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Qualitative Phytochemical Screening, Fatty Acid Profile and Biological Studies of the Bark of *Mallotus nudiflorus* (Pitali) Plant

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Abstract

In the present study, the *Mallotus nudiflorus* (L.) plant has been taken to determine the *in-vitro* analysis to find out the therapeutic value. The bioassays of the raw extract of methanol of bark of *M. nudiflorus* and by Kupchan's extraction method collecting n-Hexane (HEX), Dichloromethane (DCM), Chloroform ($CHCl_3$), Ethylacetate (EA) and Aqueous (AQ) fractions were scrutinized to find out its therapeutic value. The findings of phytochemical screening of the methanol extract of barks revealed the presence of several secondary metabolites. By using the GC-FID method the result showed that *M. nudiflorus* contained four bound fatty acids and four free fatty acids. EA fraction had the maximum phenolic content among all the fractions at (133.67±0.99) mg of GAE/g where the Folin- Ciocalteu reagent was used as an oxidizing agent. The antioxidant activity was measured in terms of its ability to scavenge free radicals (DPPH assay). Among all extractives, the greatest ability to scavenge for free radicals was shown by EA extract with an IC_{50} value (12.08±0.15) µg/ml. In the toxicity of brine shrimp test, the HEX fraction had the maximum toxicity with an LC_{50} value of (0.12±0.01) µg/ml. Cell cytotoxicity was observed for sample $CHCl_3$ and EA on both the Vero (kidney epithelial cells taken from an African green monkey) and HeLa (a human cervical cancer cell) cell line. All the fractions were subjected to *in vitro* microbial screening, which revealed that DCM, $CHCl_3$, and EA fractions showed growth inhibition, particularly against various Gram-positive and Gram-negative bacteria by disc diffusion method. The maximum zone of inhibition in the antimicrobial activity was produced by $CHCl_3$ fraction against *Staphylococcus aureus* (17 mm). The results of *in vitro* experiments have demonstrated that the extracts from the barks of *M. nudiflorus* have great potential for medicinal uses and might be studied for further chemical exploration.

Keywords: Antimicrobial Activity, Antioxidant, Cell Cytotoxicity, Fatty Acids, *Mallotus nudiflorus*, Phenolic Content, Phytochemical Screening, Toxicity

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1. Introduction

The medical system based on tradition is the primary choice of treatment for 80 per cent of the world's population, particularly in underdeveloped countries[1]. Man's consumption of pharmacological substances, in the form of food, medicines, beverages, and various industrial and household products, has significantly increased. As a result, these substances have the potential to cause both chronic and acute toxicity, ranging from mild to severe, depending on their properties[2]. Even in ancient times, the medicinal properties of plants were well-known to humans. Many of theseplants have been used for centuries in traditional medicine and have gained a reputation as effective remedies[3]. *T. nudiflora* is a member of the Euphorbiaceae family and an essential medicinal plantin Indian medical systems such as Ayurveda and Siddha[4]. The species *Mallotus* was previously named *Neotrewia, Octospermum* and *Trewia.* So, *M. nudiflorus* (L.) well known as *T. nudiflora* is a medium-sized woody tree found in Himalaya to Hainan Island[5]. It is found throughout the warmer regions, along rivers and watersheds, in moist moderately tropical woods. The plant has a long and rich history of use in Indian Ayurvedic medicine[6]. *T. nudiflora* is utilized as a stomachicas well as an astringent in the treatment of flatulence, gout, rheumatism, and cancer, notably leukaemia and hepatobiliary disorders[7]. Excessive bile and sputum are treated with an extract of *T. nudiflora* shoots and leaves[8]. Cardenolides, sterols, and triterpenoids have been found in the phytochemical screening of *T. nudiflora's* extract as well as diterpenoids, alkaloids, nitrogen-containing compounds, lignans, neolignans, simple phenolics, flavonoids, ellagic acid derivatives, phenylpropanoid glucosides, α-tocopherol and the cucurbitacin trewinine were isolated from this plant[9-11]. The bark of *T. nudiflora* was found to contain trewianin and trewioside, as well as scopoletin and indole-3-carboxylic acid. *T. nudiflora* seed has a unique glyceride oil and many new pyridinone alkaloids as well as a protein synthesis inhibitor[12]. Maytansinoids are abundant inthe seed as well. Maytansinoids were found in high concentrations in the seed and pericarp of *T. nudiflora*, according to previous research which is a prominent antifungal constituents[13]. When tested, the seed oil demonstrated its antimicrobial properties by effectively inhibiting the growthof *Mycobacterium tuberculosis*[14].

