obtained. The solution was filtered through ashless Whatman filter paper no. 41. The residue from the filtrate was transferred into a silica crucible, ignited for 2 h, then cooled, re-weighed, and the percentage of acid-insoluble ash was calculated. The same procedure was applied to all samples (AP, HE, and VE) in triplicate.

3.3.4. Extractive Values

Determination of Alcohol Soluble Extractive

The powdered sample of TC (5 g) was macerated in 95% alcohol with continuous shaking for 6 hand left to stand overnight in a flask. Twenty-five milliliters of the filtrate was evaporated to dryness in a China dish at 105 °C on a water bath. The dried sample was weighed, and the percentage of alcohol-soluble extract was calculated. The same procedure was applied to all samples (AP, HE, and VE) in triplicate.

Determination of Water-Soluble Extractive

Five gramspowder sample of TC was taken and macerated in chloroform water with continuous shaking for 6 h and stand flask overnight. 25 mL of filtrate evaporated to dryness in a China dish at 105 °C on water bath. The dried sample was weighted and the percentages of alcohol soluble extract were calculated. The same procedure was applied to all samples (AP, HE and VE) in triplicate.

3.3.5. Determination of pH

The solution of TC (10% *w*/*v*) was prepared in distilled water. The pH of the solution was then measured using a pH meter. The same procedure was applied to all samples (AP, HE, and VE) in triplicate.

3.4. High-Performance Thin-Layer Chromatography (HPTLC) Analysis

The aqueous extract of TC (100 mg) was dissolved in 1 mL of methanol and centrifuged at 1000 rpm for 5 min. The same procedure was applied to all samples (AP, HE, and VE). The upper layer of the solution was used as the test solution for HPTLC fingerprinting and quantitative analysis (densitometry). A standard sample of Gallic acid (1 mg/mL) was prepared in methanol. The chromatographic analysis was performed on a Merck HPTLC plate silica gel 60F 254 measuring 200 × 100 mm. The standard sample solution of Gallic acid (1.0–7.0 µL) and test solutions of Amalki, Vibhitki, Haritki, and Triphala (2 and 5 µL) were applied to the plates as 8 mm bands with an 11.4 mm distance between bands using Linomat 5 instruments (CAMAG, Muttenz, Switzerland). The loaded sample plate was placed in a twin developing chamber with a pre-filled solvent of Chloroform:Acetone:Formic acid (7:2:1 *v*/*v*/*v*) as the mobile phase. The plate was placed in the solvent chamber for development in the relevant mobile phase until the solvent front reached 70 mm. After development, the plate was dried with hot air to evaporate solvents. Images of the plate were captured using a CAMAG TLC Visualizer chamber (S/N: 150503) under white light, at 254 nm and 366 nm, and scanned using a TLC Scanner 4 (Version 2.5.18262.1 - Anchrom, Mumbai, India). The peak table, display, and densitogram were recorded at 281 nm [9,10]. The calibration curve of Gallic acid is shown in Figure 1.

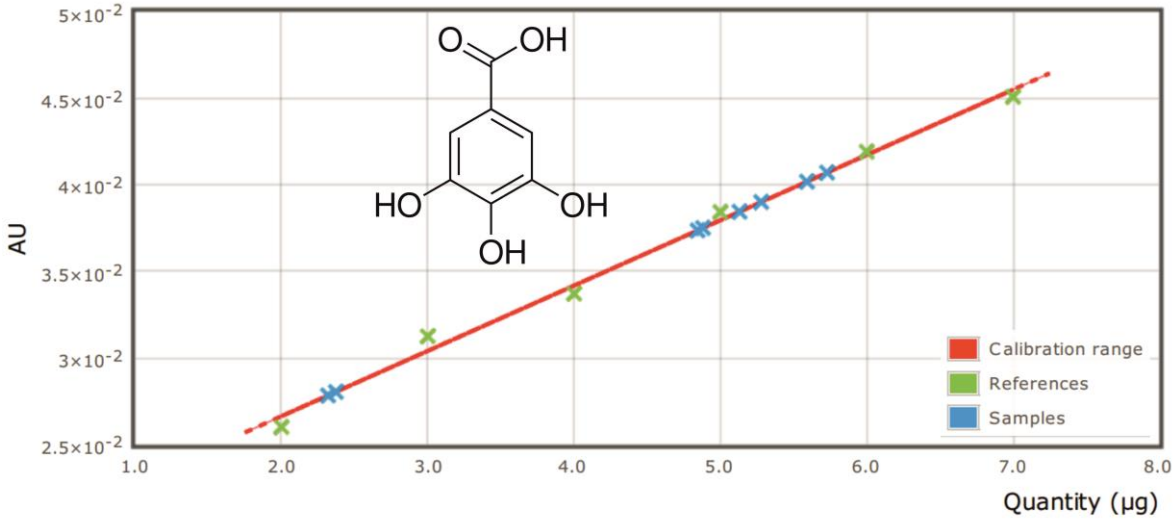


Figure 1. The calibration curve of Gallic acid.

4. Results

The present study of TC was prepared in accordance with the Ayurvedic Formulary of India to ensure the quality and efficacy of the product. The quality standardization of Ayurvedic TC was conducted using macroscopic and microscopic evaluations, physicochemical properties, physical properties, and the HPTLC method. Standard procedures as per the Ayurvedic Pharmacopoeia and other specific authorities were applied to establish suitable quality control parameters. Results were expressed as the mean (± SD) of three experiments for TC, AP, HE, and VE, as shown in Table 1.

Table 1. Standardization method for TC, AP, HE and VE.

No.	Parameters	Triphala Churna (TC)	Amalki Pulp (AP)	Epicarp of Haritki (HE)	Epicarp of Vibhitki (VE)
1.	Colour	Light Brown	Graish- Black	Yellowish Brown	Graish- Brown
2.	Odour	Characteristic	Aromatic-Sweet	Characteristic	Characteristic
3.	Taste	Characteristic	Sour - astringent	Astringent	Astringent
4.	Particle fitness	Fine to very fine powder	-	-	-
5.	Bulk density	0.4 ± 0.02 g/mL	-	-	-
6.	Tapped density	0.5 ± 0.04 g/mL	-	-	-
7.	Angle of Repose	0.25 ± 0.01	-	-	-
8.	Hausner's Ratio	1.25 ± 0.05	-	-	-
9.	Compressibility / Carr's Index	0.2 ± 0.001	-	-	-
10.	pH (10% aqueous sol.)	3.40 ± 0.07	-	-	-
11.	Loss of Drying	3.6 ± 0.06	4.55 ± 0.05	8.4 ± 0.07	4.05 ± 0.05
12.	Total Ash	6.33 ± 0.05	4.86 ± 0.03	4.43 ± 0.04	5.76 ± 0.06
13.	Acid Insoluble Ash	1.93 ± 0.04	1.63 ± 0.01	0.96 ± 0.02	1.66 ± 0.01
14.	Water soluble extractive	50.34 ± 0.08	49.4 ± 0.03	22.4 ± 0.07	43.74 ± 0.05
15.	Alcohol soluble extractive	43.68 ± 0.05	21.32 ± 0.02	12.08 ± 0.04	30.6 ± 0.03
16.	Conc. Of Gallic acid by HPTLC	2.92 ± 0.02 %	3.62 ± 0.05%	3.67 ± 0.04%	1.52 ± 0.03%

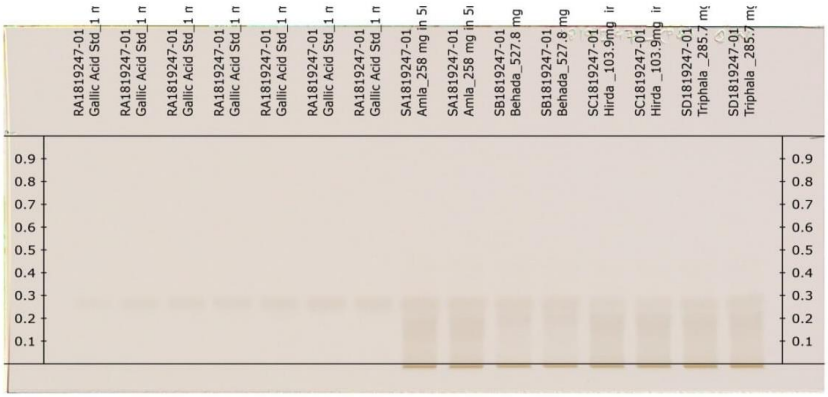
The macroscopic observation of TC reveals a reddish-brown color with a pungent odor and spicy, pungent taste. The Amalki pulp appears grayish-black, the epicarp of Vibhitki is grayish-brown, and the epicarp of Haritki exhibits a yellowish-brown color. The taste of Amalki is generally sour, while that of the other ingredients is astringent.

The physical properties of TC, such as bulk density, tapped density, and angle of repose, were measured as 0.4 ± 0.02 g/mL, 0.5 ± 0.04 g/mL, and 25 ± 1 degrees, respectively, indicating good flow properties. These flow properties were further confirmed by the Hausner ratio and Carr's index values, which were found to be 1.25 ± 0.05 and 20 ± 1%, respectively, for TC.

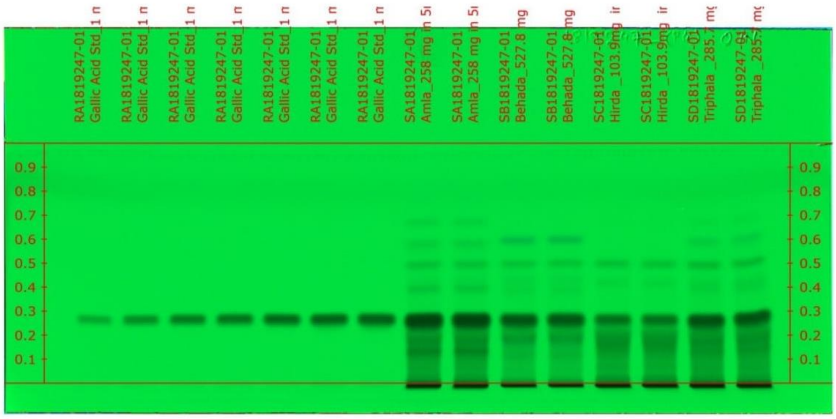
The physicochemical properties of TC, AP, HE, and VE are summarized in Table 1. The moisture content of TC, AP, HE, and VE falls within the acceptable range (3–9%), ensuring their stability during storage and protection against microbial degradation. The total ash values for TC, AP, HE, and VE were measured as 6.33 ± 0.05%, 4.86 ± 0.03%, 4.43 ± 0.04%, and 5.76 ± 0.06%, respectively, indicating the presence of inorganic components. The acid-insoluble ash value of TC (1.93%) indicates the presence of a small amount of acid-insoluble substances such as silica or sand. Moreover, the extractive values of TC, AP, HE, and VE were higher in water compared to alcohol.

The phytochemical assay was conducted using an HPTLC method for quality and quantitative analysis, with Gallic acid serving as the standard sample. HPTLC fingerprinting and quantitative analysis are effective methods for quality control in Ayurvedic medicine. Gallic acid, being a major phytoconstituent in TC, AP, HE, and VE, was used as a reference for chromatographic quantification. An optimized mobile phase (Chloroform:Acetone:Formic acid, 7:2:1 *v*/*v*/*v*) was employed to separate the phytoconstituents present in TC, AP, HE, and VE. The concentrations of Gallic acid in TC, AP, HE, and VE were determined using an HPTLC densitometer at 281 nm, yielding concentrations of 2.96 ± 0.02%, 3.62 ± 0.05%, 3.67 ± 0.04%, and 1.52 ± 0.03%, respectively. The fingerprinting at 252 nm, 366 nm, and 514 nm is shown in Figure 2, while the chromatogram of TC, AP, HE, and VE at the same wavelengths is shown in Figure 3.

RWhite



R254



R366

Figure 2. Fingerprinting of Triphala in white light,254nm, and 366nm

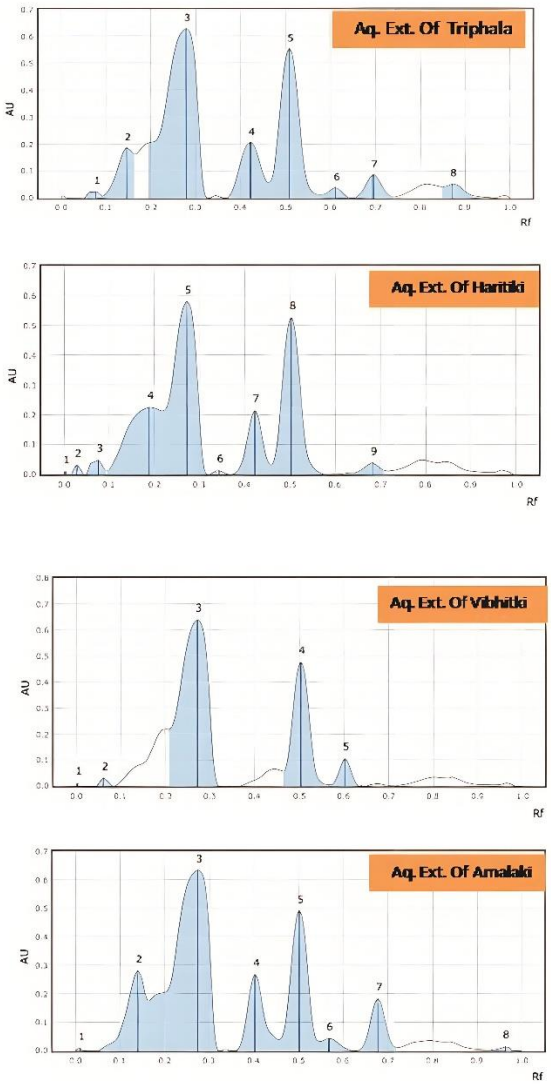


Figure 3. Chromatogram of TC, AP, HE and VE at 252, 366 and 514 nm.

5. Discussion

Ayurvedic medicine often combines herbal, mineral, and occasionally animal-based substances. Triphala Churna (TC), specifically, comprises three herbal drugs. Multiple manufacturers produce TC to treat various illnesses, but the lack of standardized parameters results in varying quality across the industry. Therefore, ensuring quality assurance for these products and their ingredients is crucial. Quality assurance is essential to guarantee consistent and reproducible medicine. TC, an Ayurvedic rasayana formulation, serves as a mild laxative for constipation and irritable bowel syndrome. Its antioxidant properties also enhance immunity and act as a detoxifying agent, making it a component in nearly 219 other Ayurvedic formulations. Thus, ensuring the qualitative standardization of these methods is necessary for safety and quality.

The World Health Organization (WHO) and India's Pharmacopeia Commission are working to establish quality standards for Ayurvedic formulations and their ingredients, addressing the challenge of standardization in this article. The organoleptic characteristics are important for identifying and ensuring the quality of TC, as they help detect adulteration, contamination, and spoilage. TC typically exhibits a reddish-brown color, but at times, it may darken to a dark brown shade due to variations in the quality or species of the herbal ingredients, namely AP, HE, and VE [11,12].

The physical characteristics of Churna include flowability, compressibility, density, and mechanical strength. Bulk density reflects the packing of particles, while the angle of repose indicates the powder's flowability and interparticle cohesion. Hausner ratio describes powder flow properties based on interparticle friction. According to Ajazuddin et al., a Hausner ratio less than 1.25 indicates good flow properties, whereas a ratio greater than 1.25 suggests poor flow [13]. The results of TC indicated good flow properties, which are crucial for the absorption and efficacy of medicines

The physico-chemical tests confirm the quality, safety, and stability of TC. Parameters such as loss on drying were observed and found to comply with ICH guidelines. No degradation was observed during the study, demonstrating the product's safety and efficacy [14,15] value and acid insoluble ash value serve as criteria to identify the purity and quality of the Ayurvedic formulation. The low acid-insoluble ash indicates minimal adulteration, such as silica or rice husk, ensuring effective absorption in the gastrointestinal tract (GIT). The water-soluble extractive was higher than the alcohol-soluble extractive, suggesting that water is a more efficient solvent for Triphala than ethanol. These values are detailed in Table 1. The correlation between pH and microbial contamination was studied suggested that a neutral or alkaline pH favors higher microbial contamination in herbal preparations [16–18].

HPTLC is crucial for qualitative and quantitative estimation of phytochemical constituents in herbal drugs and their formulations, making it vital for quality standardization [19]. TC and its ingredients contain Gallic acid, an important phytochemical easily isolated from plants, used for identification, quality control, and assay purposes. The fingerprinting of TC, AP, HE, and VE is shown in Figure 2, while their chromatograms is shown in Figure 3. Gallic acid was identified using a standard reference, and Scanner 4 quantified its percentage in TC, AP, HE, and VE. This HPTLC method serves as a valuable tool for qualitative and quantitative assays of these ingredients, playing a crucial role in their quality assessment [20].

6. Statistical Analysis

The statistical analysis applied in this work was conducted using SPSS 17 (SPSS Inc., Chicago, IL, USA). The results are presented as mean ± SD (n = 3).

7. Conclusion

Ayurvedic medicines require careful attention to manufacturing processes and quality control parameters to ensure their quality and safety. The present study focuses on developing quality control and assay methods for TC and its ingredients. This includes organoleptic and microscopic characterization, physicochemical properties, physical properties, and phytochemical qualitative and quantitative assays using HPTLC. Additionally, HPTLC densitometry proves useful for assaying Ayurvedic medicines with applied standard references.

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Article

Evaluation of *Terminalia arjuna* Bark Powder Supplementation on Isoprenaline-Induced Oxidative Stress and Inflammation in the Heart of Long Evans Rats, Understanding the Molecular Mechanism of This Old Medicinal Plant

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Abstract: This study was conducted to determine the effect of *Terminalia arjuna* bark powder supplementation on the oxidative stress of the cardiovascular system. Isoprenaline (ISO) was administered to the rats to develop the cardiac hypertrophy and myocardial infarction (MI). *Terminalia arjuna* bark powder was mixed with the food powder and provided for two weeks. At the end of the experiment, all rats were sacrificed and tissue samples were collected. ISO administration in rats increased the oxidative stress markers such as malondialdehyde (MDA), nitric oxide (NO), advanced oxidation protein product (APOP), and myeloperoxidase (MPO) in plasma and heart. *Terminalia arjuna* bark powder lowered the MDA, NO, and AOPP concentration level in ISO administered rats. Additionally, *Terminalia arjuna* restored the antioxidant enzymes (catalase and SOD) activities. Gene expression of antioxidant enzymes and inflammatory markers in the heart were studied. *Terminalia arjuna* restored Nrf-2, HO-1, HO-2, catalase, SOD, and GPx gene expression in the heart of ISO administered rats. ISO induced increased transcription levels of inflammatory genes such as IL-1, IL-6, TNF-α, TGF-β, iNOS, and NF-κB, which were decreased by *Terminalia arjuna* bark powder. Histopathology was checked and hematoxylin and eosin and Sirius red staining were performed on heart sections. ISO administration resulted in mononuclear cells infiltration and collagen deposition in the heart which were lowered by *Terminalia arjuna* bark powder. In conclusion, this study suggests that the *Terminalia arjuna* bark powder alleviated the oxidative stress by restoring the antioxidant genes and prevented the increase in inflammatory markers in the heart of ISO administered rats.

Keywords: *Terminalia arjuna*; isoprenaline; catalase; superoxide dismutase; oxidative stress

1. Introduction

The term “cardiovascular disease” (CVD) encompasses a wide range of conditions affecting the heart and blood vessels [1]. The development and progression of cardiovascular disease have been linked to inflammation and increased oxidative stress [2]. Oxidative stress may be a result of the elevated generation of oxidants or the weakened cellular defense systems. Lipid peroxidation is one of several oxidative consequences caused by forming a strong oxidant close to cell membranes. When oxygen combines with peroxyl radicals, lipid hydroperoxidation, membrane disruption, and the production of toxic compounds like malondialdehyde (MDA) occur [3]. Basic defense systems have evolved in organisms to counteract reactive oxygen species (ROS) production and injury. Superoxide dismutase (SOD), catalase, and glutathione are the detoxification systems found to be most active in the heart [4–6]. An acute state of myocardial necrosis, myocardial infarction (MI), is brought on by a disparity between coronary blood supply and myocardial demand [7]. The role of inflammation in mediating the damage to heart tissue following an ischemia event is critical. By entering the infarcted zone, neutrophils can potentially release proteolytic enzymes and produce ROS that can damage cardiac cells [8]. The pro-inflammatory cytokines interleukin-1 (IL-1) and interleukin-6 (IL-6) have been shown to be elevated when the myocardium is injured or triggered by adrenergic receptors. After myocardial infarction, pro-inflammatory cytokines and other cytokines

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like transforming growth factor-β (TGF-β) have been linked to the onset of tissue repair and wound healing [9]. However, increased ROS, ILs and TGF-β may trigger fibrosis development in the myocardium and pose potential threat for cardiac failure [10]. Isoprenaline (ISO), a β-adrenoceptor agonist, has been shown to cause myocardial infarction (MI) when administered in large doses. This is because isoprenaline undergoes auto-oxidation, releasing highly cytotoxic free radicals known to initiate the peroxidation of membrane phospholipids, severely damaging the cardiac membrane. Thus, ISO is commonly used in the development of animal models in rats since it causes MI in rats [11,12].

Prolonged inflammation may reduce blood antioxidant levels due to the chronic generation of increased ROS. Antioxidants can prevent cardiovascular disease by neutralizing reactive oxygen species (ROS) [13]. A previous report showed that antioxidant rich ratmontchi (*Flacourtia indica*) fruits extract may protect the heart in ISO induced oxidative stress in rats [14]. It has been observed that antioxidant treatment can prevent heart injury in rats by lowering lipid peroxidation and by improving antioxidant enzymes [15]. *Terminalia arjuna*, also known as arjun, is a member of the *Combretaceae* family and used as a putative cardioprotective agent. The Indo-sub-Himalayan regions of Uttar Pradesh, Southern Bihar, Chota Nagpur, Burma, Madhya Pradesh, Delhi, and the Deccan area are home to the arjuna tree, a tall deciduous tree that may grow to a height of 60–80 feet and is often found beside rivers, streams, and dry water bodies. Sri Lankan and Mauritian woods are also home to this species [16]. *Terminalia arjuna'*s bark, leaves, and fruits have been utilized in traditional medicine to treat a variety of ailments [17]. The *Terminalia arjuna* tree is revered in India for its medicinal bark, which has been utilized there for thousands of years [18]. The inotropic effect of *Terminalia arjuna* is thought to originate from the saponin glycosides present in the plant. In contrast, the flavonoids/phenolics are thought to provide antioxidant and vascular stimulating activity, validating the multifaceted role in cardio-protection. Triterpenoids are chemical ingredient present in the *Terminalia arjuna* bark, which are responsible for the cardio-active effects [19,20]. According to previous research, the bark has a substantial inotropic and hypotensive effect, implying the improvement of coronary blood flow and shields the heart from ischemic damage. Mild diuretic, hypolipidemic, antithrombotic, and prostaglandin E-enhancing effects have also been detected [21]. Ischemic mitral regurgitation (IMR) is a major cause of morbidity and mortality after an acute myocardial infarction. In healthy volunteers under the age of 70, administration of the arjun bark powder resulted in a decrease in IMR, an increase in early echocardiographic phases and late atrial phase of ventricular filling ratio (E/A) ratio, and a decrease in anginal frequency [22]. The production of reactive oxygen species is critical in the development of several illnesses. Moderate free radical scavenging activity was shown with arjungenin, and its glucoside, both of which were isolated from *Terminalia arjuna* stem bark. The antioxidant capacity of arjungenin was evaluated using the 1-1 diphenyl-2-picrylhydrazyl (DPPH) assay [23]. In response to lipopolysaccharide stimulation of macrophages, terminoside A, an oleanane triterpene isolated from the acetone fraction of the ethanolic extract of *Terminalia arjuna* stem bark, has been shown to impede nitric oxide (NO) generation and reduce inducible nitric oxide synthase (iNOS) levels. Atherosclerosis, heart failure, ischemic cardiomyopathy, and myocardial necrosis have all been linked to increase NO production by nitric oxide synthase [24]. An increase in coronary blood flow was seen after injecting an aqueous bark extract into an isolated rabbit heart preparation [25]. Arjuna bark extract has been shown to lower both total cholesterol (TC) and triglyceride levels in prior animal studies [26]. Increased hepatic clearance of cholesterol, down-regulation of lipogenic enzymes, and inhibition of HMG-CoA reductase are postulated to cause the hypolipidemic effect [27]. Research into the effects of the bark extract on cardiac and hepatic LPO in albino rats revealed a possible role on thyroid hormones (suppression of thyroid function) in the improvement of these symptoms [28]. However, there was a lack of investigation on the impact of *Terminalia arjuna* bark extract on cardiac dysfunction due to oxidative stress. In light of this literature, this experiment examined the cardioprotective effect of *Terminalia arjuna* bark powder supplementation in ISO administered rats.