2. Materials and Methods

2.1 Plant Material Collection and Extraction

M. nudiflorus bark was obtained in November 2021 at Netrakona, Bangladesh, and taxonomic identification was performed at Dhaka University Salar Herbarium (flora of Bangladesh). The authentication code was DUSH 10812. The barks were dried at ambient temperature first, then in a low-temperature oven below 45°C. The dried barks were crushed to arough powder, which was collected in an airtight box and used throughout the study. Bark powder (639 grams) was steeped in 2.5 litres of methanol for 15 days before being filtered through a cotton bud and a number 1 Whatman filter paper. The extract was condensed with a rotary evaporator at 45°C. The modified Kupchan partitioning methodology was used to partition a fraction (10 g) of the condensed methanol extract into n-Hexane (HEX), Dichloromethane (DCM), Chloroform (CHCl3), Ethyl Acetate (EA) and Aqueous (AQ) soluble fractions[15]. Even though *M. nudiflorus* has a lot of potential for disease treatment, very little scientific research has been done in Bangladesh, particularly on the stem bark of *M. nudiflorus*. As a result, the bark of this plant was chosen for the evaluation of its biological activities.

2.2 Phytochemical Screening

The raw extract of methanol of *M. nudiflorus* was evaluated for phenols, alkaloids, phlobatannins, tannoids, triterpene glycosides (saponins), steroid, flavonoids, anthraquinones, cardiac glycoside and isoprenoids using established procedures[16].

2.3 Antimicrobial Activity

DMSO was used as a control to test the antimicrobial of the methanolic raw extract and variousfractions were performed against certain gram-positive bacteria and gram-negative bacteria. The capacity of various extracts to prevent bacterial growth on discs that create an inhibition zone and using the agar diffusion technique, the antimicrobial properties were measured[17]. Blank discs and standard antibiotic (ciprofloxacin) discs serve as positive and negative controls, respectively. To ensure that the test materials are distributed throughout the surrounding medium as far as possible, these plates are maintained at low temperatures (4°C) for about 16 to 24 hours. The plates arethen inverted

and incubated at 37°C for 24 hours for maximum growth of the microorganisms[18]. The microbes' development was reduced by the antimicrobial test material, and around the discs, there was a distinct, clear, and discernible zone of inhibition. The diameter of the inhibition zone, represented in millimetres, was used to measure the antibacterial effect of the experimental substances.

2.4 Total Phenolic Content Analysis

According to the procedure published by Skerget *et al.* The Folin-Ciocalteu reagent was used as an oxidizing agent to measure the overall phenolic content of *M. nudiflorus*[19]. To persuade the entire phenolic value of the sample material, graphing absorbance vs gallic acid concentrations resulted in the creation of a standard curve. The sample's phenolic content was determined as mg of Gallic Acid Equivalent (GAE) for every gram of extract[20].

2.5 Cytotoxicity Assay on Cancer and Non-Cancer Cell Line

The Center for Advanced Research at the University of Dhaka performed cytotoxicity tests on several *M. nudiflorus* extracts against the HeLa cell line (a human cervical cancer cell) and the Vero cell line (kidney epithelial cells taken from an African green monkey). The research instruments used in this study include a Biological Bio Safety Cabinet (Model: NU-400E, Nuaire, USA), a CO_2 Incubator (Nuaire, USA), a Trinocular microscope with a camera (Optika, Italy) and Hemocytometers. The utilized materials included culture flasks, 96-well plates, 15-ml pipes, tips, and gloves. The bacteria were grown using antibiotics (P+S). The germs were likewise eradicated with the use of gentamycin. The cultures were put into the medium-filled wells using serological pipettes. Additionally, trypsin was utilized to dissolve the cultures into liquid form. The two cell lines, Vero, and HeLa were maintained in Dulbecco's Modified Eagles' Medium (DMEM), which also included 10% Fetal Bovine Serum, 1% penicillin-streptomycin (1:1), and 0.2% gentamycin (FBS). A 96-well plate was seeded with Vero cells (1.5×10^4/100 µl) and HeLa cells (2×10^4/100 µl), which were then incubated at 37°C with 5 per cent CO_2. The next day, 25 µl of the sample (filtered) was added to each well. After 48 hours of incubation, cytotoxicity was examined under an inverted light microscope. For each sample, duplicate wells were used[21].