2. Methods and Materials

2.1. Chemicals and Reagents

Creatine kinase- Muscle Brain (CK-MB), creatinine, and uric acid kits were purchased from Clinchem (Budapest, Hungary). Di-sodium hydrogen phosphate, sodium dihydrogen phosphate, sodium chloride, potassium chloride, glacial acetic acid, thiobarbituric acid, and dimethyl sulfoxide were collected from LOBA Chemie (Mumbai, India). Ethanol, xylene, and formaldehyde were obtained from Merck (Darmstadt, Germany). Isoproterenol was bought from Sigma Aldrich (St. Louis, MO, USA). Tri-sodium citrate, sulfanilamide, N-(1-Naphthyl) ethylenediamine, potassium iodide, hydrogen peroxide, DTNB-Ellman's reagent or (5, 5′-dithiobis-(2-nitrobenzoic acid), hydrochloric acid (HCl), *o*-dianisidine and pentobarbital were also obtained from Merck (Darmstadt, Germany). Adrenaline was obtained from Incepta Pharmaceuticals Limited, Bangladesh.

2.2. Plant Materials

The arjun bark was collected from the North South University campus and authenticated by the expert of Bangladesh National Herbarium, Mirpur, Dhaka. A voucher specimen was preserved for future reference (DACB-99123). The bark was cut into small pieces and dried in the open air. The dried bark was then grinded into coarse powder. This powder was supplemented with food to treat the ISO administered rats.

2.3. Animals Used for Experiments

Long-Evans male rats were used in this investigation. There were 24 rats, divided into four groups. The rats were about eight weeks old and weighed between 180 and 190 g. All rats were obtained from North South University's animal house facilities, which is part of the Department of Pharmaceutical Sciences. Each rat was housed in a standard isolated closet for a 12-h day/night cycle, in an air conditioned room (25 °C, 45% humidity). Standard chow diet and tap water supply were scrupulously maintained throughout the experiment. The Institutional Animal Use Ethical Committee (IACUC) approved the study protocol (Approval number—2024/OR-NSU/IACUC/0104).

2.4. Treatment Protocols

For this experiment, four groups were made: The control group, the ISO group (given 50 mg/kg ISO), the Control + arjun group (received arjun powder 2.5% in chow food, *W*/*W*), the ISO + arjun group (given 50 mg/kg ISO and received arjun powder 2.5% in chow food, *W*/*W*). Body weight was recorded on a daily basis, and the final body weight was reported on the 14th. On the day 14th, all rats were sacrificed with a high dose of pentobarbital (approximately 90 mg/kg), administered into the peritoneal region of each rat. Following the sacrifice, organs (heart and kidney) were taken and the moist weight of the organs was recorded. There were three sections for analysis: one for histology purposes, one for biochemical purposes and the other part was for gene expression analysis. The tissues for the histological sections were stored in neutral buffered formalin (pH 7.4), while the tissues part for biochemical analysis was frozen at −20 °C in a refrigerator. The third part was preserved carefully for mRNA extraction, at −80 °C in a refrigerator. Plasma was also extracted from blood at 4000 rpm for 15 min before being transferred to 1.5 mL microcentrifuge tubes and frozen at −20 °C for future experiments.

2.5. Heart Tissue Processing

Heart tissue was chosen for homogenization, therefore phosphate buffer with a pH 7.4, was used. These tissues were centrifuged at 8000 rpm, 4 °C for 15 min. The supernatants from the tubes were collected and preserved at −20 °C for further analysis. Clear supernatant was used to determine enzymatic activities and protein assays.

2.6. Oxidative Stress Markers Assay Procedures

2.6.1. Lipid Peroxidation Assay as Malondialdehyde (MDA)

Malondialdehyde (MDA) was determined to assess lipid peroxidation in plasma and tissues using the previously published method [29,30]. A standard curve was prepared and the MDA unit was stated as nmol/g tissue.

2.6.2. Nitric Oxide Assay in Plasma and Tissue

The level of nitric oxide (NO) was determined by measuring nitrate, and a previously reported method was used [29,30]. The absorbance of solutions at 540 nm was measured in comparison to a blank solution. The NO level was expressed in nmol/g tissue and was measured using a standard curve.

2.6.3. Advanced Oxidation Protein Product (AOPP) Assay

The degree of advanced oxidation protein product (AOPP) was evaluated using the previously described method by Sagor et al. (2015) and Ulla et al. (2017) [29,30]. In the experiment, chloramine-T standard was served in various concentrations as a positive control, and a negative control was represented by 0.2 mL acetic acid and 2 mL phosphate buffer saline. The absorbance of chloramine-T was measured at 340 nm and ranged from 0 to 100 nmol/mg. As a result, the APOP concentration unit was given as nmol mg^{-1} chloramine-T equivalents.

2.6.4. Myeloperoxidase (MPO) Activity Assay in the Heart Tissue

Myeloperoxidase (MPO) activity was measured using dianisidine-H_2O_2 system in 96-well plates [31]. For this test, three chemicals were used: H_2O_2, *o*-dianisidine dihydrochloride, and potassium phosphate buffer. The reagent amounts were as follows: potassium phosphate buffer was about 50 mM with a pH of 6, H_2O_2 was around 0.15 mM, and *o*-dianisidine dihydrochloride was about 0.53 mM. MPO absorbance was measured at 460 nm. The MPO unit was represented as U/min/mg protein.

2.7. Antioxidant Enzyme Activity Analysis

2.7.1. Catalase (CAT) Activity Assay

Catalase (CAT) activity assay protocol was explained in detail in previously published literature by Sagor et al. (2015) and Ulla et al. (2017) [29,30]. One unit of catalase activity is defined as a change in absorbance of 0.01 and expressed as units per minute.

2.7.2. Superoxide Dismutase (SOD) Activity Assay

To execute superoxide dismutase assay, the previously reported method was used [29,30]. The plasma and homogenized heart and renal tissue supernatant was used in this SOD activity assay. The capability of inhibition of epinephrine auto-oxidation was measured which was expressed as unit/mg and 50% inhibition is defined as one unit of enzyme activity.

2.8. Uric Acid and Creatinine Level Determination in Plasma

To analyze uric acid and creatinine level in plasma, the corresponding assay kits were used. All assay methods were carried out in accordance with the manufacturer's supplied instructions. The absorbance of uric acid was measured at 505 nm while creatinine absorbance was determined at 490 nm.

2.9. Analysis of Inflammation Regulatory Genes and Relative Oxidative Stress Levels by Quantitative Real Time Polymerase Chain Reaction (qRT-PCR)

The heart left ventricular tissues were used for the extraction of mRNA for qRT-PCR analysis using a previously published procedure [14]. Total mRNA isolation, cDNA synthesis, and qRT-PCR were done to measure the relative mRNA expression for inflammation and oxidative stress related genes. With the primer 3 online software, forward and reverse primers were designed and employed in this study. The genes and primers are given in Table 1. To standardize the relative transcript levels of each target gene, the housekeeping gene β-actin was employed.

Table 1. The forward and reverse sequence of the primer applied in qRT-PCR.

Name of gene	Type	Sequence
Nrf-2	Forward	5′-CCC AGCACA TCC AGACAGAC-3′
	Reverse	5′-TATCCAGGGCAAGCGACT C-3′
Heme oxigenase-1 (HO-1)	Forward	5′-TGCTCGCATGAACACTCTG-3′
	Reverse	5′-TCCTCTGTCAGCAGTGCCT-3′
Heme oxigenase-2 (HO-2)	Forward	5′-CACCACTGCACTTTACTTCA-3′
	Reverse	5′-AGTGCTGGGGAGTTTTAGTG-3′
MnSOD	Forward	5′-GCTCTAATCACGACCCACT-3′
	Reverse	5′-CATTCTCCCAGTTGATTACATTC-3
Catalase	Forward	5′-ATTGCCGTCCGATTCTCC-3′
	Reverse	5′-CCAGTTACCATCTTCAGTGTAG-3′
Glutathione peroxidase (GPx)	Forward	5′-GGGCAAAGAAGATTCCAGGTT-3′
	Reverse	5′-GGACGGCTTCATCTTCAGTGA-3′
IL-1	Forward	5′-ATGCCTCGTGCTGTCTGACC-3′
	Reverse	5′-CCATCTTTAGGAAGACACGGGTT-3′
IL-6	Forward	5′-AGCGATGATGCACTGTCAGA-3′
	Reverse	5′-GGTTTGCCGAGTAGACCTCA-3′
TNF-α	Forward	5′-ATGTGGAACTGGCAGAGGAG-3′
	Reverse	5′-CCACGAGCAGGAATGAGAAGAG-3′
TGF-β	Forward	5′-AAGAAGTCACCCGCGTGCTA-3′

	Reverse	5′-TGTGTGATGTCTTTGGTTTTGTC-3′
iNOS	Forward	5′-TGGTCCAACCTGCAGGTCTTC-3′
	Reverse	5′-CAGTAATGGCCGACCTGATGTTG-3′
NF-κB	Forward	5′-TGTGAAGAAGCGAGACCTGGAG-3′
	Reverse	5′-GGCACGGTTATCAAAAATCGGATG-3′
®-Actin	Forward	5′-GCGAGAAGATGACCCAGATC-3′
	Reverse	5′-GGATAGCACAGCCTGGATAG-3′

2.10. Histopathological Staining of the Heart of ISO Administered Rats

Initially, heart tissues were fixed in neutral buffered formalin. After being fixed, all of the tissues were embedded in paraffin. Following that, these tissues were sectioned at 5 μm with a rotary microtome, and the sliced sections were saved for staining with hematoxylin and eosin and Sirius red. Hematoxylin and eosin staining was used to reveal inflammatory cells in the heart. On the other hand, Sirius red staining was employed to assess the level of collagen deposition in the heart. After staining, photographs were taken with a Zeiss Axioscope microscope at 40× magnification.

2.11. Statistical Analysis

The mean ± standard error of the mean (SEM) was used to express all values. The results were calculated and determined using the graph pad prism program (Version 9). One-way ANOVA was used for statistical analysis and Tukey test was used as a post hoc test for multiple comparisons among the groups involved in this study. A significance level of $p < 0.05$ was adopted for all results.

3. Results

3.1. Effect of Terminalia arjuna Bark Powder on Total Heart, Left Ventricle (LV), Right Ventricle (RV) and Kidneys Wet Weight in ISO Administered Rats

In all groups of rats, ISO administration significantly increased the heart weight in rats compared to the control rats ($p < 0.05$). The result of this study demonstrates that the *Terminalia arjuna* bark powder decreased the wet weight of the entire heart (total heart weight) ($p < 0.001$) in ISO administered rats (Figure 1A). However, the wet weight of the left ventricle was not lowered in ISO administered rats by the *Terminalia arjuna* bark supplementation (Figure 1B). The heart and LV weights in control+ *Terminalia arjuna* group was not significantly impacted by the *Terminalia arjuna* bark powder supplementation (Figure 1). The kidney wet weight in Control, ISO, Control + *Terminalia arjuna* and ISO+ *Terminalia arjuna* group of rats were not significantly altered by the ISO administration as well as *Terminalia arjuna* bark supplementation (Figure 1C).

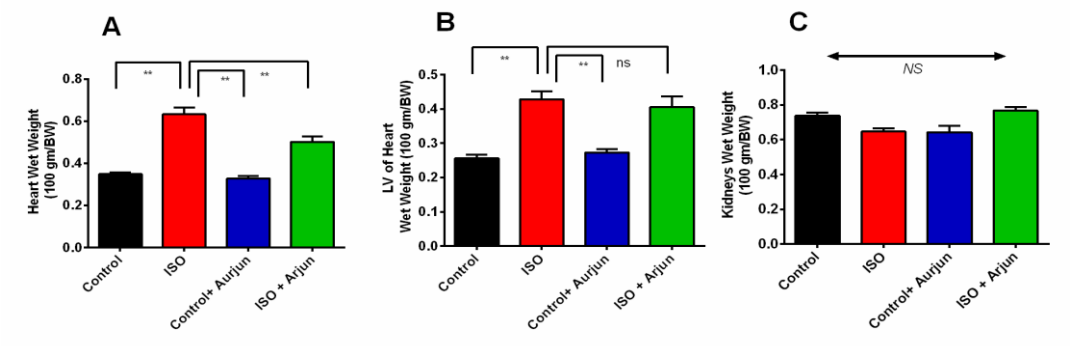

Figure 1. Effect of *Terminalia arjuna* bark powder supplementation on total heart (**A**), left ventricular (**B**) and kidney wet weights (**C**) in ISO administered rats. Data are expressed as mean± standard error of mean, $n = 6$. Statistical analysis was done by One-way ANOVA followed by Tukey post hoc test for the comparison between the groups used in this study. Statistical significance was considered as $p < 0.05$ in all cases. ** sign means $p < 0.001$, ns—not significant.

3.2. Effect of Terminalia arjuna Bark Extract on MDA in Plasma, Heart of ISO Administered Rats

As a result of lipid peroxidation, the first parameter, called MDA, was found to be considerably higher in the plasma and heart of ISO administered rats than in the controls ($p < 0.001$) (Figure 2A,B). The ISO + *Terminalia arjuna* group showed considerably decreased MDA concentrations in the heart and plasma compared to the ISO group, ($p < 0.001$) (Figure 2A,B). The MDA levels in the plasma and heart did not change in the Control + *Terminalia arjuna* group compared to control rats (Figure 2A,B).

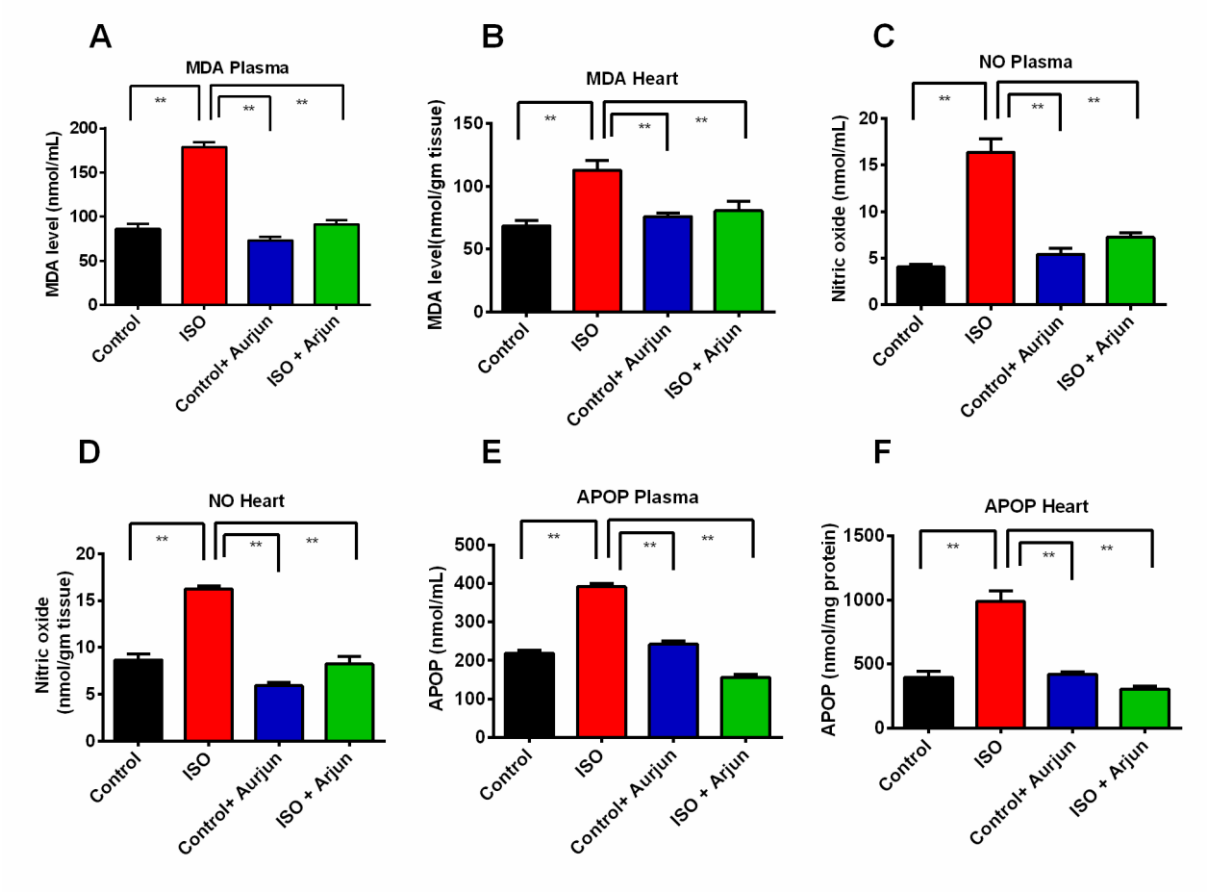

Figure 2. Effect of *Terminalia arjuna* bark powder supplementation on oxidative stress parameters such as MDA, NO and APOP level in plasma and heart of ISO administered rats. The picture represented the following data, MDA plasma (A), MDA Heart (B), NO plasma (C), NO heart (D), APOP plasma (E) and APOP heart (F). Data are expressed as mean ± standard error of mean, $n = 6$. Statistical analysis was done by One-way ANOVA followed by Tukey post hoc test for the comparison between the groups used in this study. Statistical significance was considered as $p < 0.05$ in all cases. ** sign means $p < 0.001$.

The next oxidative stress parameter measured was NO. When compared to the control group, the NO concentration in the heart and plasma was increased significantly ($p < 0.001$) in the ISO administered group (Figure 2C,D). The rats in the ISO + *Terminalia arjuna* group showed lower NO concentrations in the heart and plasma ($p < 0.001$) compared to the ISO administered rats. This data demonstrated that even after administering ISO, *Terminalia arjuna* bark powder may return the NO concentrations to normal level (Figure 2C,D). Moreover, in comparison to the control group, the Control + *Terminalia arjuna* did not alter the NO concentration in plasma, heart and kidneys ($p < 0.001$) (Figure 2C,D).

AOPP is another parameter of oxidative stress. The AOPP level in plasma and tissues was increased significantly in ISO administered rats compared to the control group (Figure 2E,F). *Terminalia arjuna* bark powder supplement in ISO administered rats showed significantly declined levels of AOPP in plasma, and heart ($p < 0.001$) compared to the rats which were only administered with ISO (Figure 2E,F). Control + *Terminalia arjuna* group did not show any changes in AOPP level in plasma, and heart compared to the control rats (Figure 2E,F).

3.3. Effect of Terminalia arjuna Bark Powder on SOD, Catalase and Glutathione in Plasma and Heart of ISO Administered Rats

The SOD enzymatic activity was significantly lowered in plasma and heart of ISO administered rats compared to the controls ($p < 0.001$) (Figure 3A,B). On the other hand, the *Terminalia arjuna* bark powder recovered the SOD enzyme activity considerably ($p < 0.001$) in the plasma and heart of ISO administered rats. This data suggests that *Terminalia arjuna* bark powder can enhance the SOD action in ISO administered rats (Figure 3A,B). In comparison to the control group, the plasma and cardiac SOD enzyme activities were not altered in the Control + *Terminalia arjuna* group ($p < 0.001$) (Figure 3A,B).

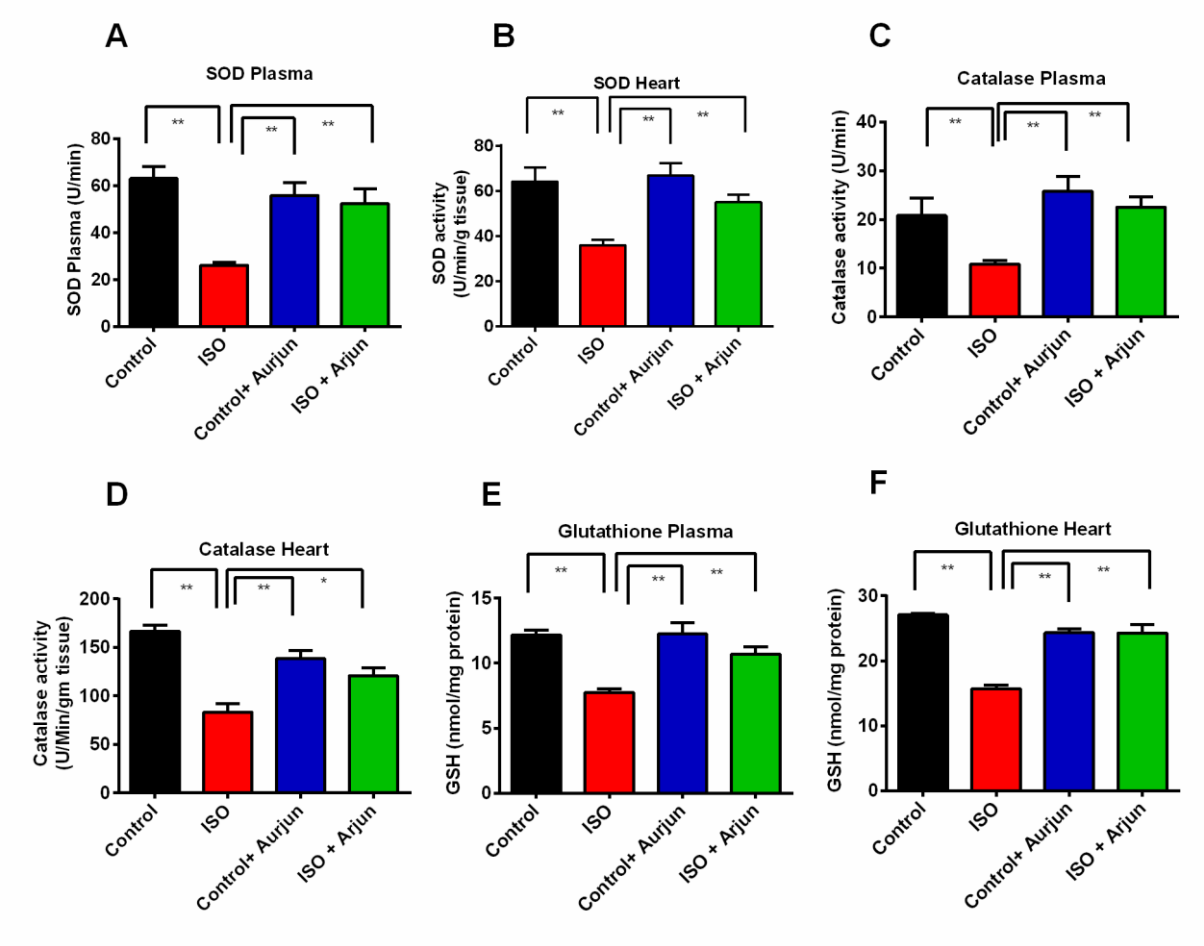

Figure 3. Effect of *Terminalia arjuna* bark powder supplementation on SOD and catalase activity and GSH concentration in plasma and heart of ISO administered rats. The picture represented the following data, SOD plasma (A), SOD Heart (B), catalase plasma (C), catalase heart (D), glutathione plasma (E) and glutathione heart (F). Data are expressed as mean ± standard error of mean, $n = 6$. Statistical analysis was done by One-way ANOVA followed by Tukey post hoc test for the comparison between the groups used in this study. Statistical significance was considered as $p < 0.05$ in all cases. * means $p < 0.05$ and ** sign means $p < 0.001$.

The ISO administered rats showed improvement of catalase activity in the plasma and heart compared to the controls ($p < 0.001$) (Figure 3C,D). Rats in the ISO + *Terminalia arjuna* group exhibited a considerable ($p < 0.001$) improvement of catalase enzyme activity in both plasma and the heart when compared to the ISO group (Figure 3C,D). The Control + *Terminalia arjuna* group also showed normal catalase enzyme activity in the heart and plasma (Figure 3C,D).

GSH is an additional indicator of antioxidant enzymes. The concentration of GSH in the heart and plasma was reduced significantly ($p < 0.001$) in ISO administered rats in comparison to the control group (Figure 3E,F). ISO administered rats given *Terminalia arjuna* bark powder supplement showed considerably ($p < 0.001$) restored plasma and cardiac GSH concentrations than the ISO group (Figure 3E,F). When compared to the control rats, the GSH level in the plasma and heart did not alter in the Control + *Terminalia arjuna* group (Figure 3E,F).

3.4. Effect of Terminalia arjuna Bark Powder Supplementation on CK-MB Activities in the Plasma and MPO Activity in the Heart of Isoprenaline (ISO) Administered Rats

Rats given ISO showed higher levels of CK-MB activities in the plasma ($p < 0.001$) when compared to the control group (Figure 4A). Rats treated with *Terminalia arjuna* in ISO administered rats showed significantly ($p < 0.001$) lower CK-MB activity in the plasma (Figure 4A).

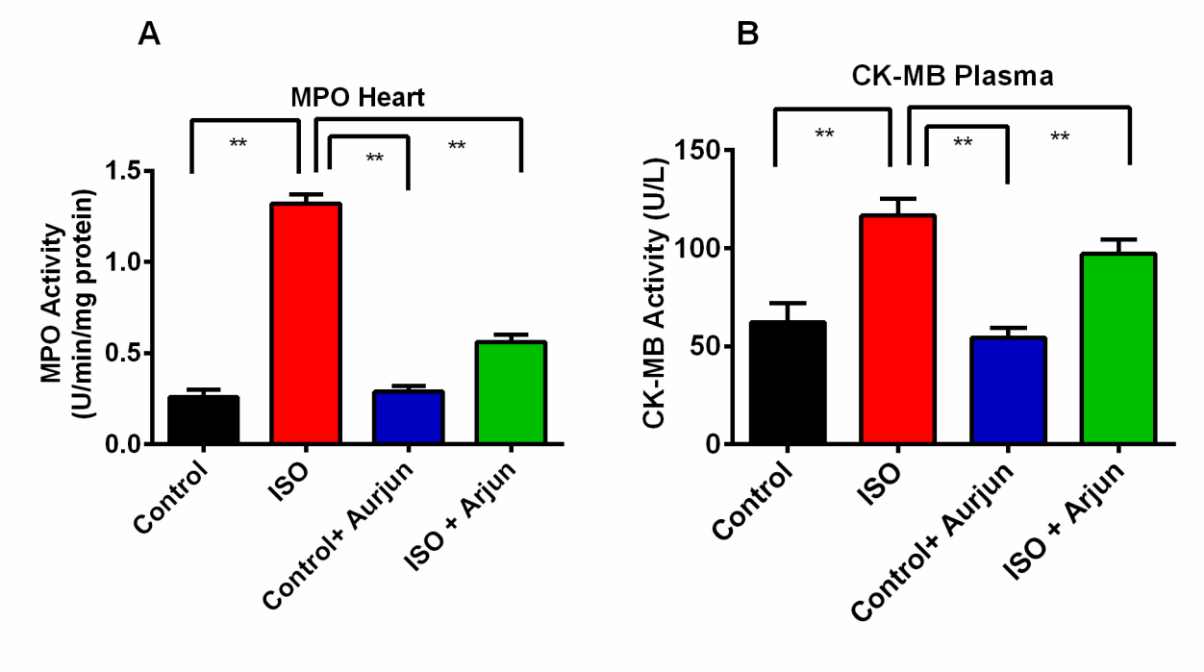

Figure 4. Effect of *Terminalia arjuna* bark powder supplementation on MPO and CK-MB activity in ISO administered rats. The picture represented the following data, MPO heart (A), and CK-MB plasma (B). Data are expressed as mean ± standard error of mean, $n = 6$. Statistical analysis was done by One-way ANOVA followed by Tukey post hoc test for the comparison between the groups used in this study. Statistical significance was considered as $p < 0.05$ in all cases. ** sign means $p < 0.001$.

ISO administration in rats resulted in significantly increased MPO activities in ISO administered rats significantly ($p < 0.05$) compared to control rats. *Terminalia arjuna* bark powder supplementation in ISO administered rats showed considerably decreased MPO activity ($p < 0.001$) than ISO administered rats (Figure 4B). *Terminalia arjuna* bark powder supplementation did not change the MPO activities in control+ *Terminalia arjuna* rats (Figure 4B).

3.5. Effect of Terminalia arjuna Bark Powder Supplementation on Antioxidant Genes Expression in the Heart of ISO Administered Rats

The ISO administered rats showed lower Nrf-2 transcript levels in the heart than control rats (Figure 5). *Terminalia arjuna* bark powder supplementation restored the Nrf-2 expression in the heart of ISO administered rats significantly (Figure 5). A considerable ($p < 0.001$) up-regulation of HO-1 and HO-2 transcript levels was also seen in ISO administered rats, which received the *Terminalia arjuna* bark powder supplementation (Figure 5). Moreover, ISO administered rats showed lower gene expression for endogenous antioxidant enzymes including SOD, catalase, and GPx compared to the control rats (Figure 5). Additionally, the *Terminalia arjuna* bark powder supplementation in ISO-administered rats resulted in a considerable restoration in the gene expression of those antioxidant enzymes (Figure 5).

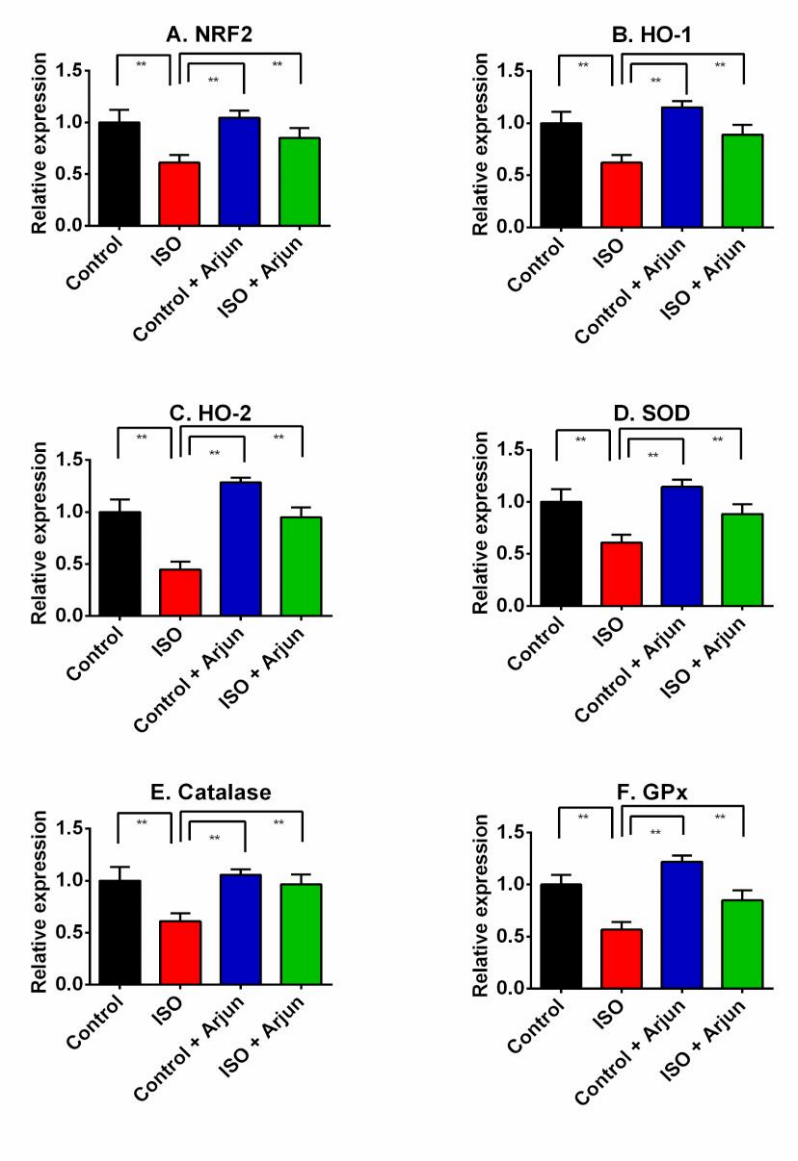

Figure 5. Effect of *Terminalia arjuna* bark powder supplementation on mRNA expression related antioxidants in the heart of ISO administered rats. The picture represented the following data, Nrf-2 (**A**), HO-1 (**B**), HO-2 (**C**), SOD (**D**), catalase (**E**) and GPx (**F**). Data are expressed as mean ± standard error of mean, $n = 6$. Statistical analysis was done by One-way ANOVA followed by Tukey post hoc test for the comparison between the groups used in this study. Statistical significance was considered as $p < 0.05$ in all cases. ** sign means $p < 0.001$.

3.6. Effect of Terminalia arjuna Bark Powder Supplementation on Inflammatory Genes Expression in the Heart of ISO Administered Rats

This study assessed the expression of six genes, including interleukin-1 (IL-1), interleukin-6 (IL-6), transforming growth factor beta-1 (TGF-β1), tumor necrosis factor alpha (TNF-α), nuclear factor kappa B (NF-κB), and inducible nitric oxide synthase (iNOS), that cause inflammation and fibrosis in the heart of ISO administered rats (Figure 6). The heart showed considerably ($p < 0.001$) higher levels of IL-1, IL-6, and TNF-α gene expression in ISO administered rats compared to the control rats (Figure 6). *Terminalia arjuna* bark powder supplementation in ISO-administered rats resulted in a lower level of these gene expression in the heart compared to the ISO administered rats (Figure 6).

Again, rats given ISO doses showed considerably higher expression of TGF-β1, iNOS, and NF-κB in the heart than the control group (Figure 6). *Terminalia arjuna* bark powder supplementation lowered the expressions of all these pro-inflammatory and inflammatory genes in the heart of ISO administered rats significantly ($p < 0.05$) (Figure 6).

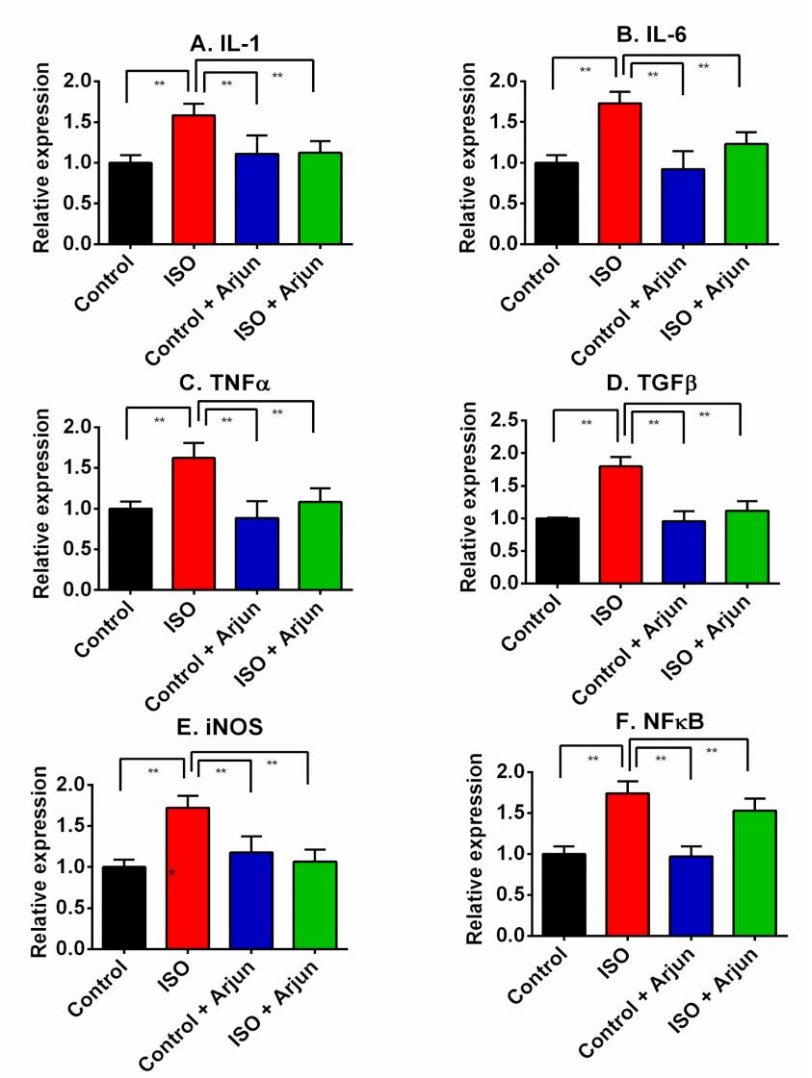

Figure 6. Effect of *Terminalia arjuna* bark powder supplementation on mRNA expression related to inflammation in the heart of ISO administered rats. The picture represented the following data, IL-1 (A), IL-6 (B), TNF-α (C), TGF-β (D), iNOS (E) and NF-κB (F). Data are expressed as mean ± standard error of mean, $n = 6$. Statistical analysis was done by One-way ANOVA followed by Tukey post hoc test for the comparison between the groups used in this study. Statistical significance was considered as $p < 0.05$ in all cases. ** sign means $p < 0.001$.

3.8. Effect of Terminalia arjuna Bark Powder Supplementation on Plasma Uric Acid and Creatinine Level in ISO Administered Rats

The plasma uric acid concentration was measured for every group present in this study. The uric acid concentration was increased significantly ($p < 0.001$) in ISO administered rats than that of the control group (Figure 7A). As compared to the ISO group, the uric acid plasma concentration was found considerably lower ($p < 0.001$) in rats, which received *Terminalia arjuna* bark powder supplementation (Figure 7A). The Control + *Terminalia arjuna* group did not exhibit any anomalies in plasma uric acid concentration (Figure 7A).

Moreover, the ISO administered rats showed higher levels of plasma creatinine concentration ($p < 0.001$) compared to the control rats (Figure 7B). *Terminalia arjuna* bark powder supplementation lowered the creatinine plasma levels significantly ($p < 0.001$) in the ISO + *Terminalia arjuna* group (Figure 7B). Creatinine plasma concentrations were found normal in the Control + *Terminalia arjuna* group compared to the control group (Figure 7B).

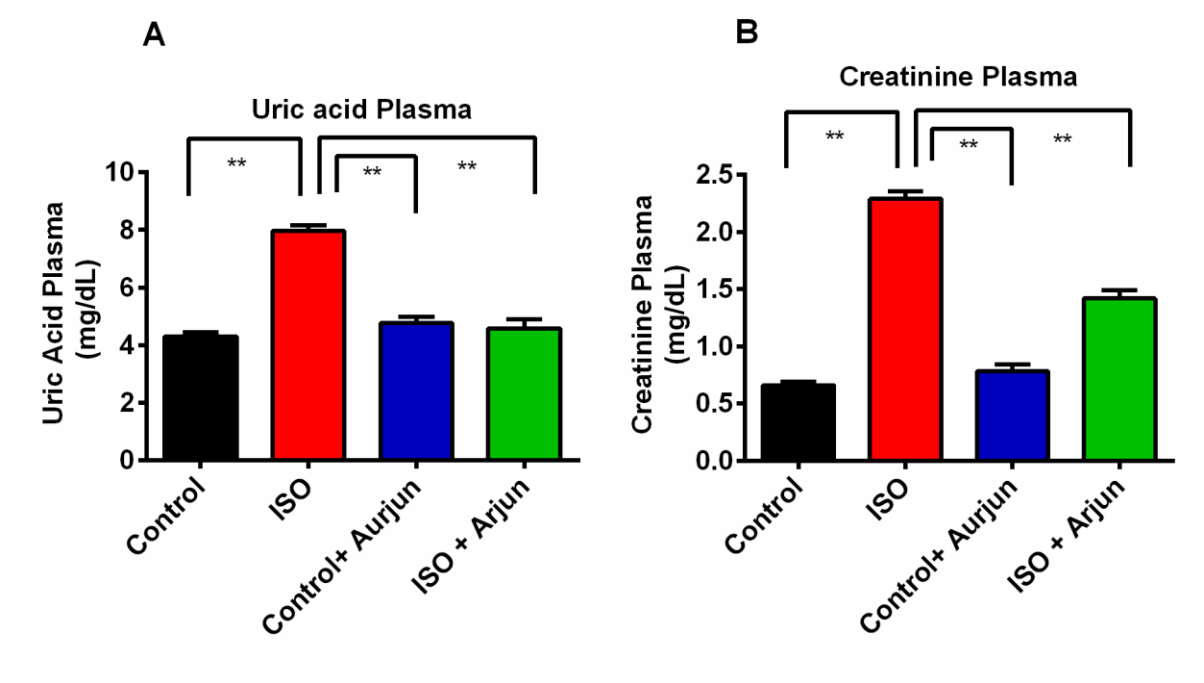

Figure 7. Effect of *Terminalia arjuna* bark powder supplementation on uric acid and creatinine level in plasma of ISO administered rats. The picture represented the following data, uric acid plasma (**A**) and creatinine plasma (**B**). Data are expressed as mean ± standard error of mean, $n = 6$. Statistical analysis was done by One-way ANOVA followed by Tukey post hoc test for the comparison between the groups used in this study. Statistical significance was considered as $p < 0.05$ in all cases. ** sign means $p < 0.001$.

3.9. Effect of Terminalia arjuna Bark Powder Supplementation on Histological Assessment in the Heart of ISO Administered Rats

To check the abnormalities of the cardiac tissues due to ISO administration, the hematoxylin and eosin (H and E) staining as well as Sirius red staining were performed. H and E staining revealed that ISO administered rats showed increased scar formation, inflammatory cells infiltration and myocytes hypertrophy in the heart compared to the control rats (Figure 8). *Terminalia arjuna* bark powder supplementation lowered these pathological changes in the heart of ISO administered rats (Figure 8). *Terminalia arjuna* bark powder supplementation did not produce any deleterious changes in the heart of control rats (Figure 8).

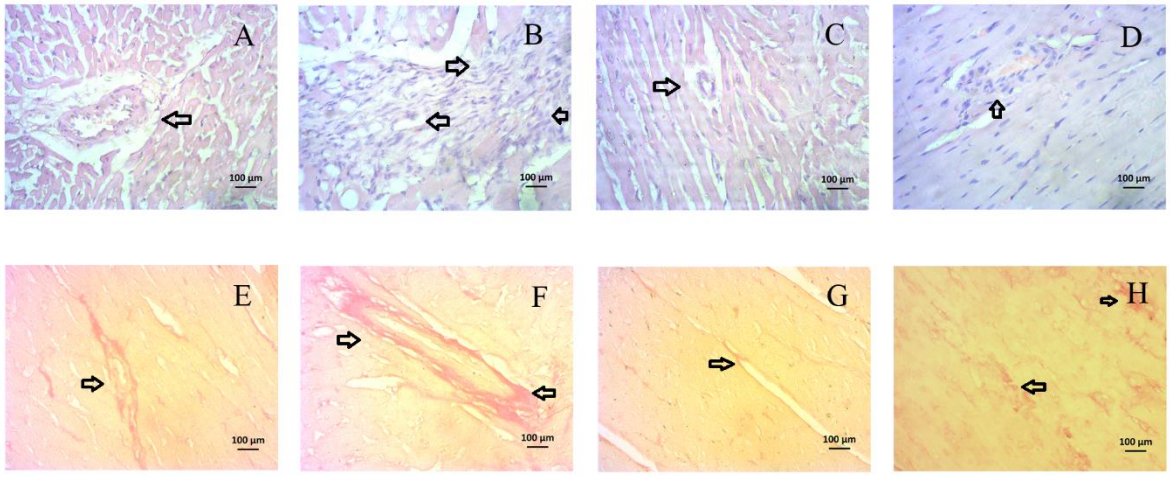

Figure 8. Effect of *Terminalia arjuna* bark powder supplementation on cardiac structure and fibrosis level in ISO administered rats. Upper panel Hematoxylin and eosin staining, (**A**) control; (**B**) ISO; (**C**) Control + *Terminalia arjuna*; (**D**) ISO + *Terminalia arjuna*. Lower panel- Sirius red staining, (**E**) control; (**F**) ISO; (**G**) Control+ *Terminalia arjuna;* (**H**) ISO + *Terminalia arjuna*. The arrow head showed the inflammatory cells infiltration and collagen deposition in the heart section.

Sirius red staining in the heart section demonstrated increased levels of collagen deposition and fibrosis in the left ventricle of ISO administered rats compared to the control rats (Figure 8). *Terminalia arjuna* bark powder supplementation decreased the collagen deposition and fibrosis in the heart of ISO administered rats (Figure 8). This staining also revealed that *Terminalia arjuna* bark powder supplementation did not alter the normal architecture of the heart (Figure 8).

4. Discussion

Oxidative stress is considered as a key regulator for the development of cardiac dysfunction in case of MI. This investigation showed that ISO administration in rats developed lipid peroxidation and oxidative stress in the heart. The antioxidant enzymes function also declined because of the ISO administration. *Terminalia arjuna* bark powder supplementation prevented the lipid peroxidation and improved the oxidative stress in ISO administered rats. Moreover, the inflammation and cardiac fibrosis was also prevented by the *Terminalia arjuna* bark powder supplementation.

Oxidative stress is particularly responsible for the tissue damage in the heart and therefore an efficient treatment is necessary to reduce this stress. MDA is a final product of oxidative stress in the tissues. In our experiment, ISO raised MDA concentration in tissues, which was decreased by *Terminalia arjuna* bark powder supplementation. This result is also consistent with the prior study showing that *Terminalia arjuna extract* reduced MDA levels due to its antioxidant activity [32]. Another marker related to oxidative stress is NO. In this study, ISO administration in rats raised NO concentration, which was lowered by *Terminalia arjuna* bark powder supplementation. In a prior investigation, rotenone was used to induce neurotoxicity accompanied by elevated NO level which was reduced with *Terminalia arjuna* extract therapy [33]. Severe oxidative stress may lead to the development of protein oxidation and generate dysfunctional essential proteins. AOPP is thus considered as another component of the oxidative stress. This investigation showed that ISO administration in rats caused increased AOPP levels in the plasma and tissues. This finding is supported by previous report suggests that ISO administration may increase the AOPP level in kidney tissues [34]. *Terminalia arjuna* bark powder supplementation reduces AOPP concentration in the plasma and tissues in ISO-administered rats.