2.6 Free Radical Scavenging Activity Measurement

The free radical scavenging abilities of bark extracts were determined using the Brand-Williams *et al.*, technique on the stable radical 1,1-diphenyl-2-picrylhydrazyl (DPPH)[22]. The antioxidant potential of the bark extract was determined by comparing a purple-coloured methanolsolution of diphenylpicrylhydrazyl (DPPH) radical by the extract to that of Butylated Hydroxytoluene (BHT). To estimate the % of DPPH free radical inhibition, the following equationwas used:

% of inhibition = [1 (AM sample/AM blank)] × 100

The reaction mixture's and the DPPH MeOH solution's absorbance are denoted by AM blank and AM sample. Then, using the % of inhibition vs. concentration curve, the 50% inhibition (IC_{50}) of the DPPH radical was computed.

2.7 Bioassay for Brine Shrimp Toxicity

The fatality action of extracts of *M. nudiflorus* bark on brine shrimp was tested using the outlinedprocedure[23,24]. As a control, vincristine sulfate was employed.

2.8 Analysis of Fatty Acids

Analysis of fatty acids was carried out on the bark of *M. nudiflorus* n-Hexane fraction. The bark of the *M. nudiflorus* tree was used to prepare both free fatty acids and bound fatty acids and transformed into respective methyl ester counterparts so that the volatile could be evaluated using gas-liquid chromatography. The retention time of the produced methyl ester of free fatty acids and bound fatty acids, as well as standard fatty acids ester samples, was measured using gas- liquid chromatography (Shimadzu 9A, Column-BP-50, Detector-FID, 170°C-1min/4°C - 270°C - 30 min). The peak area of the gas chromatograms was used to calculate the relative ratios of free fatty acids and bound fatty acids[25].

In all bioassays, three replicates of every sample have been used for statistical analysis, and the results' data were expressed as an average.

3. Results and Discussion

3.1 Phytochemical Screening

The phytochemical screening of the methanolic extract of the plant sample resulted in the presence of various secondary metabolites such as alkaloids, tannoids, flavonoids, phlobatannins, steroids,cardiac glycosides, and isoprenoids (terpenoids) which have been shown in Table 1.

Table 1. Qualitative analysis for phytochemical screening of barks of *M. nudiflorus*

Plantpart (Bar k)	Phytochemicals							
	Tannins	Phlobatannins	Saponin	Triterpenoids	Alkaloids	Flavonoids	Steroids	Cardiac glycosides
T. nudiflora	+	+	-	+	+	+	+	+

The negative symbol (-) implies that phytochemicals are absent, and the positive symbol (+) implies that phytochemicals are present.

Potential therapeutic and metabolic effects can be produced by these phytochemicals. Alkaloids are substances found in plants that deter parasites and predators. Alkaloids found in plants are also potent analgesics. The astringent substance tannins are used to treat gastrointestinal diseases like diarrhoea and dysentery[26]. Flavonoids are potent anticancer substances that search for freeradicals and shield cells from oxidative stress[27]. Additionally important for their aromatic properties, plantterpenoids are used in traditional herbal remedies[28]. The study demonstrates that

M. nudiflorus barks are abundant in phytonutrients, and the use of these materials for therapeutic purposes mightbe strongly suggested.

3.2 Fatty Acid Analysis

Fatty acids play vital pharmacological, structural, and functional properties in human biologyand physiology. They serve as the primary elements of the cellular membranes in addition to serving as a form of energy[29]. The fatty acid content of the n-Hexane (HEX) extract was analyzed, and the amounts of BFA and FFA were measured and indicated in Figure 1.

The percentage of bound fatty acids found in *M. nudiflorusis* is as follows: Caprylic acid (12.894%), stearic acid (33.553%), oleic acid (28.062%), and Linoleic acid (6.543%). Similarly, Analysis also showed that *M. nudiflora* contained Palmitic acid (5.190%), Palmitoleic acid (39.938%), Stearic acid (33.689%), and Oleic acid (10.101%) as free fatty acids.

It can be noted that *M. nudiflorus* contained a higher percentage of Stearic acid both as bound fattyacid and free fatty acid, which is a saturated long-chain fatty acid. Similarly, Oleic acid, a mono-unsaturated omega-9 fatty acid, is also present in both BFA and FFA forms. The inclusion of stearic acid in a wide variety of commercial and academic compositions is due to the advantages it provides on the structural and functional qualities of consumer products. The basic value of a material is its capacity to act as a lubricant by improving its flow ability and to work with a varietyofsurfaces through chemical

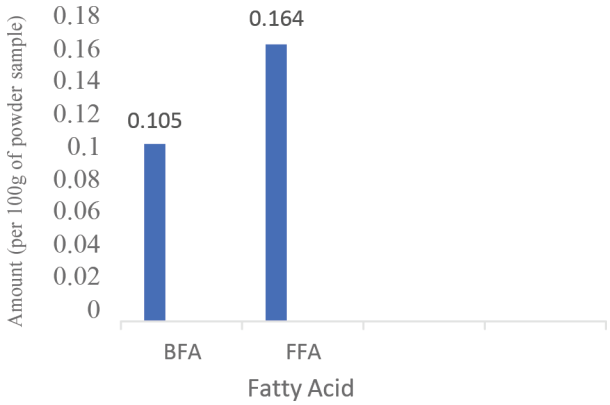


Figure 1. BFA and FFA quantity in the sample.