Increased oxidative stress is a direct consequence of declined antioxidant enzymes function in the tissues. Several antioxidant systems are available to fight against the free radicals production and oxidative stress. Catalase and SOD activities are measured for assessing the antioxidant capacities in tissues. It was demonstrated that ISO reduced the antioxidant enzyme activities such as SOD and catalase [14,15]. Declined SOD and catalase could be a result of decreased gene expression in the tissues level. Previous reports suggest that SOD and catalase gene expression were found decreased in the heart of ISO administered rats [14]. *Terminalia arjuna* bark powder supplementation restored the SOD and catalase activities by stimulating the gene expression in the heart of ISO administered rats. These findings are in line with previous investigational report suggesting that *Terminalia arjuna* boosted antioxidant activity in lymphoma bearing AKR mice [35].

ISO administration in rats also developed cardiac damage and oxidative stress as discussed above. Significant hypertrophy and increased wet weight of heart in ISO administered rats were observed in this study. The cardiac damage is confirmed by the elevation of CK-MB activities in plasma of ISO administered rats. Previous reports also suggest that ISO administration develop MI like symptoms and cardiac damage which is evident by the raised CK-MB activities [14]. *Terminalia aurjuna* powder supplementation prevented the cardiac damage and normalized the CK-MB activities in plasma of ISO administered rats. Previous investigation showed that antioxidant rich plant extracts may prevent cardiac damage and lowered the CK-MB activities suggesting the cardiac protection of antioxidants [14]. *Terminalia aurjuna* also possess strong antioxidants which may be responsible for the cardiac protection in ISO administered rats.

Nuclear factor erythroid 2–related factor 2 (Nrf-2), heme oxygenase 1 (HO-1), and heme oxygenase 2 (HO-2), which are mostly related to oxidative stress, were all investigated in this study. The Nrf-2 gene senses the oxidative stress and enhances the expression of other antioxidant genes such as SOD, catalase and glutathione peroxidase (Gpx) etc. to counter the oxidative stress. Thus, decline expression of Nrf-2 may further jeopardize the antioxidant system in the tissues. In this study, ISO administration in rats showed declined Nrf-2 with its other regulator genes such as HO-1 and HO-2 [14]. This phenomenon can be seen in other studies showing that increased oxidative stress may lead to the decline of Nrf-2 expression [34,36]. In this study, *Terminalia arjuna* bark powder enhanced the Nrf-2 transcript levels in the heart, which were reduced by ISO administration. In this investigation, ISO reduced the transcript level of HO-1, which was regained by *Terminalia arjuna* bark powder supplementation. This report again supported by a previous investigation showed that *Terminalia arjuna* bark powder

supplementation boosted catalase, SOD, and GPx enzyme activities, which was exacerbated by the administration of N-nitrosodiethylamine [37].

Interleukin-1 (IL-1), interleukin-6 (IL-6), tumor necrosis factor alpha (TNF-α), inducible nitric oxide synthase (iNOS), transforming growth factor beta (TGF-β), and nuclear factor kappa B (NF-κB) are important inducers for inflammation and fibrosis in tissues. In this investigation, ISO administration raised IL-1 and IL-6 transcription factors, which were normalized by *Terminalia arjuna* bark powder supplementation. This finding is in consonance with the previous one showing that IL-6 was downregulated by the treatment of *Terminalia arjuna* extract [38]. TNF-α is another inflammatory agent that was raised by ISO administration. However, *Terminalia arjuna* bark powder supplementation decreased TNF- expression in the heart of ISO administered rats which was also supported by a previous study [32]. In this investigation, *Terminalia arjuna* bark powder supplementation also normalized the elevated levels of iNOS, TGF β, and NF-κB transcript levels in ISO administered rats. It is evident that *Terminalia arjuna* extract decreased iNOS in murine macrophages [39]. MPO, which serves as an important marker for inflammation in the tissues, is particularly present in the neutrophils. In this study, ISO boosted the MPO activities in the heart. *Terminalia arjuna* bark powder supplementation reduces increased MPO activity in rats given ISO. *Terminalia arjuna* extract prevented the rise of MPO activity in rotenone induced neurotoxicity [40].

Cardiac fibrosis is evident in case of oxidative stress and in ISO administered rats [36]. Inflammatory cell infiltration and inflammatory mediators may influence the cardiac fibroblast to increase the production of extracellular matrix proteins, mainly collagen [41]. The genes expression of IL-1, IL-6 and TNF-α are highly correlated with the production of cardiac fibrosis in MI and in ISO administered rats [42]. These inflammatory gene expressions may raise the TGF-β signaling which is the master regulator of fibrosis development and also cardiac hypertrophy [43]. Enlarged heart and fibrosis scar leads to a dysfunctional heart and ultimately turn into heart failure. In this study, histological assessment confirms that ISO administration may induce mononuclear cell infiltration in the heart and causes collagen deposition as a marker of fibrosis. *Terminalia arjuna* bark powder prevented the mononuclear cells infiltration and collagen deposition in the heart of ISO administered rats. This finding is supported by previous report showed that inflammatory gene expression and TGF-β signaling may jeopardize the cardiac function which may be modulated by *Terminalia arjuna* bark extract [44].

In this investigation, plasma creatinine and uric acid were also measured. ISO administration in rats raised plasma creatinine and uric acid levels. In this study, it was found that creatinine and uric acid levels were normalized/reduced in rats given ISO by *Terminalia arjuna* bark powder supplementation. These findings are correlated with a previous study reporting that *Terminalia arjuna* bark powder supplementation reduces both serum creatinine and uric acid levels in cyclosporine A- induced cardiotoxicity in rats [45].

5. Conclusions

This investigation revealed the protective effect of *Terminalia arjuna* bark powder in the heart of ISO administered rats through biochemical and histological assessment. The molecular mechanism of the protective effect in the heart of ISO administered rats were also elucidated in this study probably by suppressing the mediators of oxidative stress and inflammation. However, more clinical and preclinical research is warranted for elucidating the benefits in human. There should also be a toxicological assessment, required for the safety assurance of *Terminalia arjuna* bark use. Considering these aspects, it could be a future choice of medicine for the treatment of cardiac disorders associated with inflammation and oxidative stress.

Author Contributions: The concept and design of this study was generated by N.S. and A.A. A.A. also trained M.A., N.R., K.A., P.S., S.S. (Sumaia Sarif), K.F.M., I.J. and S.S. (Shahnaz Siddiqua) on all the research related activities and supervised and coordinated the whole study. M.A., P.S., N.R., and S.S. (Sumaia Sarif) carried out animal handling, animal experimentation and animal sacrifice. M.A., N.R., P.S., K.F.M., I.J. and S.L. also performed the biochemical analysis. K.A., S.S. (Sumaia Sarif), I.J. and S.S. (Shahnaz Siddiqua) performed the histological analyses. M.A., P.S., N.R., I.J. and F.K. performed the gene expression analysis. Statistical analysis and result interpretation were done by A.A., N.S., K.A. and F.K. The draft manuscript was prepared by N.S., A.A., K.A., and F.K., that was read and approved by all authors. All authors have read and agreed to the published version of the manuscript.

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Institutional Review Board Statement: The study was conducted according to the guidelines of the Declaration of Helsinki, and approved by the Institutional Review Board (or Ethics Committee) of North South University (2024/OR-NSU/IACUC/0104).

Informed Consent Statement: Not applicable

Data Availability Statement: All experimental data of this study are stored in the hard disk drive of laboratory computer which will be attainable upon request.

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Conflicts of Interest: The authors declare no competing interests regarding the publication of this paper.

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Article

Phytochemical Analysis of Bangladeshi Medicinal Plants Led to the Isolation of Anti-Staphylococcal Compounds

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Abstract: Antibacterial resistance is a major threat to global health. Due to its new resistance mechanisms, it is spreading and emerging widely, thereby threatening the treatment of common infectious diseases. Ancient history and ethnopharmacological studies highlighted the importance of natural sources in treating resistance infections. This study involved bioassay-directed phytochemical investigation on Bangladeshi medicinal plants selected by an ethnopharmacological survey to explore antibacterial compounds against Methicillin resistance *Staphylococcus aureus* (MRSA). In 2016, an ethnopharmacological survey conducted in Bangladesh led to the recommendation of 71 medicinal plants by 127 respondents (71 Ayurvedic/Unani practitioners, 21 Ayurvedic patients and 35 local inhabitants) for the treatment of infectious diseases. Based on the literature review, data analysis of the ethnopharmacological survey and ease of availability of the plants, 18 plants were initially selected and collected from Bangladesh. After the initial antibacterial screening of 18 plants, five plants with Minimum Inhibitory Concentration (MIC) of 32–512 µg/mL were chosen based on potential antibacterial activity. These are (*Zingiber montanum*, *Uraria picta*, *Diospyros malabarica*, *Cynometra ramiflora*, *Swertia chirayita*. Extensive phytochemical work using different chromatographic and spectroscopic techniques on five Bangladeshi medicinal plants led to the isolation and identification of 24 compounds. Eight terpenes (zerumbol (**3**), zerumbone (**4**), buddledone A (**5**), germacrone (**6**), furanodienone (**7**), (−) borneol (**1**)**,** camphor (**2**) and 8(17), 12-labdadiene-15, 16-dial) (**8**) were isolated from *Zingiber montanum* with the MIC (32– >128 µg/mL). Eugenol (**14**) and steroids were isolated from *Uraria picta* (MIC 64– >128 µg/mL). Lupane-type triterpenoids (Lupeol (**20**), betulin (**21**)**,** betulinaldehyde (**23**)**,** betulone (**24**) and messagenin (**22**) were isolated and identified from *Diospyros malabarica* with the MIC (64– >128 µg/mL), while pentacyclic triterpene (glutinol (**10**)**,** glutinone (**11**)), simple phenolic (ethyl 4-ethoxybenzoate (**9**)) and steroids were isolated from *Cynometra ramiflora* with MIC (64– >128 µg/mL). A series of xanthones (swerchirin (**16**), swertiaperenin (**17**), bellidifolin (**18**) and decussatin (**19**)) were identified from *Swertia chirayita* with MIC (>128 µg/mL). 4-ethoxybenzoate (9) and messagenin (22) were identified as new natural compounds among these compounds. In terms of activity, 8(17), 12-labdadiene-15, 16-dial (**8**) (32 µg/mL against ATCC 5941) and zerumbol (**3**) (32 µg/mL against EMRSA 15) exhibited potential antibacterial activity. Phytochemical discoveries of Bangladeshi medicinal plants gave a new dimension to exploring anti-staphylococcal compounds.

Keywords: antibacterial resistance; MRSA clinical strains; ethnopharmacological survey; phytochemistry; isolation; column chromatography; identification of chemical structure; NMR; mass spectrometry; medicinal plant extract

1. Introduction

The incidence of the number of bacterial pathogens bestowing antimicrobial and multidrug resistance to antibiotics has remarkably accelerated over the past few decades. The mishandling, misuse and abuse of antimicrobial agents are the main reasons for the emergence of the resistant genes in microorganisms [1]. Apart from manifestation of the antimicrobial agent, antimicrobial resistance may occur due to the mutations in bacterial DNA or the acquisition of bacterial resistance gene through horizontal gene transfer [2]. Antimicrobial resistance is now a serious and complex problem for global health, requiring a multi-disciplinary approach involving partners from all health sectors, including public health authorities and the scientific community.

Resistant infection has become the third leading cause of mortality worldwide [3]. The statistics indicate that around 33,000 people die each year in Europe due to AMR (antimicrobial resistance), and more than 670,000 people are affected with antimicrobial resistance infections [4]. AMR has made cancer chemotherapy, organ transplantation, diabetes management and major surgery like caesarean sections more challenging [2]. Unless

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appropriate action is taken to tackle the threat, drug-resistant infections will kill an extra 10 million people annually worldwide by 2050, which is more than the predicted death of cancer, and it will cost 100 trillion USD of the world's economic output [5].

Medicines from natural sources have contributed to humankind's pharmacy since antiquity; hence, plants may be a source for developing new novel antimicrobial compounds followed by subsequent pharmacological, chemical and clinical studies [6]. For example, the most prominent anti-inflammatory agent, acetylsalicylic acid, commercially known as the drug aspirin, is isolated from the bark of the willow tree *Salix alba* L. [7]. Quinine isolated from the bark of *Cinchona succirubra* has been used traditionally to treat malaria, indigestion, fever, mouth and throat diseases for centuries [6].

An ethnopharmacological survey is a strategy to select medicinal plants for scientific exploration of biologically active agents. An ethnopharmacological survey was conducted in Bangladesh during the autumn of 2016 to document indigenous knowledge regarding the treatment of infections. The aim was to identify plants that could be selected for the phytochemical investigation to identify vital secondary metabolites responsible for their anti-infective properties.

Based on the outcome of the ethnopharmacological survey [8] and the availability of plants,18 plants were initially chosen and collected from various parts of Bangladesh. Five plants were chosen for further phytochemical study based on the potential antibacterial activity of the plant extracts. The research involved finding potential anti-infective secondary metabolites from Bangladeshi medicinal plants using bioassay-directed chromatographic and spectroscopic methods. The author reported the isolation and identification of 24 pure compounds and their antibacterial activity against a panel of clinical isolates of methicillin-resistant *Staphylococcus aureus* (MRSA) strains.

2. Material and Methods

2.1. Ethnopharmacological Survey

The ethnopharmacological survey was carried out in Bangladesh during autumn 2016. A total of 127 respondents, including 71 practitioners, 21 Ayurvedic hospital patients, and 35 village inhabitants, participated in the survey [8].

2.2. Collection of Plant Materials

Eighteen plants (Section 3.1.) selected through the ethnopharmacological survey were collected from various parts of Bangladesh, including the Bangladesh National Botanical Garden (Mirpur, Dhaka), the Medicinal Plant Garden of Govt. Unani and Ayurvedic Hospital (Mirpur, Dhaka), and the Sundarbans mangrove forest (Khulna), Rajshahi University Botanical Garden (Rajshahi).

2.3. Drying and Grinding

All the samples were sun-dried (40 °C) for 2–3 days in Bangladesh. The selected plants' bark, stems, leaves, or fruits were the samples. After bringing the samples to the United Kingdom, they were sun-dried at the University of East London, United Kingdom, before being cut into pieces with a plant pruner and oven-dried at 30–40 °C for 30 min before grinding with a grinder. Subsequently, all the samples were ground into fine powders.

2.4. Extraction and Bioassay

Initially, small-scale (10–20 g) plant material was extracted sequentially with 80–100 mL solvents of increasing polarity (hexane, chloroform, and methanol), which offers efficient extractions for preliminary bioassay against MRSA clinical strains.

Each extract was subjected to antibacterial screening by microtiter assay using 96-well plates [9]. Five plants were selected for bioassay-guided phytochemical study to identify potential antibacterial secondary metabolites based on potential antibacterial activity against MRSA clinical strains (EMRSA-15, SA1199B, ATCC25941, XU212).

Large-scale extraction of 5 selected plants was completed with 230–300 g of plant material (Section 3.2.) with Soxhlet apparatus with solvents (approximately 800–900 mL) of increasing polarity Hexane < Chloroform < Methanol. Each extract was concentrated using a rotary evaporator under reduced pressure at a maximum temperature of 40 °C to afford crude extracts as semi-solid mass.

2.5. Fractionation of the Plant Extracts

Column chromatography with silica and Sephadex LH20, Vacuum liquid chromatography, SPE (Solid Phase Extraction) and preparative thin layer chromatography were used to isolate pure compounds. Thin layer chromatography and reagent 1% Vanillin in sulfuric acid were applied to monitor the fractions. The chromatography and combination of solvents used in isolating the compounds are listed in Tables S1–S6.

2.6. Identification of Pure Compounds

Isolated compounds were identified by 1D and 2D Nuclear Magnetic Resonance, High-Resolution Mass Spectrometry, and Infrared Spectroscopy.

2.7. Antibacterial Activity of the Pure Isolated Compounds

Antibacterial screening of the plant extracts and pure secondary metabolites was conducted using a minimum inhibitory concentration (MIC) assay against MRSA clinical strains.

Antibacterial screening was expressed in terms of μg/mL [9]. The antibacterial activities were evaluated against a panel of clinical strains of MRSA, including XU212, EMRSA15, SA1199B, MRSA 340702 and MRSA 24821, along with MRSA standard strain ATCC 25941. These efflux strains are resistant to common antibiotics such as SA1199B, which is resistant to fluoroquinolones, and XU212, which is resistant to tetracycline. The antimicrobial activities of the active compounds were compared to the standard antibiotic, norfloxacin. The MIC of norfloxacin was found to be in the range of 16–128 μg/mL.

The starting concentration was 512 μg/mL (four times more diluted than the stock concentration). The plant extracts with a MIC concentration ≤256 μg/mL were mainly chosen for further phytochemical investigation.

3. Results

3.1. List of Plants

Table 1 is the list of plants collected from various regions of Bangladesh. Plant parts such as leaf, fruit, bark, rhizome, or thorn of the selected plants were collected from 7 to 13 September 2016.

Table 1. List of the plants collected for the study.

Scientific Name	Plant Part(s) Collected	Place of Collection	Date of Collection
Paedaria foetida	Leaf	NBG	7 September 2016
Aegle marmelos	Fruit	NBG	8 September 2016
Justicia adhatoda	Leaf	NBG	8 September 2016
Terminalia arjuna	Bark	GUAH	9 September 2016
Diospyros malabarica	Leaf	NBG	9 September 2016
Andrographis periculata	Leaf	NBG	9 September 2016
Tinospora cordata	Leaf	NBG	10 September 2016
Zingiber montanum	Rhizome	NBG	10 September 2016
Azadirechta indica	Bark and Leaf	NBG	11 September 2016
Tylophora indica	Leaf	NBG	11 September 2016
Tribulus terrestris	Thorn	NBG	12 September 2016
Uraria picta	Leaf	RUBG	12 September 2016
Cynometra ramiflora	Leaf	SF	13 September 2016
Swertia chirayita	Leaf and thorn	NBG	13 September 2016
Terminalia chebula	Fruit	NBG	13 September 2016
Glyccrrhiza glabra	Fruit	NBG	13 September 2016
Abroma augusta	Leaf with fruit	GUAH	13 September 2016
Feronia limonia	Bark and Leaf	NBG	13 September 2016

3.2. Dry Plant Material

The chart of the powdered plant material indicated that the starting material of *Uraria picta* (300 g) was the highest among all other selected medicinal plants for phytochemical study (Figure 1).

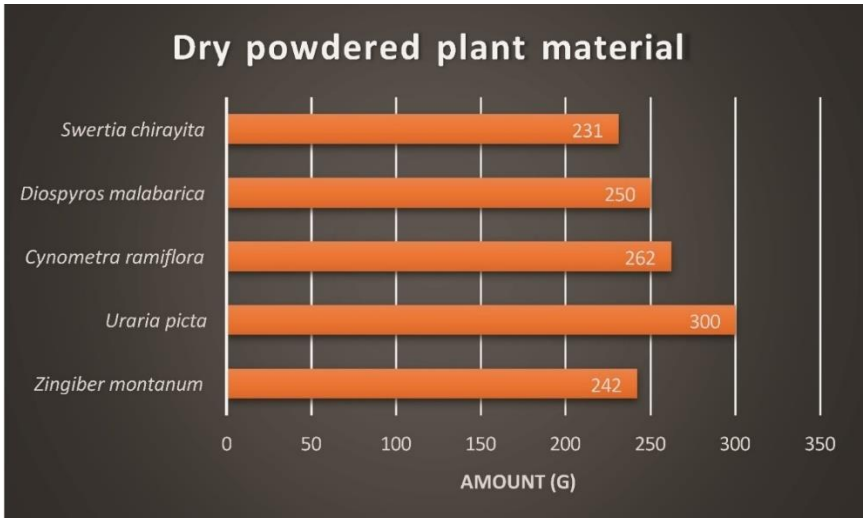

Figure 1. The starting amount of the plant material used for isolation and identification of pure compounds.

3.3. Percentage Yield of Extracted Plant Material

The Figure 2 shows the percentage yield of the plants' extraction with hexane, chloroform, and methanol of increasing polarity using the Soxhlet apparatus. Methanol extract of *Z. montanum* had the highest percentage yield (9.5%).

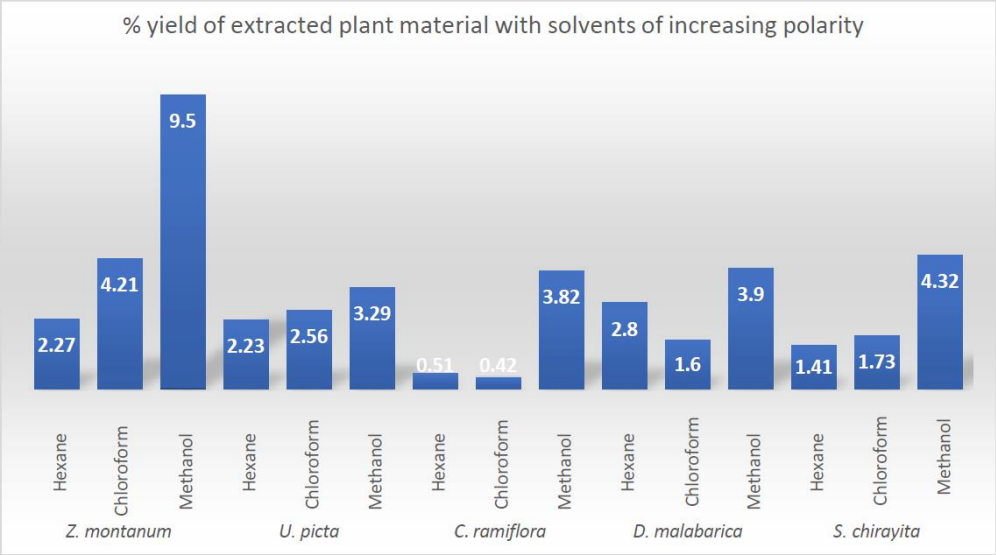

Figure 2. the percentage yields of the plant extracts with solvents of increasing polarity.

3.4. The Chemical Structures of the Isolated Compounds Are Given Below

3.4.1. Compounds isolated from *Z. montanum*

Eight compounds were isolated from *Z. Montanum.* Their chemical structures were listed in Figure 3.

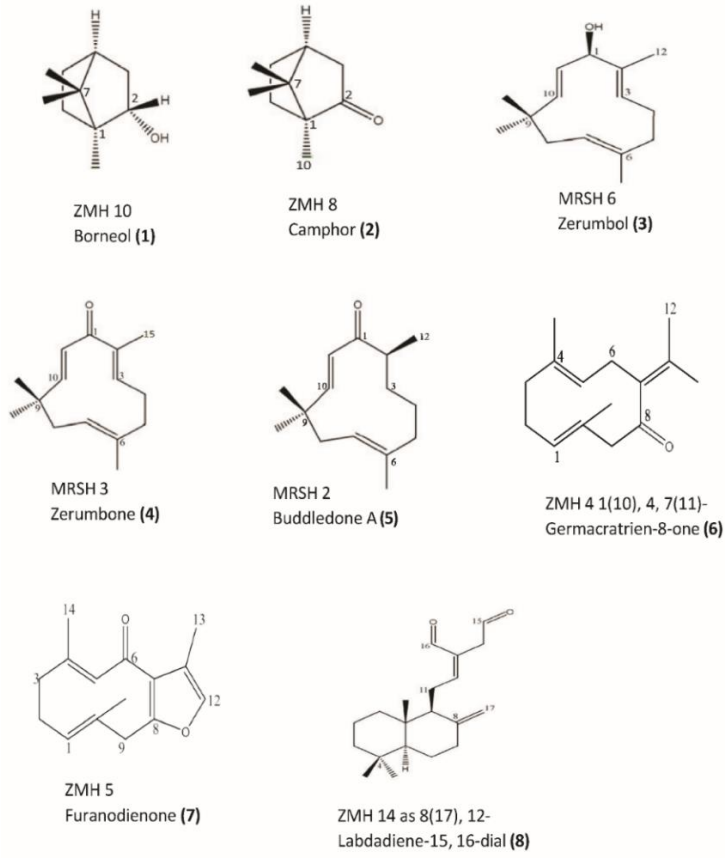

Figure 3. Chemical structures of compounds isolated from *Z. montanum*.

3.4.2. Compounds isolated from *C. ramiflora*

Five compounds were isolated from *C. ramiflora.* Their chemical structures were listed in Figure 4.

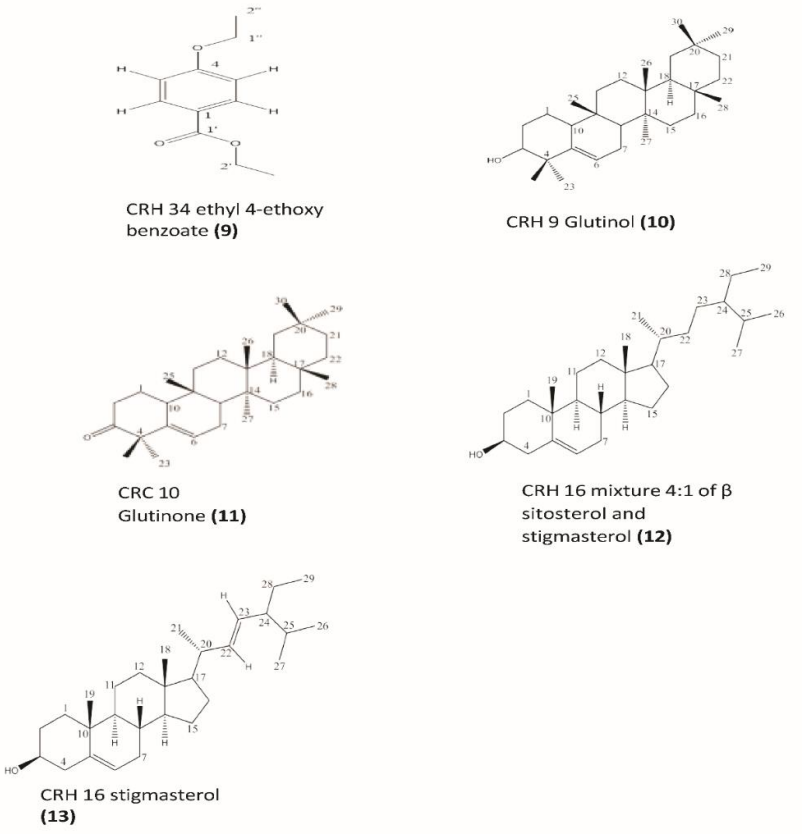

Figure 4. Chemical structures of compounds isolated from *C. ramiflora.*

3.4.3. Isolation of compounds from methanol extract of *U. picta*

Two compounds were isolated from *C. ramiflora.* Their chemical structures were listed in Figure 5.

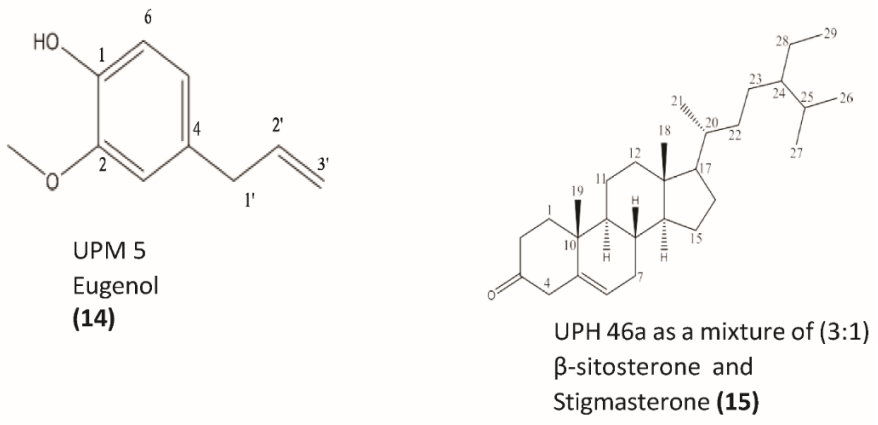

Figure 5. Chemical structures of compounds isolated from *U. picta.*

3.4.4. Compounds Isolated from *S. chirayita*

Four compounds were isolated from *C. ramiflora.* Their chemical structures were listed in Figure 6.

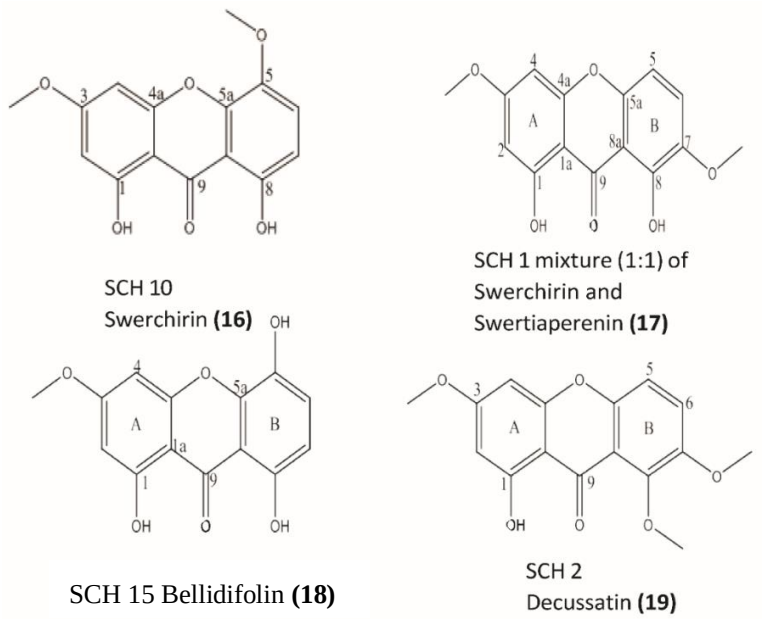

Figure 6. Chemical structures of compounds isolated from *S. chirayita.*

3.4.5. Compounds isolated from *D. malabarica*

Five compounds were isolated from *C. ramiflora.* Their chemical structures were listed in Figure 7.

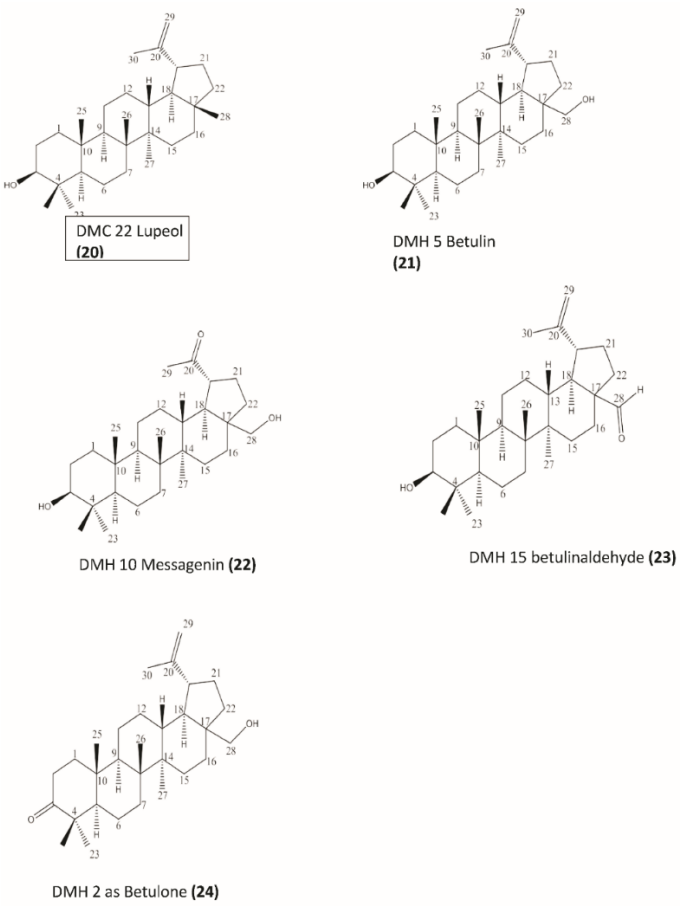

Figure 7. Chemical structures of compounds isolated from *D. malabarica.*

3.5. Antibacterial Screening of Eighteen Plant Extracts

Antibacterial screening of the eighteen medicinal plants resulted in the selection of five plants for further phytochemical investigation based on their potent antibacterial activity (MIC concentration ≤ 256 µg/mL) (Table 2).

Table 2. The antibacterial screening result of 18 selected plants against clinical strains of Methicillin-resistant *Staphylococcus aureus* in µg/mL.

Plant Extract/Standard	MRSA Strains in µg/mL			
	EMRSA 15	ATCC 25941	SA1199B	XU212
Paedaria foetida				
Hexane	512	512	512	512
Chloroform	512	512	512	512
Methanol	>512	>512	>512	>512
Justicia adhatoda				
Hexane	128	512	128	512
Chloroform	128	512	128	512
Methanol	256	512	512	512
Aegle marmelos				
Hexane	128	64	128	512
Chloroform	128	64	128	512
Methanol	256	512	512	512
Abroma augusta				
Hexane	>512	>512	>512	>512
Chloroform	>512	>512	>512	>512
Methanol	>512	>512	>512	>512
Terminalia arjuna				
Hexane	>512	>512	>512	>512
Chloroform	>512	>512	>512	>512
Methanol	>512	>512	512	128
Diospyros malabarica				

Hexane	64	128	>512	512
Chloroform	64	128	>512	512
Methanol	>512	>512	>512	>512
Andrographis periculata				
Hexane	256	512	512	512
Chloroform	256	512	512	512
Methanol	256	512	512	>512
Tinospora cordata				
Hexane	>512	>512	>512	>512
Chloroform	>512	>512	>512	>512
Methanol	>512	>512	>512	>512
Azadirechta indica				
Hexane	>512	>512	>512	>512
Chloroform	>512	>512	>512	>512
Methanol	>512	>512	>512	>512
Tylophora indica				
Hexane	512	512	>512	>512
Chloroform	512	512	>512	>512
Methanol	>512	>512	>512	>512
Tribulus terrestris				
Hexane	64	>512	32	64
Chloroform	64	>512	32	64
Methanol	>512	>512	512	>512
Uraria picta				
Hexane	128	64	32	64
Chloroform	128	64	32	64
Methanol	64	64	128	128
Cynometra ramiflora				
Hexane	128	64	128	64
Chloroform	128	64	128	64
Methanol	256	>512	512	256
Swertia chirayita				
Hexane	256	512	>512	>512
Chloroform	256	512	>512	>512
Methanol	256	512	512	>512
Terminalia chebula				
Hexane	128	256	512	512
Chloroform	128	256	512	512
Methanol	>512	>512	>512	>512
Glyccrrhiza glabra				
Hexane	512	512	128	256
Chloroform	512	512	128	256
Methanol	>512	>512	>512	>512
Feronia limonia				
Hexane	512	>512	>512	>512
Chloroform	512	>512	>512	>512
Methanol	128	>512	512	>512
Zingiber montanum				
Hexane	256	128	128	128
Chloroform	128	256	64	128
Methanol	>512	>512	>512	>512
Norfloxacin	16	16	32	64

3.5.2. Antibacterial Screening of Isolated Compounds

Potential antibacterial activity of isolated compounds against Methicillin-resistant *Staphylococcus aureus* clinical isolates listed in Table 3.

Table 3. Showed the results of antibacterial screening of the pure compounds.

Compounds (Number)	**MRSA Strains (MIC Values in µg/mL)**					
	XU212	**ATCC 25941**	**EMRSA 15**	**MRSA 340702**	**MRSA 24821**	**SA1199B**
Z. montanum						
ZMH 10 (**1**)	>128	128	>128	>128	>128	>128
ZMH 8 (**2**)	128	128	>128	>128	>128	>128
MRSH 6 (**3**)	128	64	32	128	128	128
MRSH 3 (**4**)	>128	128	64	>128	>128	64

MRSH 2 (**5**)	128	128	128	128	128	128
ZMH 4 (**6**)	>128	64	>128	>128	>128	>128
ZMH 5 (**7**)	>128	>128	>128	>128	>128	>128
ZMH 14 (**8**)	64	32	64	64	128	64
			C. ramiflora			
CRH 34 (**9**)	>128	64	128	>128	>128	64
CRH 9 (**10**)	128	>128	128	>128	>128	64
CRC 10 (**11**)	>128	64	>128	>128	>128	128
			U. picta			
UPM 5 (**14**)	64	128	128	128	>128	>128
			S. chirayita			
SCH 10 (**16**)	>128	>128	>128	>128	>128	>128
SCH 1 (**17**)	>128	>128	>128	>128	>128	>128
SCH 15 (**18**)	>128	>128	>128	>128	>128	>128
SCH 2 (**19**)	>128	>128	>128	>128	>128	>128
			D. malabarica			
DMC 22 (**20**)	64	128	>128	>128	>128	128
DMH 5 (**21**)	>128	64	>128	>128	>128	>128
DMH 10 (**22**)	64	>128	64	>128	>128	>128
DMH 15 (**23**)	>128	64	>128	>128	>128	64
DMH 2 (**24**)	>128	128	>128	>128	>128	>128
Control (Norfloxacin)	64	32	16	64	64	32

4. Discussion

Z. montanum (Fam. Zingiberaceae) is one of the five chosen plants. It is indigenous to Bangladesh, where it is widely used in the northern part of the country. The ethnopharmacological survey's outcome indicated that *Z. montanum* was being used by traditional practitioners and local village people for the treatment of gastrointestinal infections [8].

The phytochemical analysis led to the isolating of eight terpenes/terpenoids isolated from hexane and chloroform extracts of *Z. montanum*. These compounds were characterized as zerumbone (**4**), zerumbol (**3**), buddledone A (**5**), camphor (**2**), borneol (**1**), furanodienone (**7**), germacrone (**6**), (*E*)-8(17),12-labdadiene-15,16-dial (**8**) (Figure 3) using 1D and 2D NMR spectroscopy and mass spectrometry [10]. (*E*)-8(17),12-labdadiene-15,16-dial (**8**) and zerumbol (**3**) showed potential antibacterial activity (MIC) values 32–128 µg/mL against a series of clinical isolates of multi-drug resistant (MDR) and MRSA [10] (Table 3). Terpenes are a potential molecule to exert antibacterial activity via a membrane disruption mechanism [11]. Zerumbol (**3**) was isolated for the first time in this study. Previously, Takashi and his colleagues [12] synthesised optically active zerumbol from zerumbone as the starting material for conversion to useful chiral products such as paclitaxel.

Sesquiterpene zerumbol (**3**) showed moderate antibacterial activity with an MIC result of 32–128 µg/mL (Table 3). The presence of a hydroxyl group in zerumbol (**3**) instead of the carbonyl group might make zerumbol (**MRSH 6**) more active than zerumbone (**4**) [13]. The antibacterial activity against SA1199B MRSA strains was the same as the crude hexane extract of *Z. montanum*. Still, the compound showed 3-fold stronger antibacterial activity against XU212 and 2-fold stronger antibacterial activity against ATCC clinical strain of MRSA compared to the crude extract. Isolated fraction zerumbol (**3**) was proved to be bioactive compared to crude extract because the fraction contained a concentrated active principle but might be diluted in other fractions [14].

(*E*)-8(17),12-Labdadiene-15,16-dial (**8**) (Table 3) exhibited the most potent antibacterial activity with the MIC value 32–128 µg/mL. The compound (MIC value 64 µg/mL) showed the same activity as the control (Norfloxacin, MIC 128 µg/mL) against XU212 and MRSA 340702 bacterial strains. The compound is a labdadiene diterpene with exomethylene, olefin and two aldehyde groups. The presence of these groups and unsaturation could account for the significant antibacterial activity against MRSA strains. Other isolated compounds ZMH 4 (**6**) and ZMH-5 (**7**), ZMH 8 (**2**) and ZMH 10 (**1**) exhibited moderate activity against MRSA clinical strains. Two isolated fractions of *Z. montanum* exhibited antibacterial activity, which supports its traditional use to treat infectious diseases.

C. ramiflora belongs to the family Fabaceae and is used in herbal medicine [15]. It is Indigenous to India and Bangladesh, predominantly in the mangrove forest Sundarbans, Sri Lanka, and tropical areas of Africa and Australia [16]. The therapeutic activity, such as antihyperglycemic [17], anti-ulcer [18], anti-oxidant [19], antibacterial [20], and cytotoxicity [21] of the leaf and bark of the plant were investigated in-vitro, but the secondary metabolites responsible for the activity was still under research. Therefore, three triterpenoids β sitosterol (**12**), glutinol (**10**), glutinone (**11**), and ester ethyl 4- ethoxy benzoate (**9**) **(**Figure 4), were isolated for the first time from *C.*

ramiflora. Among these compounds, ethyl 4-ethoxybenzoate (**9**) appeared to be a new natural product. The isolated compounds exerted moderate antibacterial activity against MRSA clinical strains.

During this study, eugenol (**14**), and a mixture of (3:1) β-sitosterol and stigmasterol (**15**) (Figure 5) were isolated from *U. picta*. Eugenol (**14**) is the major chemical constituent in clove (*Syzygium aromaticum*) oil and manifests a versatile pharmacological action. It was reported for the first time from *U. picta*. Eugenol (**14**) exerted moderate antimicrobial activity with a MIC of 64–128 µg/mL against XU212, ATCC 5941, EMRSA 15, and MRSA 340702 (Table 3). In 2010, Qiu and his colleagues investigated the effect of eugenol on MRSA clinical strains; they suggested eugenol as the base of the new antibacterial drug to combat infectious diseases associated with *S. aureus* [22].

S. chirayita, the popular ethnomedicinal herb indigenous to the Himalayas, has been well-documented in Ayurveda, Unani, Siddha, and other conventional medical systems for its wide spectrum of pharmacological properties [23]. Xanthones are abundant in *S. chirayita*. The current study has led to the isolation of xanthones, including swerchirin (**16**), swertiaperenin (**17**), decussatin (**19**), and bellidifolin (**18**), from the hexane extract of *S. chirayita*. Bellidifolin (**18**) and decussatin (**19**) (Figure 6) were first isolated in 1973 from *S.Chirayita* [24]. The hypoglycemic and antimalarial activity of swerchirin were investigated previously [25,26]. *S. chirayita* contains 40 xanthone derivatives [23]. Ethanolic, aqueous and methanolic extracts of *S. chirayita* documented as exhibiting antimicrobial activity against both Gram-positive and Gram-negative bacteria [27–31]. However, in the current study, xanthone derivatives did not exert imperative antibacterial activity against MRSA clinical strains. Therefore, the author concluded that the crude extract of *S.chirayita* may exert antibacterial activity synergistically compared to individual compounds.

D. malabarica, indigenous to India, Pakistan and Bangladesh, produces edible seasonal fruit [32]. The genus *Diospyros* has been widely researched in terms of phytochemistry. The genus *Diospyros* is dominated by the production of lupane series triterpenoids and naphthoquinones. [33]. In this current study, the lupane series triterpenoids- lupeol (**20**), betulone (**24**), betulin (**21**), betulinaldehyde (**23**) and messagenin (**22**) (Figure 7) were isolated and reported for the first time from the hexane leaf extract of *D. malabarica* species. Among the Lupane series, Messagenin (**22**) has been synthesised in previous studies, but there is no literature on isolating this compound from natural sources [34]. Therefore, the natural compound messagenine (**22**) was reported for the first time in this study. The lupane series are common natural compounds mostly abundant in fruit and vegetables like green pepper, mangoes, grapes, white cabbage and in medicinal plants such as *Celastrus paniculatus, Tamarindus indica, Himatanthus sucuuba, Zanthoxylum riedelianum, Leptadenia hastata, Sebastiania adenophora* and *Bombax ceiba* [35].

The lupine series was isolated from *D. malabarica* and was tested against MRSA strains. Betulone (**24**) was inactive against all the MRSA strains, while lupeol (**20**) exerted moderate antimicrobial activity against XU212 (64 µg/mL) and ATCC 5941 (128 µg/mL). Lupeol (**20**) is a triterpene with an exomethylene and hydroxyl group, conferring better antimicrobial activity than Betulone (**24**). Another compound such as messagenin (**22**), displayed activity against XU212 and EMRSA 15 with MIC 64 µg/mL, betulinaldehyde **(23)** exerted activity against ATCC 5941 and SA1199B with the MIC of 64–128 µg/mL, while betulin (**21**) exerted activity against ATCC 5941 (64 µg/mL) only (Table 3).

5. Conclusion

The ethnopharmacological knowledge of Bangladeshi medicinal plants and the demonstration of their anti-infective activity contributed to preserving medicinal plants in decline. In this study, the combination of traditional knowledge and extensive scientific work using a wide range of chromatographic and spectroscopic techniques led to the identification of a total of 24 compounds, including terpenes and simple phenolic compounds with potential antibacterial activity against clinical isolates of a panel of MRSA. This study gives an insightful approach to the scientific explanation of using medicinal plants as primary health care to tackle infectious diseases. In the future, investigating the activity of the plant extracts and their active compounds to understand virulence or pathogenesis will provide useful knowledge of alternative bacterial targets for natural compounds.

Supplementary Materials: The following supporting information can be downloaded at: https://www.sciltp.com/journals/jmnp/2024/1/405/s1, Table S1: Isolation of compounds from hexane extract of *Zingiber montanum*.; Table S2: Isolation of compounds from chloroform extract of *Zingiber montanum.* (The starting amount was 242 g); Table S3: Isolation of compounds from methanol extract of *Uraria picta.*; Table S4: Isolation of compounds from Hexane extract of *Cynometra ramiflora.*; Table S5: Isolation of compounds from Hexane extract of *Diosphyros malabarica.*; Table S6: Isolation of compounds from Hexane extract of *Swertia chirayita*.

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Institutional Review Board Statement: In this study, participants participated in an ethnopharmacological survey, which was ethically approved by the University of East London's research ethics committee (UREC) (Reference number: UREC—1516 154).

Informed Consent Statement: Not applicable.

Data Availability Statement: All experimental data of this study are stored in the hard disk drive of a laboratory computer and will be attainable upon request.

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Abbreviations

AMR	Antimicrobial Resistance
NBG	National Botanical Garden
GUAH	Govt. Unani and Ayurvedic Hospital
RUBG	Rajshahi University Botanical Garden
SF	Sundarbans mangrove forest
UEL	University of East London
MRSA	Methicillin-resistant Staphylococcus aureus
MIC	Minimum inhibitory concentration
NMR	Nuclear Magnetic Resonance

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Article

Unveiling the Bioactive Phytochemicals of *Momordica charantia* Leaves and Their Antibacterial Effects

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Abstract: *Momordica charantia* is widely cultivated in Zaria, Nigeria, and holds a prominent place in traditional medicine. Its leaves, fruits, and seeds are known to be rich in bioactive compounds and are commonly employed to treat various infections and diseases. This study aimed to investigate the bioactive components and antibacterial properties of methanolic leaf extracts and their fractions. Phytochemical analysis of the methanolic extract revealed the presence of alkaloids, saponins, flavonoids, glycosides, tannins, steroids, and terpenoids. The methanolic extract was fractionated into n-butanol, ethyl acetate, and chloroform fractions. The methanolic extract exhibited superior antibacterial activity compared to its fractions, suggesting potential synergistic effects among the plant's constituents. Antibacterial efficacy was evaluated using well-diffusion, minimum inhibitory concentration (MIC), and minimum bactericidal concentration (MBC) assays. *Salmonella typhi* was the most susceptible bacterium, with zones of inhibition of 25.00 ± 0.10 mm, MIC of 12.5 ± 0.82 mg/mL, and MBC of 50 ± 0.22 mg/mL. This was followed by *Escherichia coli* (18.77 ± 0.25 mm, MIC: 50 ± 0.53 mg/mL, MBC: 100 ± 0.82 mg/mL) and *Staphylococcus aureus* (14.13 ± 0.91 mm, MIC: 50 ± 0.23 mg/mL, MBC: 100 ± 0.48 mg/mL). Among the fractions, the n-butanol fraction demonstrated the highest antibacterial activity. Subsequent analysis of this fraction using GC-MS identified key compounds, including 2-pentanone, 4-hydroxy-4-methyl-, n-amyl isovalerate, 2(5*H*)-furanone, 3,5,5-trimethyl-, furan, tetrahydro-2,2,4,4-tetramethyl-, and 3-tetradecanol acetate. In conclusion, the methanolic extract followed by n-butanol fraction of *M. charantia* exhibited significant antibacterial activity, particularly against Gram-negative bacteria such as *S. typhi* and *E. coli*. Further research is recommended to isolate and characterize the bioactive compounds responsible for this activity.

Keywords: secondary metabolites; bacteria, natural product; medicinal plants; GC-MS analysis

1. Introduction

For millennia, *Homo sapiens* have recognized the therapeutic benefits of certain plants in treating various illnesses. Fossil records from Iraq, dating back 60,000 years, provide evidence of this practice. Nonetheless, the use of medicinal herbs likely extends even further, given that anatomically modern humans emerged in Africa around 300,000 years ago [1], and throughout ancient civilizations herbs are being used in the treatment of diseases and revitalize the body system [2]. Plants are recognized not only for their nutritional benefits but also for providing remedies. As such they are recognized for their medicinal and healing properties, which are often commonly referred to as medicinal plants [3]. Medicinal plants are characterized by the presence of bioactive metabolites that can be utilized for medicinal purposes for synthesis of drugs or medicinal agent [4]. Several herbs frequently used in traditional treatments include *Picrorhiza* sp., garlic, cloves, neem (both fruit and leaves), nutmeg, cinnamon, ginger, peppermint, sage, thyme, mustard, and fenugreek [5,6]. These plants serve various purposes such as promoting digestion, alleviating diarrhoea, acting as antiseptics and anti-inflammatories, combating parasites, and enhancing appetite in both humans and animals [3].

Thousands of compounds, including phytochemicals and other bioactive polyphenolic compounds, have been isolated from various varieties of medicinal plants and showed potent bioactive properties [7]. The phytochemicals isolated in various plants include phenolic acids, tannins, flavonoids, saponins, cardiac glycosides, anthocyanins, anthraquinones, and terpenoids [4]. Some of them, like tannins, flavonoids and saponins were reported to possess good antimicrobial activity [8]. However, microbes, including bacteria, have developed resistance against antimicrobial agents, leading to emerging multi-drug resistant bacteria. The growing ineffectiveness of

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chemotherapeutics and the rise in antibiotic resistance of pathogenic microbes have spurred the screening of various medicinal plants for their potential antimicrobial activities [9]. Despite pharmaceutical breakthroughs in producing new antibiotics, bacterial resistance persists leading to ongoing development of resistance to current antibacterial drugs which underscores the need for exploring new antimicrobial agents [10].

Nanotechnology is a rapidly advancing field with broad applications across science and technology, particularly in the synthesis and development of nanomaterials and nanoparticles. nanoparticle technology has demonstrated the ability to overcome bacterial drug resistance mechanisms by inhibiting biofilm formation and disrupting other key processes linked to bacterial virulence [11]. Green-synthesized silver nanoparticles (AgNPs) have demonstrated promising antibacterial activity against a wide spectrum of Gram-positive and Gram-negative pathogenic bacteria. These include *Salmonella* spp., *Pseudomonas* spp., *Staphylococcus aureus*, *Streptococcus* spp., *Escherichia coli*, *Bacillus* spp., *Vibrio* spp., and many more [12]. *S. typhimurium*, among others, are the most common agents causing bacterial diseases, especially associated with unhygienic practices [13]. These species are known to develop strains that are resistant to available antibiotics. Due to challenges in antibacterial chemotherapy, our goal is to address multi-drug resistance through testing clinical bacterial isolates by leveraging medicinal plants as the primary source of natural antibacterial agents. A previous finding on the study of efficacy of *Moringa oliefera* leaves extract showed significant antibacterial activity against *S. aureus* and *E. coli* [14]. The further suggest that the efficacy of the plant's extract against *Shigella* sp. is concentration dependent. In literature, Adamu et al. [15], have documented the antibacterial potential of *Citrus sinensis* leaf extract against *S. typhi* and *S. paratyphi* and showed the potentiality of citrus leaves extract in management of Salmonella's infections. Bashir et al. [16], have also reported the effectiveness of Baobab leaves and stem bark extract against the growth of clinical bacterial isolates (*S. typhi, S. aureus* and *E. coli*) with more activity recorded in leaf extract against these bacteria.

Little is known about the biological properties of *Momordica charantia* grown in Nigeria. *Momordica charantia* (*M. charantia*) common name is Bitter melon, is belonging to the Cucurbitaceae family, is renowned for its bitter taste. The unripe fruit is believed to contribute to optimal health. The plant, particularly its fruits, has shown numerous indications of therapeutic properties [17]. The plant is grown in tropical and subtropical regions, including India, Asia, South America, and Nigeria. In various parts of the world, including South American countries. *M. charantia* is extensively cultivated for both culinary and medicinal purposes because of its properties such as hypoglycemic activity, anti-HIV, antitumor, antidiabetic, antileukemic, anticancer, anti-inflammatory, antioxidant; In African countries it has been used for its antimalarial property [18,19]. A previous study by Hassan et al. [20], have reported antifungal efficacy of methanolic extract of *M. charantia* fruit. Another study conducted on the anticancer effect of *M. charantia* fruits documented the potentiality of the methanolic extract in the inhibition of cancer cell line growth [21]. Similarly, Zeyp et al. [22], cited anti-hyperglycemic effects of *M. charantia* fruits. Gultom et al. [23], reported a findings that have demonstrated strong antibacterial potential of the ethanolic leaf extract of *M. charantia*. However, the authors did not record much activities against *S. aureus* and *S. typhi* which may likely be due to the type of solvents used in the extraction process. Despite researchers' efforts to harness the medicinal attributes of *M. charantia* leaves. In general, little is known about its antibacterial properties, especially when utilizing different solvents and fractions in the extraction. Therefore, the current study aimed to evaluate the antibacterial potentials of methanolic extract and three fractions of *M. charantia* leaves grown in Nigeria against some bacterial pathogens, beside, unveiling its phytochemical contents.

2. Materials and Methods

2.1. Plant Samples

The *M. charantia* leaves were collected from the Zaria Local Government Area in Kaduna State, located at coordinates 11°04′ N and 7°42′ E. Collection was conducted during the dry season, with a temperature of 38 °C. The leaves were sent to the laboratory in sanitized polythene bags. A taxonomist from the Herbarium Unit, Department of Botany, Ahmadu Bello University, Zaria, verified the plant, awarding them voucher number 1697. The fresh leaves were washed with distilled water, air-dried in the shade at ambient temperature, then crushed into a fine powder using a mortar and pestle. The powdered samples were preserved in sterile polythene bags for further examination.

2.2. Preparation of Extract

The methanolic extract of the plant leaves was prepared following the method described by Yusuf et al. [24]. A total of 100 g of plant material was suspended in 500 mL of absolute methanol in conical flasks and vigorously shaken for a few minutes. The mixture was left to stand for 72 h, then filtered first with muslin cloth and

subsequently with filter paper. The filtrate was concentrated in a water bath at 40 °C for 48 h. The resulting dry crude extract was stored in a sterile container and kept in a refrigerator at 4 °C until needed for use.

2.3. Qualitative Phytochemical Screening

The methanolic extract of *M. charantia* leaves was screened for the presence of secondary metabolites following the methods outlined by Trease and Evans [25]. The metabolites tested for included carbohydrates, flavonoids, saponins, tannins, terpenoids, steroids, anthraquinones, alkaloids, and cardiac glycosides:

2.3.1. Test for Carbohydrates

Molisch's test was used to assess carbohydrate content. Three drops of Molisch's reagent were added to the methanolic leaf extract in a test tube, followed by concentrated sulfuric acid. The formation of a reddish ring at the interface indicated the presence of carbohydrates.

2.3.2. Test for Saponins

Two milliliters of the extract were mixed with 10 mL of distilled water and shaken vigorously for 30 s. The formation of a persistent 2 cm layer of foam after 5 min indicated the presence of saponins.

2.3.3. Test for Flavonoids

To test for flavonoids, 1 mL of NaOH was added to 3 mL of the extract. The appearance of a yellow color confirmed the presence of flavonoids.

2.3.4. Test for Tannins

The presence of tannins was tested by adding 3 drops of 0.1% ferric chloride to 2 mL of the extract. A brownish-green precipitate confirmed the presence of tannins.

2.3.5. Test for Terpenoids

Two milliliters of chloroform were added to 5 mL of the extract, followed by 3 mL of concentrated sulfuric acid. A reddish coloration at the interface indicated the presence of terpenoids.

2.3.6. Test for Alkaloids

Wagner's reagent was added to 2 mL of the extract. The formation of an orange-brown precipitate indicated the presence of alkaloids.

2.3.7. Test for Anthraquinones

The sample was dissolved in chloroform, filtered, and shaken with 10% ammonia solution. The appearance of a bright pink color in the upper aqueous layer indicated the presence of anthraquinones.

2.3.8. Test for Cardiac Glycosides

A portion of the extract was dissolved in glacial acetic acid containing traces of ferric chloride, and 1 mL of sulfuric acid was carefully added. The presence of a brown ring at the interface indicated the presence of cardiac glycosides.

2.4. Identification of Bactreia

Standard experimental procedures were employed to identify the bacterial strains. A full loop of the stock cultures of *Staphylococcus aureus*, *Escherichia coli*, and *Salmonella typhi* was streaked onto both blood agar and nutrient agar plates, followed by incubation for 18–24 h at 37 °C. Colony morphology was observed and documented, after which individual colonies were selected for further confirmation tests. These tests included Gram staining and culture on various differential media such as MacConkey agar, xylose lysine deoxycholate agar, Salmonella-Shigella agar, mannitol salt agar, and eosin methylene blue agar. In addition, biochemical identification assays, including the indole test, oxidase test, coagulase test, catalase test, motility test, Simmon's citrate test, MRVP (methyl-red, Voges-Proskauer) test, and TSI (triple sugar iron) test, were conducted following established protocols [26].

2.5. Antibacterial Activity

The antibacterial activity of the plant extract was evaluated using the agar well diffusion method. A standardized bacterial suspension was used to prepare an inoculum, of which 0.1 mL was applied onto Mueller-Hinton agar plates, with each test conducted in triplicate. The inoculum was uniformly spread across the plate surface using a sterile cotton swab. After allowing the plates to rest for 10 min, wells with a 6 mm diameter were punched into the agar using a sterile cork borer. Each well was filled with 0.1 mL of the plant extract at varying concentrations (100 mg/mL, 50 mg/mL, 25 mg/mL, and 12.5 mg/mL). Wells containing dimethyl sulfoxide (DMSO) were used as negative controls. The plates were left at room temperature for 10 min to enable diffusion of the extract into the agar, followed by incubation at 37 °C for 24 h. After incubation, inhibition zones were observed, and the diameters of these zones were measured in millimeters using a ruler. The means and standard deviations were then statistically calculated [27].

2.6. MIC and MBC Assays

To determine the minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) of the plant extracts against the tested bacteria were determined following a modified version of previously published methods [28]. In summary, 1 mL of Mueller-Hinton broth was dispensed into five sterilized test tubes. A 50% extract concentration was prepared by adding 1 mL of the plant extract to the first test tube, followed by a series of twofold dilutions to create concentrations of 50%, 25%, 12.5%, 6.25%, and 3.12%. Subsequently, 100 µL of a standardized bacterial culture was added to each tube. The lowest concentration that exhibited no turbidity, indicating inhibition of bacterial growth, was recorded as the MIC. For the MBC determination, 50 µL from each tube showing no turbidity was plated on nutrient agar and incubated at 30 °C to 35 °C overnight. The lowest concentration at which no bacterial growth was observed on the agar plates was identified as the MBC.

2.7. Fractionation of the Extract

The most active components of the extract were fractionated using different solvents: chloroform, ethyl acetate, and n-butanol. *M. charantia* methanol extract was subjected to fractionation to separate its active components. The process involved dissolving approximately 100 g of the crude extract in 1 litre of distilled water using a separator funnel. Then, 250 mL of chloroform was mixed with 250 mL of the extract solution (1:1 *v*/*v*). The funnel was vigorously shaken, and after allowing it to settle for 15 min, the organic solvent layer was separated from the aqueous layer. The chloroform layer was isolated and collected in a clean flask by carefully opening the funnel knob. This separation process was repeated sequentially with ethyl acetate and n-butanol. The collected soluble fractions were labelled and concentrated to dryness under vacuum using a rotary evaporator. Each extract from the soluble fractions was securely sealed in a clean container and stored in a refrigerator at 4 °C until further use [26].

2.8. GC-MS Analysis

The extract was dissolved in ethanol along with a mixture of solvents, including toluene, chloroform, ethanol, and ethyl acetate, before being subjected to GC-MS analysis. The analysis was performed at Kaduna State University, 800283, Kaduna State, Nigeria. Helium (99.999%) served as the carrier gas, with a flow rate of 1 mL/min. The separation was carried out using an HP5 column with a length of 30 mm, an internal diameter of 0.32 mm, a film thickness of 0.25 mm, and a temperature range from −60 °C to 325 °C (maximum 350 °C). The total GC run time was 35 min, with the oven temperature increasing from 70 °C to 280 °C at a rate of 8 °C per minute. A sample volume of 4 µL was injected, and the MS was operated at 70 eV. Compound identification was based on comparing the spectra of unknown compounds with those in the reference library, allowing for probable determination of their names, molecular weights, and structures [29].

2.9. Statistical Analysis

The agar well diffusion assay was performed in triplicate, and the results were reported as means ± standard deviation (SD). Analysis of Variance (ANOVA) was utilized to evaluate significant differences among the isolates compared to the control. A p-value of < 0.05 was considered statistically significant for assessing the observed differences.

3. Results and Discussion

The Percentage

Phytochemical analysis of the methanolic leaf extract of *M. charantia* (Table 1) demonstrated the presence of steroids, alkaloids, saponins, flavonoids, carbohydrates, cardiac glycosides, tannins, and terpenoids, while anthraquinones were notably absent. This study corroborates previous findings regarding the presence of saponins, flavonoids, and alkaloids [14,24]. Alkaloids exhibit antibacterial properties by interfering with the peptidoglycan structure in bacterial cells, inhibiting cell wall synthesis and leading to cell death. Additionally, alkaloids function as DNA intercalators, hindering the activity of bacterial cell topoisomerase enzymes [21,30]. The qualitative analysis of the methanolic extract also indicated the presence of phenolic compounds. Valizadeh et al. [31] and Rahmi and Sari [32] similarly reported the presence of flavonoids, saponins, and phenols in the ethanolic extracts of *M. charantia* fruits and leaves.

Table 1. Phytochemical Constituents of *M. charantia* methanolic leaf extract.

Bioactive Components	*M. charantia*
Alkaloids	+
Saponins	+
Flavonoids	+
Steroids	+
Terpenes	+
Cardiac glycosides	+
Anthraquinones	−
Tannins	+
Carbohydrates	+

KEY:+: Presence; − : Absence.

Saponins act as antibacterial agents by reducing surface tension, enhancing permeability, and promoting cell leakage. This increased permeability allows for the diffusion of intercellular substances through the cytoplasmic membrane, compromising cell membrane integrity and resulting in cytoplasmic leakage and cell death [33]. The discrepancies observed in these investigations may be attributed to the solvents utilized in Soxhlet extraction, as the choice of solvent and plant component yield varying results during preliminary phytochemical screenings. Notably, saponins exhibit anti-inflammatory properties, hemolytic activity, and cholesterol-binding capabilities, while flavonoids mitigate cellular oxidative stress and demonstrate beneficial effects as anticancer and antibacterial agents [34].

Table 2 presents the mean inhibition zones of *M. charantia* against Staphylococcus aureus, Salmonella typhi, and Escherichia coli at varying concentrations of 100 mg/mL, 50 mg/mL, 25 mg/mL, and 12.5 mg/mL. The results indicate that the antibacterial activity of *M. charantia* extract is concentration-dependent, with the highest mean inhibition zone observed against *S. typhi* (25.0 mm), followed by *E. coli* (18.77 mm) and *S. aureus* (14.13 mm). These findings are consistent with recent research by Lawrence and Olusola [35], which also demonstrated significant antibacterial activity against *S. aureus* and *E. coli*. Notably, high inhibition zones were recorded for *S. typhi* and *E. coli*. The observed variations in inhibition may come from the structural differences in bacterial membranes; *S. aureus*, as a Gram-positive organism, possesses an extracellular envelope that can confer resistance to certain antibacterial agents. The differing susceptibility among these bacteria may be attributed to the permeability barriers presented by the lipopolysaccharide-rich outer membranes of Gram-negative bacteria [36]. Another study found that *Momordica charantia* exhibited no inhibitory effects against the tested bacterial isolates, including *E. coli, Klebsiella pneumoniae*, *Proteus mirabilis,* and *S. typhi* [37]. The notable inhibition of *S. typhi* is likely related to the presence of alkaloids and flavonoids in *M. charantia*, which have been documented to inhibit cellular membrane synthesis and act as bacterial cytotoxins [19]. In contrast, Hassan et al. [20] reported no inhibition zone for *S. typhi* at concentrations of 25 mg/mL and 50 mg/mL of *M. charantia* fruit extract. The discrepancies in these studies suggest that *M. charantia* fruit may possess lower concentrations of phytonutrients compared to its leaves, despite the same solvent being used for extraction.

Table 2. Antibacterial activity of *M. charantia* methanolic leaf extract against bacterial isolates.

	Mean Zones of Inhibition (mm) ± Standard Deviation of *M. charantia*				
	Concentration (mg/mL)				
Bacterial	**100**	**50**	**25**	**12.5**	**Ciprofloxacin**
Staphylococcus aureus	14.13 ± 0.91 ^{c}	11.83 ± 0.31 ^{c}	5.90 ± 0.10 ^{c}	3.03 ± 0.15 ^{c}	35.43 ± 0.31 ^{c}
Salmonella typhi	25.00 ± 0.10 ^{a}	20.43 ± 0.12 ^{a}	13.97 ± 0.41 ^{a}	7.76 ± 0.46 ^{a}	39.13 ± 0.47 ^{b}
Escherichia coli	18.77 ± 0.25 ^{b}	16.23 ± 0.25 ^{b}	12.70 ± 0.44 ^{b}	6.90 ± 0.10 ^{b}	42.47 ± 0.15 ^{a}

Results are means ± standard deviation of triplicate (n = 3) determinations. Values with different superscripts in the column differ significantly at $p < 0.05$ using the Duncan Multiple Range Test.

The MIC and the MBC results of the methanolic leaf extracts of *M. charantia* against the bacterial isolates are summarized in Table 3. The MIC values recorded were 50 mg/mL for *S. aureus*, 12.5 mg/mL for *S. typhi*, and 50 mg/mL for *E. coli*, while the MBC values were 100 mg/mL for *S. aureus*, 50 mg/mL for *S. typhi*, and 100 mg/mL for *E. coli*. The lowest zone of inhibition was noted for *S. typhi* at 12.5 mg/mL compared to the other bacterial species. The reduced inhibition observed for *S. typhi* may be due to characteristics inherent to its bacterial cell membrane. The MIC results for *S. typhi* are in agreement with recent findings by Shahrajabian et al. [38] (2023), who also reported high efficacy of *M. charantia* fruit extract against this pathogen. However, our study demonstrated significant inhibition at a lower concentration of 12.5 mg/mL compared to the 100 mg/mL reported previously. Furthermore, a study on *M. charantia* seed extract showed effective inhibition against *S. aureus* at concentrations as low as 50 mg/mL [8]. Despite the same solvent being used for extraction, our results indicated lower inhibition zones, suggesting that the leaves of *M. charantia* may contain higher concentrations of phytonutrients capable of eliciting stronger antibacterial effects compared to seeds and fruits. Sherekar et al. [18] recommend a concentration of 100 mg/mL as sufficient for exerting antibacterial activity against various bacterial strains, indicating that extract of *M. charantia* can demonstrate antibacterial effects against both Gram-negative and Gram-positive bacteria, contingent upon the solvent and extraction method utilized.

Table 3. Minimum Inhibitory Concentration and Minimum Bacterial Concentration of *M. charantia* leaf extract.

Bacteria	**MIC (mg/mL)**	**MBC (mg/mL)**
S. aureus	50 ± 0.23 ^{a}	100 ± 0.48 ^{b}
S. typhi	12.5 ± 0.82 ^{c}	50 ± 0.22 ^{a}
E. coli	50 ± 0.53 ^{a}	100 ± 0.82 ^{b}

Results are means ± standard deviation of triplicate (n = 3) determinations. Values with different superscripts in the column differ significantly at $p < 0.05$ using the Duncan Multiple Range Test (DMRT).

The results of antibacterial tests for the n-butanol, ethyl acetate, and chloroform fractions of *M. charantia* against *S. aureus*, *S. typhi*, and *E. coli* (Tables 4–7) were evaluated at concentrations of 100 mg/mL, 50 mg/mL, 25 mg/mL, and 12.5 mg/mL, respectively. The results revealed that the antibacterial activity of the extract is concentration-dependent, which agrees with previous studies on various medicinal plants tested [39,40]. The highest mean inhibition observed against *S. typhi* in both n-butanol and ethyl acetate fractions, while no significant difference ($p < 0.05$) was noted in the chloroform fraction. Both fractions exhibited the lowest zone of inhibition for *S. typhi*. Similar findings by Singh et al. [41] also reported reduced zones of inhibition for *S. typhi*. Previous studies indicate that anthraquinones possess intercalating activity against plasma phospholipids, inducing oxidative stress and increasing the likelihood of bacterial cell rupture [33]. This suggests that chloroform may not be a good solvent for extracting antibacterial compounds from *M. charantia*.

Table 4. Antibacterial Activity of n-butanol fraction of *M. charantia.*

	Mean Zones of Inhibition (mm) ± Standard Deviation of *M. charantia*				
	Concentration (mg/mL)				
Bacterial	**100**	**50**	**25**	**12.5**	**Ciprofloxacin**
Staphylococcus aureus	10.67 ± 0.15 ^{b}	7.50 ± 0.10 ^{c}	4.67 ± 0.21 ^{c}	3.33 ± 0.15 ^{c}	35.50 ± 0.50 ^{c}
Salmonella typhi	12.63 ± 0.15 ^{a}	11.10 ± 0.10 ^{a}	9.63 ± 0.12 ^{a}	6.10 ± 0.10 ^{a}	38.80 ± 0.44 ^{b}
Escherichia coli	9.87 ± 0.25 ^{c}	8.40 ± 0.10 ^{b}	6.40 ± 0.10 ^{b}	4.33 ± 0.06 ^{b}	42.63 ± 0.15 ^{a}

Values are means ± standard deviation of triplicate (n = 3) determinations. Values with different superscripts in the column differ significantly at $p < 0.05$ using the Duncan Multiple Range Test (DMRT).

Table 5. Anti-bacterial Activity of ethyl acetate fraction of *M. charantia* against bacterial isolates.

	Mean Zones of Inhibition (mm) ± Standard Deviation of *M. charantia*				
	Concentration (mg/mL)				
Bacterial	**100**	**50**	**25**	**12.5**	**Ciprofloxacin**
Staphylococcus aureus	7.43 ± 0.05 [b]	4.60 ± 0.10 [b]	3.53 ± 0.06 [b]	2.23 ± 0.15 [b]	35.5 ± 0.50 [c]
Salmonella typhi	8.07 ± 0.06 [a]	6.23 ± 0.21 [a]	5.20 ± 0.26 [a]	3.23 ± 0.06 [a]	38.80 ± 0.44 [b]
Escherichia coli	6.60 ± 0.10 [c]	4.43 ± 0.06 [b]	2.93 ± 0.06 [c]	1.53 ± 0.06 [c]	42.63 ± 0.15 [a]

Values are means ± standard deviation of triplicate (n = 3) determinations. Values with different superscripts in the column differ significantly at $p < 0.05$ using the Duncan Multiple Range Test (DMRT).

Table 6. Anti-bacterial activity of chloroform fraction of *M. charantia* against bacterial isolates.

	Mean Zones of Inhibition (mm) ± Standard Deviation of *M. charantia*				
	Concentration (mg/mL)				
Bacterial	**100**	**50**	**25**	**12.5**	**Ciprofloxacin**
Staphylococcus aureus	5.13 ± 0.06 [a]	3.20 ± 0.10 [b]	2.36 ± 0.15 [b]	1.27 ± 0.06 [c]	35.50 ± 0.50 [c]
Salmonella typhi	5.10 ± 0.10 [a]	4.20 ± 0.10 [a]	2.57 ± 0.06 [a]	1.90 ± 0.10 [a]	38.80 ± 0.44 [b]
Escherichia coli	5.10 ± 0.10 [a]	4.03 ± 0.12 [a]	2.27 ± 0.06 [b]	1.53 ± 0.15 [b]	42.63 ± 0.15 [a]

Values are means ± standard deviation of triplicate (n = 3) determinations. Values with different superscripts in the column differ significantly at $p < 0.05$ using the Duncan Multiple Range Test (DMRT).

Table 7. Minimum Inhibitory Concentration (MIC) and Minimum Bactericidal Concentration (MBC) of fractions of *M. charantia* against bacterial isolates.

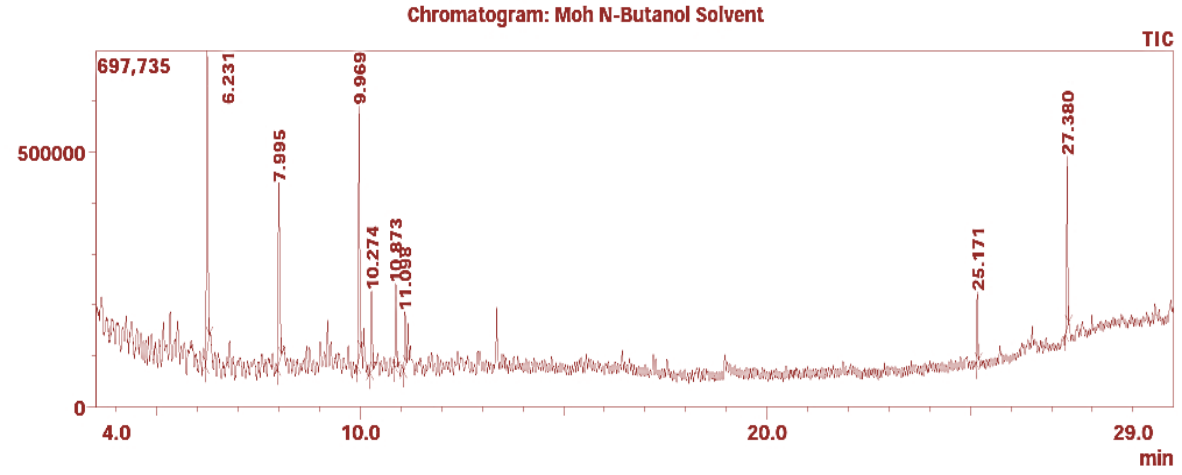
Bacteria	**MIC (mg/mL)**			**MBC(mg/mL)**		
	N-Butanol	**Ethyl Acetate**	**Chloroform**	**N-Butanol**	**Ethyl Acetate**	**Chloroform**
S. aureus	50 ± 0.61 [a]	100 ± 0.34 [a]	100 ± 0.28 [a]	100 ± 0.61 [a]	-	-
S. typhi	25 ± 0.32 [b]	50 ± 0.12 [b]	50 ± 0.31 [b]	50 ± 0.14 [b]	100 ± 0.28 [c]	100 ± 0.88 [b]
E. coli	50 ± 0.42 [a]	100 ± 0.88 [a]	100 ± 0.18 [a]	100 ± 0.89 [a]	-	-

Values are means ± standard deviation of triplicate (n = 3) determinations. Values with different superscripts in the column differ significantly at $p < 0.05$ using the Duncan Multiple Range Test (DMRT).

The MIC and MBC assays are critical for evaluating the inhibitory effects of plant extracts [42], and sometimes MBC/MIC ratio is calculating to understand the mode of action [43]. Our findings indicate that the n-butanol fraction of M. charantia (Table 4) exhibited the highest antibacterial potency, with the lowest MIC values of 50 mg/mL against *S. aureus*, 25 mg/mL against *S. typhi*, and 50 mg/mL against *E. coli*. This fraction also demonstrated bactericidal activity, as evidenced by the MBC of 100 mg/mL against both *S. aureus* and *S. typhi*. Overall, our assays revealed inhibition of all tested organisms at extract concentrations below 100 mg/mL for the n-butanolic fraction. This MBC result corroborates our preceding MIC findings. A related study by Rahmi and Sari [32] also demonstrated the efficacy of fresh leaf extracts of *M. charantia* against *S. aureus*, while the ethyl acetate extract from the same plant part showed no significant antibacterial effect. Likewise, Validezh et al. [31] reported on the efficacy of *M. charantia* fractions against *E. coli*. The observed differences suggest that the leaves of *M. charantia* may contain higher concentrations of phytonutrients compared to seeds. Previous studies have also confirmed the superior antibacterial efficacy of leaf extracts over fruits and seeds of *M. charantia* [20,22].

The results of GC-MS are presented in Figure 1 and Table 8. The GC-MS analysis of the n-butanol fraction of *Momordica charantia* identified a diverse array of compounds, including 2-pentanone, 4-hydroxy-4-methyl- ($C_6H_{12}O_2$), n-amyl isovalerate ($C_{10}H_{20}O_2$), and squalene ($C_{30}H_{50}$), each exhibiting distinct molecular weights and high similarity indices, indicative of their potential biological activities. The presence of esters and ketones suggests significant antibacterial properties, with compounds such as 2(5*H*)-furanone and di-n-octyl phthalate highlighting the extract's complexity and potential for broader pharmacological applications. Notably, squalene's high similarity index underscores its recognized antioxidant and health-promoting benefits, positioning *M. charantia* as a promising source of natural bioactive agents. These findings warrant further exploration of the synergistic interactions among these compounds and their mechanisms of action in antimicrobial therapies and functional applications. Previous research indicated that GC-MS analysis of the leaves and fruits of *M. charantia* resulted in the identification of 18 compounds, whereas hydrodistillation yielded 21 compounds. Notably, benzaldehyde, linalool, and β-cyclocitral were detected using both techniques, with linalool being the predominant compound in each method reflecting their potential medicinal applications and common use in herbal practices [44]. It was also reported that there is a strong correlation of the GC-MS-based metabolite profile of *Momordica*

charantia fruit and its antioxidant activity [45]. To the best of our knowledge, there is currently no study that links experimentally the correlation between the GC-MS-based metabolite profile of *Momordica charantia* and its antibacterial potential. Therefore, we recommend conducting further studies on this topic. Our study provides preliminary evidence that these metabolites may act either independently or synergistically to contribute to the observed antibacterial activity of *Momordica charantia*.

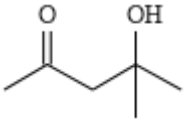
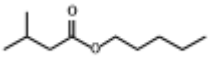
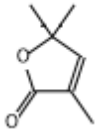
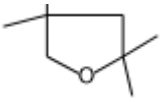
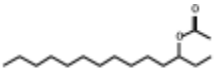
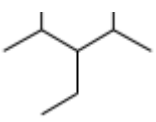


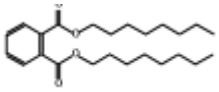
Peak Report TIC

Peak#	R.Time	I.Time	F.Time	Area	Area%	Height	Height%	A/H	Name
1	6.231	6.190	6.290	1531838	29.63	591280	25.21	2.59	2-Pentanone, 4-hydroxy-4-methyl-
2	7.995	7.960	8.065	982302	19.00	354992	15.13	2.77	
3	9.969	9.925	10.015	947603	18.33	493668	21.05	1.92	2(5H)-Furanone, 3,5,5-trimethyl-
4	10.274	10.240	10.330	369414	7.15	158104	6.74	2.34	Furan, tetrahydro-2,2,4,4-tetramethyl
5	10.873	10.840	10.920	319913	6.19	163303	6.96	1.96	3-Tetradecanol acetate
6	11.098	11.065	11.135	206082	3.99	109235	4.66	1.89	Pentane, 3-ethyl-2,4-dimethyl-
7	25.171	25.140	25.205	214461	4.15	137044	5.84	1.56	Di-n-octyl phthalate
8	27.380	27.340	27.420	598275	11.57	337926	14.41	1.77	Squalene
				5169888	100.00	2345552	100.00		

Figure 1. Gas Chromatography, Mass Spectroscopy analysis of n-butanol fraction of *M. charantia.*

Table 8. Molecular formula and structures of probable compounds identified in the n-butanol fraction of *M. charantia* by GC-MS.

Compound	Formula	Molecular Weight	Similarity Index (SI)	Structure
2-Pentanone, 4-hydroxy-4-methyl-	$C_6H_{12}O_2$	116	87	
n-Amyl isovalerate	$C_{10}H_{20}O_2$	172	89	
2(5*H*)-Furanone, 3,5,5-trimethyl-	$C_7H_{10}O_2$	126	84	
Furan, tetrahydro-2,2,4,4-tetramethyl-	$C_8H_{16}O$	128	81	
3-Tetradecanol acetate	$C_{16}H_{32}O_2$	256	80	
Pentane, 3-ethyl-2,4-dimethyl-	C_9H_{20}	128	81	

Di-n-octyl phthalate	$C_{24}H_{38}O_4$	390	86	
Squalene	$C_{30}H_{50}$	410	90	

4. Conclusion

This study highlights the antibacterial potential of *Momordica charantia* methanolic leaf extract and its fractions, particularly the n-butanol fraction, against gram-negative pathogens like *Salmonella typhi* and *Escherichia coli*. The presence of bioactive compounds such as alkaloids, saponins, flavonoids, glycosides, and terpenoids, confirmed through phytochemical screening, supports its use in traditional medicine. Notably, the antibacterial activity observed, especially the good inhibition against *S. typhi* and *E. coli*, underscores the potential of *M. charantia* as a source of antibacterial molecules. However, this preliminary study recommends further advanced techniques such as nuclear magnetic resonance (NMR) spectroscopy, high-performance liquid chromatography (HPLC), and mass spectrometry could be employed to identify and purify these bioactive compounds. Additionally, in vivo studies should be conducted to assess the safety, toxicity, and therapeutic efficacy of the bioactive components of *M. charantia*. Such research would offer valuable insights into the pharmacokinetics and pharmacodynamics of these compounds, guiding their potential clinical application. The development of novel dosage forms, such as nano-formulations or topical applications, could also enhance the delivery and effectiveness of *M. charantia* molecules in controlling bacterial infections.

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Article

Evaluation of Therapeutic Activity of *Physalis angulata* (*In Vitro Studies*)

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Abstract: *Physalis angulata* L. family Solanaceae, commonly known as ground cherry, cape gooseberry, or bladder cherry, has a long history of traditional use in various regions around the world. The primary goal of this study is to investigate the different pharmacological effects produced by the ethanolic leaf extracts of *Physalis angulata.* The leaf extract was prepared in two different dosages: 250 mg/kg body weight and 500 mg/kg body weight, which were administered according to the body weight of the mice. In yeast-induced pyrexia in mice, after 4 h, positive control (Paracetamol 150 mg/kg), *Physalis angulata* 250 mg/kg, *Physalis angulata* 500 mg/kg expressed temperature were 98.78 ± 0.051 °F, 97.4 ± 0.213 °F and 96.56 ± 0.177 °F respectively. In the evaluation of acetic acid-induced peripheral analgesic activity, *P. angulata* extract exhibited 43% and 63% inhibition of writhing at 250 mg/kg and 500 mg/kg body weight, respectively. Whereas the standard Diclofenac-Na inhibited 76% at a dose of 25 mg/kg body weight. In castor oil-induced diarrhea, plant extract inhibited defecation by 59.65% at 250 mg/kg body weight and 72.45% at 500 mg/kg b.w., whereas standard loperamide at a dose of 3 mg/kg b.w. inhibited 83.50% of defecation. Ethanolic extract of *Physalis angulata* at the dose of 300 mg/kg, 2000 mg/kg and 5000 mg/kg showed average weight 21.2 ± 1.56 gm, 21.8 ± 0.82 gm and 24.45 ± 1.51 gm respectively at 2nd day. The disc diffusion method has been adopted for the evaluation of antimicrobial activity. The ethanolic extracts of *Physalis angulata* leaf exhibited inhibitory activity against fourteen strains, including Bacillus megaterium, Salmonella paratyphi, Candida aibicans, Vibrio mimicus, and Staphylococcus aureus.

Keywords: *Physalis angulata*; analgesic; antipyretic; antidiarrheal; acute toxicity; antimicrobial

1. Introduction

Since the dawn of civilization, people have relied heavily on the therapeutic qualities of medicinal plants. The World Health Organization estimates that even now, almost 80% of the world's population, particularly in less developed countries uses traditional medicine as their major source of healthcare [1]. There are roughly 2000 different ethnic groups in the world, and nearly all of them have unique traditional medicinal practices [2]. *Physalis angulata* (Family: Solanaceae) commonly referred to as the cut-leaf ground cherry or bladder cherry, is an herbaceous shrub that is native to tropical America but is currently found as a weed around the world. This erect herb boasts smooth leaves with deeply cut edges. *Physalis angulata*, is a bit of a bully in the garden. It spreads easily, handles most weather conditions, and shrugs off weed killers. This bushy annual herb stands roughly 50 cm tall and has smooth or slightly hairy stems [3]. Beyond its well-known ability to strengthen the immune system, this plant surprisingly finds a role in the kitchen, particularly when making sauces [4]. Standardised medicinal plant extracts are used in a variety of traditional and mainstream medications across the globe. Natural remedies have been shown to regulate the aberrant cell division process and have a very good therapeutic index against various tumour cell. *Physalis angulata* boasts a rich history of medicinal use in Japan, particularly for fevers. This plant's extracts or infusions are used as dermatitis, asthma, and anti-malarial remedies in many parts of the world

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[5,6]. Scientific investigations have revealed a diverse range of molecules within the plant, including carbohydrates, minerals, vitamins, and fats. Interestingly, the entire plant contains various steroidal lactones that belong to physaline and withanolide, such as physalins A–I, physagulin A–G, withangulatin A, and withanolide T. Withanolides have a C-28 ergostane-type steroid structure with a δ-lactone group at C-22 and C-26. It also contains a flavonol glycoside named myricetin 3-*O*-neohesperidoside [7–10]. Interestingly, people in some places actually eat the leaves and fruits, and parts of the plant might even have medicine in them. Scientists are looking into these special molecules called anolides to see if they can fight cancer and inflammation [11]. Furthermore, in vitro studies have shown promise for the plant's potential as an anticancer agent. Specifically, *P. angulata* leaf extracts have demonstrated cytotoxic effects against various cancer cell lines, including Y79, HeLa, DLD-1, MCF-7, and HGC-27 [12]. These findings instigate further investigation to explore the potential therapeutic applications of *P. angulata* extracts and their isolated bioactive compounds. *P. angulata* leaves have a rich history of traditional use as medicine in Southeast Asia, North America, and South America, particularly for treating bacterial infections. This widespread use suggests the potential antibacterial properties of the plant. However, this potential remains largely unexplored by modern science. Crucially, no in vivo studies, meaning studies conducted within living organisms like mice or humans, have been documented to definitively confirm the effectiveness of *P. angulata* against bacteria. Further scientific exploration through in vivo research is necessary to bridge the gap between traditional knowledge and validate the potential of *P. angulata* as a bactericidal agent [13]. Ingesting significant amounts of *Physalis angulata* might be harmful to one's health because it contains poisonous chemicals, mainly glycoalkaloids and steroidal lactones like physalins. In particular, the unripe fruits and leaves contain glycoalkaloids, which have been linked to neurological side effects including headaches and dizziness as well as gastrointestinal symptoms like nausea, vomiting, and diarrhoea. High doses or extended exposure can, in extreme circumstances, cause toxicity to the kidneys and liver. The cytotoxic effects of physalins can be hazardous, particularly at elevated dosages, despite their therapeutic benefits. It can be dangerous to consume too much of the plant or to eat its unripe sections, even though it has medical uses [14–16].

Thus, we have tried to investigate the pharmacological antipyretic, analgesic, antidiarrheal, acute toxicity, and antimicrobial activity, of the plant extracts that could be beneficial in further drug discovery.

2. Materials and Methods

2.1. Flowchart outlining the experimental steps

The flowchart below outlines the complete experimental workflow, beginning with plant collection and concluding with the various testing methods (Figure 1).

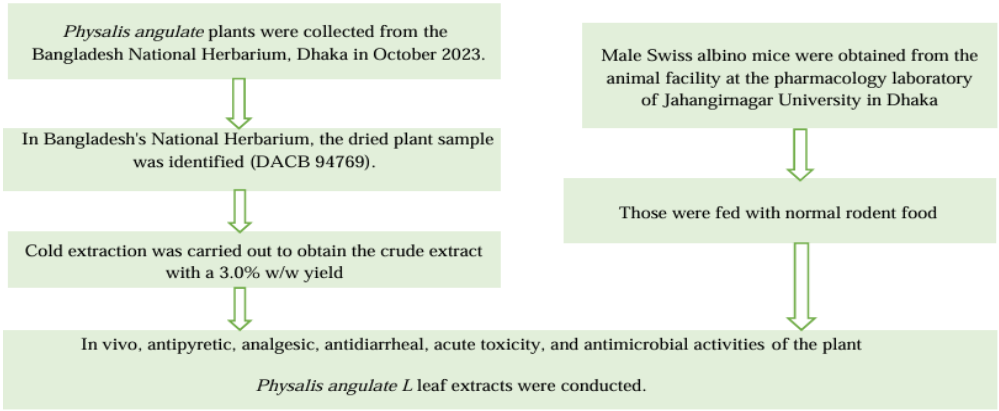

Figure 1. Diagrammatic representation of the experimental protocol.

2.1.1. Plant Material Collection

Physalis *angulata* (Voucher specimen: 94769 DACB) was collected in October 2023 from the Bangladesh National Herbarium, Dhaka. After removing unwanted materials, the plant's leaves were dried in the shade and ground into a coarse powder.

2.1.2. Extraction

For 14 days, 350 g of the coarse powder was soaked in of ethanol with a 10:1 solvent to dry weight ratio in sealed glass containers, and shaken intermittently [17,18]. The mixture was filtrated by a clean cloth followed by

cotton, and then filter paper [19]. The ethanol extract was filtered and concentrated through a rotary evaporator. 10.2 g of crude extract from 350 g of dry powder with a yield rate of approximately 3%.

2.2. Experimental Animals

The trials were performed on young Swiss Albino mice that were 4–5 weeks old and weighed between 20–30 gm. The animals were obtained from the animal facility at the pharmacology laboratory of Jahangirnagar University in Dhaka. The subjects had one week of acclimatization in the animal facility of the Department of Pharmacy at Dhaka International University, located in Bangladesh. The mice were provided with regular laboratory food and water without any restrictions, and their natural day-night cycle was preserved. The research was conducted with proper ethical approval (Ref No: CPP/DIU/EC/005) at Dhaka International University.

2.3. Experimental Design for Antipyretic Activity

To assess the antipyretic properties of *Physalis angulata* extract, a previously established method using yeast-induced fever in mice was employed described by Subedi et al. [20]. The experiment involved four groups of five mice each; Group I (control) received saline (10 mL/kg), Group II (standard) received paracetamol (150 mg/kg), Group III received *Physalis angulata* extract (250 mg/kg), and Group IV received the same extract (500 mg/kg). All preparation was made by dissolving the materials in distilled water.

The mice were weighed to establish the appropriate dosage, and their baseline body temperatures were measured using a digital thermometer. Fever was induced by administering a subcutaneous injection of a 15% Brewer's yeast suspension at a dose of 10 mL/kg. Following an overnight fast with unrestricted access to water, rectal temperatures were recorded 24 h post-injection [21]. Animals with a temperature rise of less than 0.5 °C were excluded, while those with a rise above 0.5 °C were confirmed to have pyrexia. Each group received its respective treatment, and rectal temperatures were measured 1,2,3, and 4 h post-medication.

2.4. Experimental Design for Analgesic Activity

Using the model of acetic acid-induced writhing in mice, the analgesic efficacy of *Physalis angulata* extract was investigated according to the method described by Debnath et al. [22,23]. The study included four groups with every group containing 5 mice; Group I (negative control): 1% Tween-80 in distilled water, 10 mL/kg orally given, Group II (positive control): Diclofenac Na in distilled water, 25 mg/kg orally given, Group III: *Physalis angulata* leaf extract in distilled water, 250 mg/kg d orally given, Group IV: *Physalis angulata* leaf extract in distilled water, 500 mg/kg orally given.

Mice were administered test and control solutions using a feeding needle and injected intraperitoneally with 0.7% acetic acid at 30-min intervals. Writhing frequency was recorded over 15 min as a measure of distress [24,25]. Any unfinished writhing was regarded as partial writhing; hence, two partial writhing were seen as one complete writhing.

The percentage of writhing inhibition relative to the control group was used as a measure of analgesia and was determined using the following formula:

$$\text{Inhibition of writhing \%} = [(W_c - W_t) / W_c)] \times 100$$

where, W_t is the average number of writhing in the test group and. W_c is the average number of writhings in the control group.

2.5. Evaluation of In-vivo Antidiarrheal Activity

The mice were divided into four groups to assess the antidiarrheal effects of *Physalis angu*lata extract in a model of castor oil-induced diarrhea described by Jahan et al. [26,27]. Each group contained five animals. The groups were as follows: Group I (control group, treated orally with 1% Tween-80 in distilled water), Group II (standard group, administered Loperamide at a dose of 3 mg/kg body weight), Group III (test group-I, given *Physalis angulata* leaf extract at 250 mg/kg body weight), and Group IV (test group-II, given *Physalis angulata* leaf extract at 500 mg/kg body weight). One hour prior to receiving a 0.5 mL oral dose of castor oil, each group was given their respective treatments. The mice were then individually housed in separate cages lined with blotting paper to observe for signs of diarrhea. After administering the castor oil, the occurrence of diarrhea was monitored and recorded hourly for four hours. The number of fecal deposits or any other fluid that stained the blotting paper was counted and noted for each mouse. The latency period for the onset of diarrhea was also recorded for each mouse. Fresh blotting papers were replaced every hour.

2.6. Evaluation of Acute Toxicity Test

The oral acute toxicity study of ethanolic extract of *Physalis angulata* was evaluated according to Organization for Economic Cooperation and Development (OECD) guideline 423 on BALB/c mice (20–30 g) [28,29]. Prior to the experiment, all the animals were housed in an overnight fasting state with unrestricted access to water. There were four groups, each consisting of five animals. Group I received an oral administration of a normal saline solution containing 0.9% sodium chloride (NaCl). The mice in this group were given a solution of (0.01 times their body weight) milliliters on the first day. Groups II and III and, IV received the extract orally at doses of 300, 2000, and 5000 mg/kg body weight (dissolved in purified water). The mice in this group were given a solution of (0.01 times their body weight) milliliters on the first day. The animals were monitored for any toxicological impact throughout the initial 4-h period following treatment [30]. In addition, animals were examined for a period of 3 days to determine if there were any harmful consequences [31]. Furthermore, alterations in behavior and several factors like body mass, urine patterns, food consumption, body temperature, and alterations in eye and skin pigmentation were observed.

2.7. Determination of Antimicrobial Activity

The minimum inhibitory concentration of the *Physalis angulata* extract was evaluated using the broth dilution method following Clinical and Laboratory Standards Institute (CLSI) guidelines (document M26-A). *Pseudomonas aureus*, *Bacillus megaterium*, *Salmonella paratyphi*, *C. albicans*, *Salmonella typhi*, *Staphylococcus aureus*, *Vibrio Parahaemolyticus* and *E. coli* were used in the experiment.

To prepare bacterial suspensions for testing, a small quantity of each organism in its active growth stage was transferred to vials with fresh, nutrient-rich liquid and incubated in a controlled environment to enhance growth. After several hours, a tiny amount from each vial was moved to fresh broth in test tubes and mixed thoroughly. The bacterial suspensions were then aseptically transferred to individual Petri dishes, ensuring uniform distribution by gently rotating the dishes. Five petri dishes were prepared for each of the five bacterial strains, and all dishes were appropriately labeled.

Ten 5 mm filter paper discs were placed on the agar surface of each petri dish using sterile forceps. The discs were positioned at least 15 mm from the edge and spaced to avoid overlapping inhibitory zones. In a sterile laminar flow hood, 10 µL of extract solutions (250 µg/10 µL and 500 µg/10 µL) were added to the discs with a micropipette, resulting in final concentrations of 250 µg and 500 µg per disc for testing [32].

Kanamycin antibiotic discs (30 µg/disc) were used as a positive control, placed on each petri dish to confirm test organism susceptibility and compare responses with the test samples. After applying the discs, the plates were inverted and incubated at 37 °C for 16–18 h. Post-incubation, the antibacterial activity was assessed by measuring the diameter of the inhibition zones in millimeters with a calibrated scale [32].

2.8. Statistical Analysis

Every test parameter's mean and SEM were utilized to calculate the data. All of the study's data were analyzed using GraphPad Prism 9 and Microsoft Excel, utilizing a one-way ANOVA and the Dunnett test. All differences were considered for statistical significance at $p < 0.05$.

3. Results

3.1. Evaluation of Antipyretic Activity

There is a significant change in rectal temperature (In Fahrenheit) over time. The changes in body temperature for negative control, positive control (Paracetamol 150 mg/kg), *Physalis angulata* 250 mg/kg, and *Physalis angulata* 500 mg/kg extracts were shown in at different time intervals (Figure 2).

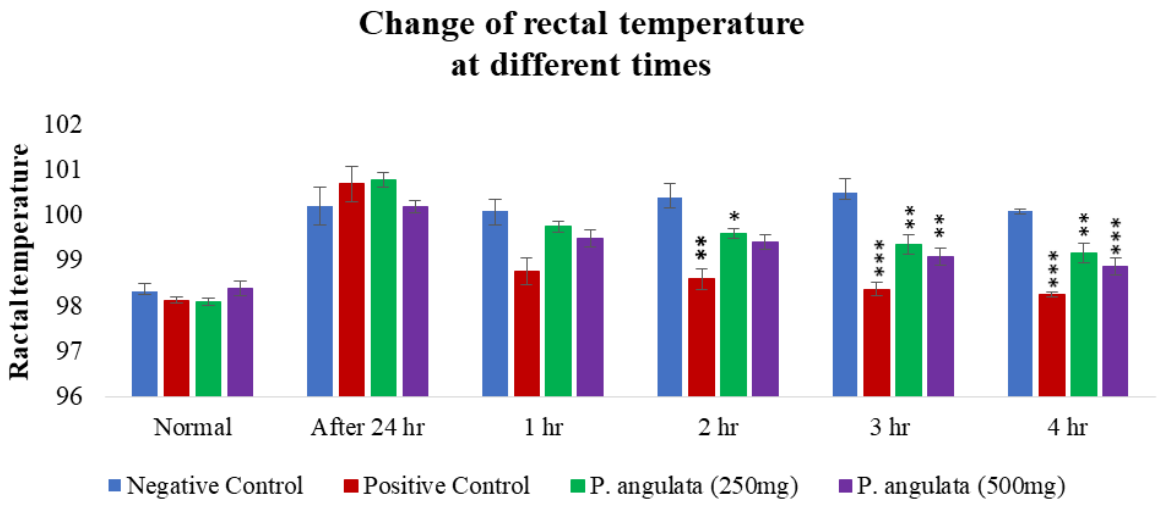

Figure 2. Change of rectal temperature at different times in different samples of *P. angulata* (Significance: * P<0.05, ** p<0.01, ***p<0.001).

3.2. Determination of Analgesic Activity

In a model of the writhing reflex, *Physalis angulata* extract showed analgesic properties. The analgesic effect of different doses, standard and negative control groups are shown in Figure 3. Comparing the two doses (250 mg/kg and 500 mg/kg) to the negative control group revealed a significant ($p < 0.05$) decrease in writhing. Furthermore, the traditional drug diclofenac sodium shown a highly substantial effect ($p < 0.0001$) (Figure 3).

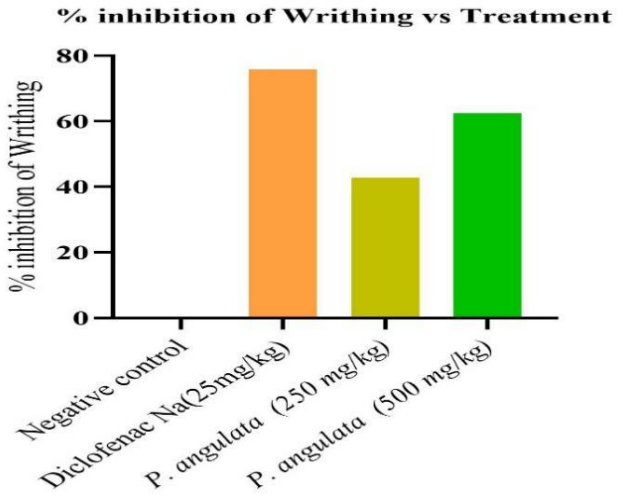

Figure 3. Percentage inhibition of writing of different groups on acetic acid-induced writing in mice (standard was Diclofenac Na).

3.3. Evaluation of In-Vivo Antidiarrheal Activity

The latent durations for the control, standard (Loperamide 3 mg/kg), plant extract 250 mg/kg, and 500 mg/kg were 48 min, 172.2 min, 118.8 min, and 130.2 min, respectively (Figure 4). The percentages of defecation inhibition were 83.50, 59.65, and 72.45% for the standard (loperamide 3 mg/kg), the *Physalis angulata* 250 mg/kg, and the *Physalis angulata* 500 mg/kg (Figure 5).

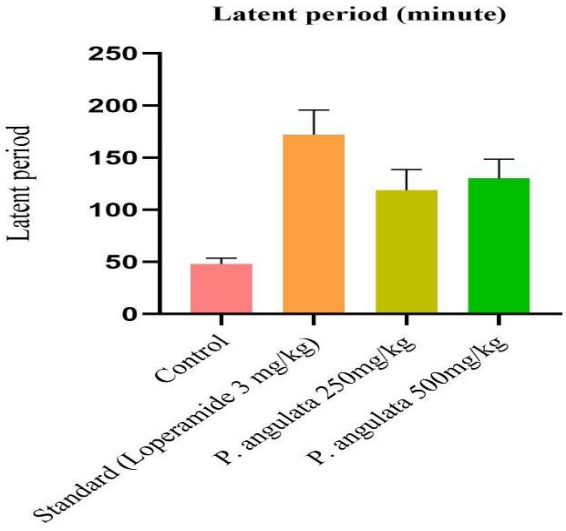

Figure 4. Effect of the extract of *Physalis angulata* on prolongation of the latent period in castor oil-induced diarrheal episodes in mice.

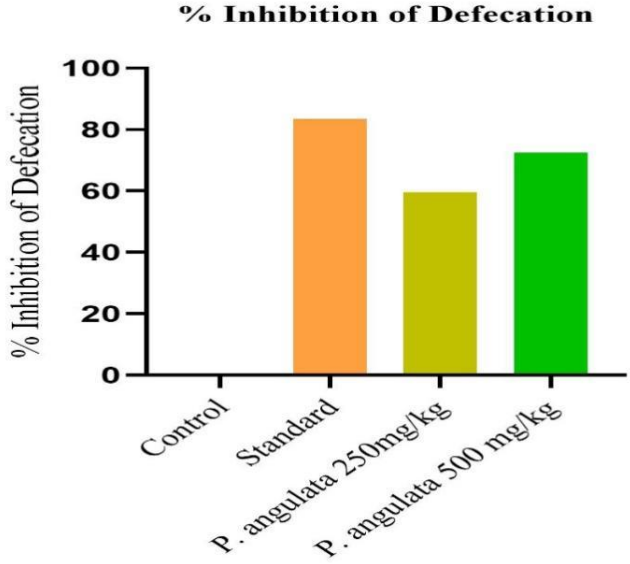

Figure 5. Percentage inhibition of defecation of four different groups**.**

3.4. Evaluation of Acute Toxicity Test

The mean weight of mice in the control group (Group-I) experienced a modest increase from 26.6 ± 1.76 gm to 28.3 ± 1.82 gm, indicating normal growth. However, Group-II (300 mg/kg) exhibited a weight gain that was comparable to the control group. On the other hand, Groups III (2000 mg/kg) and IV (5000 mg/kg) experienced lesser weight gains, ranging from 15.8 ± 0.92 gm to 21.8 ± 0.82 gm and 22 ± 1.48 gm to 24.45 ± 1.51 gm, respectively (Figure 6).

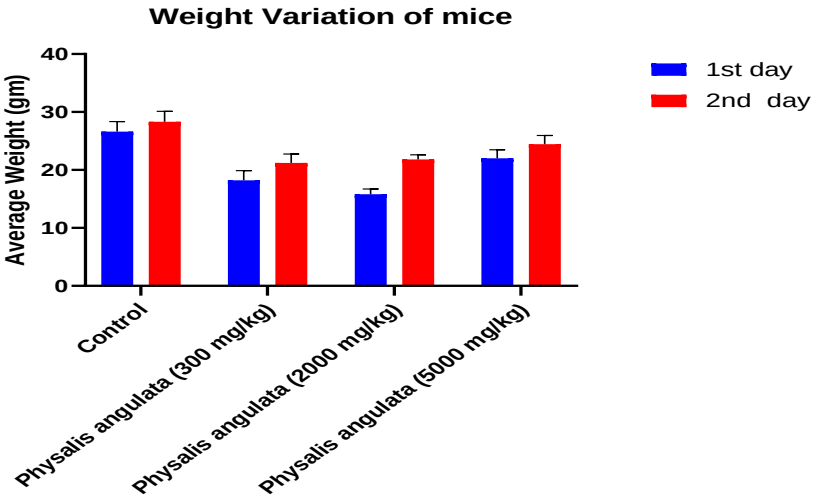

Figure 6. Weight variation of different groups.

3.5. Determination of Antimicrobial Activity

The crude 250 µg/disc plant extract exhibited a good zone of inhibition against *Pseudomonas aureus* (10 mm), *Bacillus megaterium* (15 mm), *Salmonella paratyphi* (10 mm), *C. albicans* (10 mm), *Salmonella typhi* (13 mm), *Staphylococcus aureus* (12 mm), but *Vibrio Parahaemolyticus* and *E. coli* showed no zone of inhibition while the dose of *Physalis angulata* 500 µg/disc sample showed good to moderate zone of inhibition against most of the strains notably *Shigella boydii* (11 mm), *Shigella dysenteriae* (12 mm), *Pseudomonas aureus* (11 mm), *Bacillus megaterium* (19 mm), *Bacillus subtilis* (12 mm), *Salmonella paratyphi* (20 mm), *Candida aibicans* (13 mm), *Aspergillus niger* (11 mm), *Salmonella typhi* (11 mm), *Vibrio mimicus* (13 mm), *Staphylococcus aureus* (15 mm) (Figure 7).

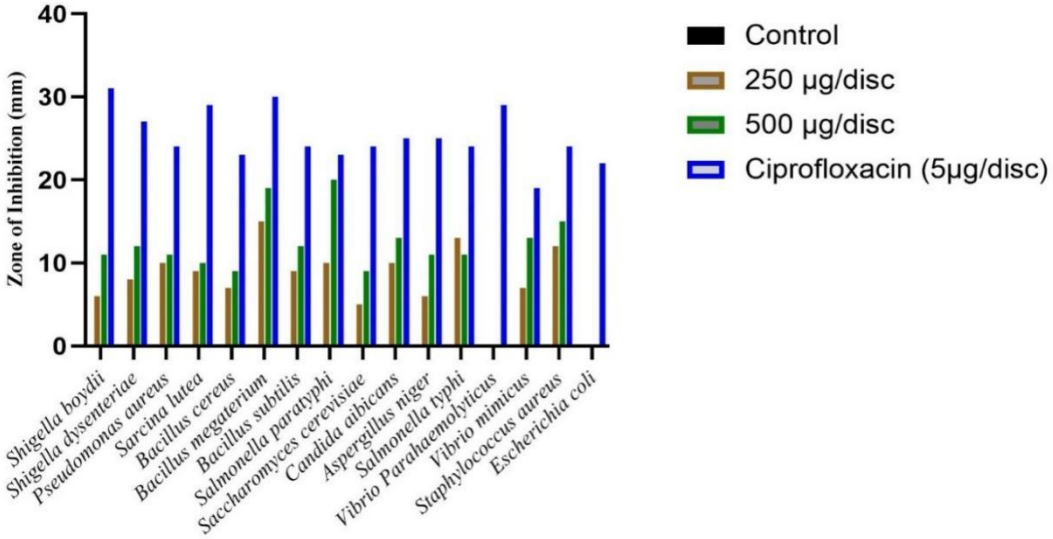

Figure 7. Zone of inhibition of *P. angulata* leaf extracts against sixteen strains.

4. Discussion

A number of plants have been utilized in traditional medicine for many years. Certain treatments appear to be effective even if there may not be enough scientific evidence (double-blind trials, for instance) to support this claim. These plants ought to be considered medicinal herbs [33]. The leaves of *Physalis angulata* possess antimicrobial, cytotoxic, and antioxidant activity. This study gives information about the antipyretic, analgesic, antidiarrheal, antimicrobial, and anti-toxic effects of *Physalis angulata* leaves.

Using ethanol, *Physalis angulata* leaves were extracted, producing a yield of about 3% by weight. Many existing anti-inflammatory and fever-reducing medications (both steroid and non-steroid based) cause a variety of

side effects. This is mainly because these drugs block the activity of two enzymes, COX-1 and COX-2, in a non-specific way [34]. As a result, researchers are actively searching for drugs that selectively target COX-2 or work through entirely different mechanisms to minimize side effects [35,36]. In this study, *Physalis angulata* leaf extract exhibited a statistically significant effect in antipyretic, analgesic, antidiarrheal, acute toxicity and antimicrobial tests. After 4 h of extract administration, *Physalis angulata* 250 mg/kg, *Physalis angulata* 500 mg/kg expressed temperatures of 97.4 ± 0.213 °F and 96.56 ± 0.177 °F, respectively. When comparing *Physalis angulata* to other medicinal plants like; *Launaea sarmentosa* and *Aegialitis rotundifolia*, *Physalis angulata* shows a clear dose-dependent antipyretic effect. At 250 mg/kg, *Physalis angulata* reduced the temperature to 97.4 ± 0.213 °F after 4 h, while at 500 mg/kg, it further decreased to 96.56 ± 0.177 °F. In contrast, *Launaea sarmentosa* at 400 mg/kg lowered the temperature to 97.48 °F after 5 h, comparable to the 250 mg/kg dose of *Physalis angulata*. *Aegialitis rotundifolia*, on the other hand, showed a more moderate effect, reducing the temperature to 97.9 °F, which is higher than both doses of *Physalis angulata*. Therefore, *Physalis angulata* at 500 mg/kg demonstrates stronger antipyretic activity, achieving a temperature close to the standard acetyl salicylic acid [29].

Analgesics are substances that, either centrally or peripherally, act on the sensory nerve system to lessen or remove pain without appreciably changing awareness. Two categories of analgesics exist: non-opioid/non-narcotic/antipyretic/anti-inflammatory analgesics, and opioid/narcotic/morphine-like analgesics, which depress the central nervous system. Non-opioids mainly affect peripheral pain pathways and increase the pain threshold in the central nervous system [37]. Any substance that reduces the amount of writhing will exhibit analgesia through the peripheral route of prostaglandin production suppression. Our findings from the experiment for acetic acid-induced belly constriction showed a significant decrease in writhing reflux. The analgesic effect seen at doses of 250 mg/kg and 500 mg/kg was similar to that of the NSAID diclofenac sodium standard medication. The peripheral analgesic efficacy of *Physalis angulata* leaf extract may be mediated by cyclooxygenase inhibition, which inhibits local peritoneal receptors. *Physalis angulata* showed dose-dependent analgesic effects, inhibiting writhing by 43% at 250 mg/kg and 63% at 500 mg/kg. This is comparable to medicinal plants like; *Launaea sarmentosa* (AELS) at 400 mg/kg, which also inhibited writhing by 63.1%, while *Aegialitis rotundifolia* (AEAR) showed 57.1% inhibition at the same dose. At lower doses, AELS (200 mg/kg) and AEAR showed 55.36% and 47.86% inhibition, respectively, which aligns with Physalis angulata at 250 mg/kg. The standard drug at 50 mg/kg showed the highest inhibition of 69.23% [29].

Castor oil causes diarrhea due to its active metabolite, ricinoleic acid, which stimulates intestinal peristalsis and fluid secretion [38]. This causes alterations in the intestinal mucosa's electrolyte permeability and increases peristaltic movement in the small intestine [39]. Castor oil has also been shown to boost the release of endogenous prostaglandin [40]. *Physalis angulata* extract possesses significant anti-diarrheal activity, decreasing the rate of defecation, and increasing the percentage inhibition of defecation. Consequentially, the *Physalis angulata* extract at 500 mg/kg showed a better effect than the 250 mg/kg extract dose. The extract reduced the amount of defecation by castor oil-induced diarrhea by 69.65% at 250 mg/kg body weight and 72.45% at 500 mg/kg body weight.

An essential component of determining the safety of synthetic or natural compounds meant for ingestion by humans or animals—as well as any potential environmental effects—is the assessment of acute toxicity. Determining the harmful effects of a material after a single or brief exposure, usually over a period of 24 to 48 h, is known as acute toxicity testing [31]. The average body weight of the control group considerably dropped while the average body weight of the *Physalis angulata* leaf extract group showed no alteration in appearance or behavior and increased average body weight, indicating that it had no harmful effect. On the second day, the average weight of the *Physalis angulata* ethanolic extract at doses of 300 mg/kg, 2000 mg/kg, and 5000 mg/kg was 21.2 ± 1.56 gm, 21.8 ± 0.82 gm, and 24.45 ± 1.51 gm, respectively. An earlier investigation demonstrated the *Physalis angulata* leaves's antitoxin properties by treating HeLa cell cultures with an ethanolic extract. In addition, the anticancer cell's notable decline and the cell's morphological alterations [5].

An ethanolic extract from *Physalis angulata* has shown efficacy against various bacterial strains. It previously demonstrated antibacterial activity against *Escherichia coli* (ATCC 25922) and *Staphylococcus aureus* (ATCC 25923). The minimum bactericidal concentration (MBC) and minimum inhibitory concentration (MIC) values for *E. coli* were 5 mg and 10 mg, respectively, while for *S. aureus*, the MBC and MIC were 2.5 mg and 5 mg, respectively [5]. A further study assessed the antibacterial activity of *Physalis angulata* leaf extracts against ATCC strains of *Pseudomonas aeruginosa* (27853), *Klebsiella pneumoniae* (13883), and *Staphylococcus aureus* (25923), and demonstrated the extracts' efficacy against these organisms [3]. Furthermore, experiments revealed that *P. angulata* leaf extracts were efficient against a variety of bacteria, such as Salmonella species, *Staphylococcus aureus*, and *Escherichia coli*. [41]. This study differs from prior research in that it showed better antibacterial activity against the strains mentioned: *Bacillus megaterium, Salmonella paratyphi, Candida aibicans, Vibrio*

mimicus, and *Staphylococcus aureus*. All microorganisms except *Escherichia coli* and *Vibrio Parahaemolyticus* were susceptible to the effects of *Physalis angulata* plant extracts at doses of 250 mg/kg and 500 mg/ kg.

The study on *Physalis angulata* ethanolic leaf extracts has several limitations that can be addressed in future research. Only two dosages were tested, so exploring a wider range of doses could provide a better understanding of the dose-response relationship. Long-term toxicity studies should be conducted to assess the safety of prolonged use. Investigating the specific molecular mechanisms and identifying the bioactive compounds responsible for the observed effects would deepen understanding of its pharmacological properties. Expanding the antimicrobial evaluation to include more microbial strains, especially drug-resistant ones, could offer a broader insight into its therapeutic potential. Additionally, a detailed phytochemical analysis and comparison with other medicinal plants could help highlight the uniqueness and efficacy of *Physalis angulata* extracts.

5. Conclusion

This work involved the effective extraction of *Physalis angulata* leaf and subsequent pharmacological tests utilizing the ethanolic extract of dried leaf. The materials examined exhibited noteworthy analgesic, antipyretic, antidiarrheal, antibacterial, and cytotoxic actions during our analysis. It is crucial to emphasize that the pharmacological activity detected in the plant extract is entirely dependent on the dosage. Further investigation is necessary to determine and separate the active components accountable for these actions, which could potentially result in the creation of innovative medications aimed at treating a range of disorders and advancing the biomedical sciences. A biochemical analysis might be performed to assess plasma levels of AST, ALT, and ALP in mice with CCl_4-induced hepatotoxicity. Plasma levels of oxidative stress indicators, including MDA, NO, and APOP, can be assessed to determine the analgesic activity in mice.

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