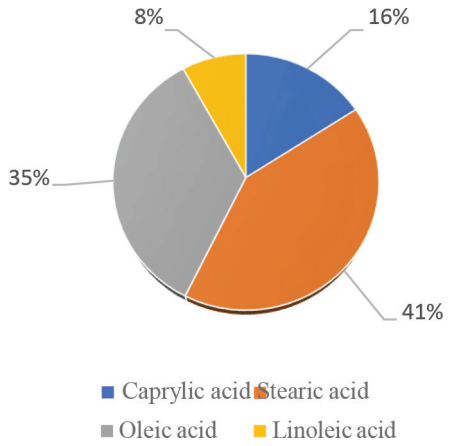


Figure 2. Relative percentages of BFA.

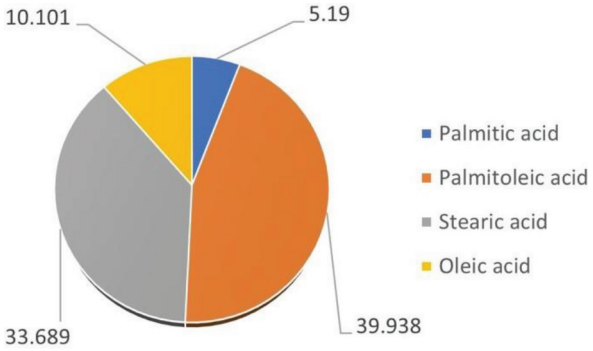


Figure 3. Relative percentages of FFA.

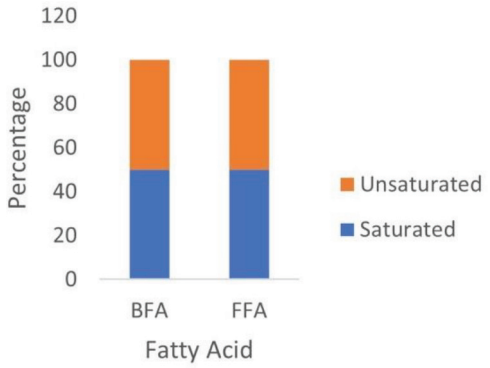


Figure 4 Saturated and unsaturated acid content in FFA and BFA as a percentage.

or physical methods by forming layer structure[30]. The highest amount of Palmitoleic acid (39.938%) was present as free fatty acid which is a popular monounsaturated fatty acid. Additionally, it was claimed that it might have anti-thrombotic qualities that could help prevent strokes[31]. Among bound fatty acids, *M. nudiflorus* contains 12.894% of Caprylic acid and it helps treat excessive cholesterol, skin diseases, digestive problems, and fungal infections. The likelihood of antibiotic resistance may be reduced by using it as a disinfectant. A trace amount of linoleic acid (6.543%) was found since it cannot be produced in the body. Linoleic acid is the most popular fatty acid used in beauty and skincare products. Linoleic acid is alwaysutilized as an emollient or moisturizer for skin, fingernails, and hair. According to several studies,consuming foods high in unsaturated fats rather than saturated fats lowers cholesterol levels in blood which minimizes the risk of cardiac type-2 diabetes[32]. So, it can be concluded that *M. nudiflorus* is a great source of economically important fatty acids especially Stearic acid acid and Palmitoleic acid which can be extracted commercially from this source. The plant also contains a few other important fatty acids and these fatty acids used to have great applications in medical and industry.

3.3 Cytotoxicity

Various fractions of *M. nudiflorus* were subjected to Vero and HeLa cell lines to assess the Cytotoxicity and the results are reported in Table 2.

3.4 Antimicrobial Activity

The Methanolic Extract (ME) of *M. nudiflorus* barks and various fractions including Aqueous(AQ), Hexane (HEX), Ddichloromethane (DCM), Chloroform ($CHCl_3$), and Ethyl Acetate (EA) of the methanolic extract were tested for the presence of microorganisms at a concentration of 400µg/disc in all cases. The findings are shown in Table 3.

The results of the disc diffusion method indicate that the *M. nudiflorus* bark extract exhibits substantial antibacterial activity having a range of 7-17 mm zone of inhibition for variousfractions. The use of *M. nudiflorus* as an antibacterial agent may have a lot of promise and can be a viable tool for further study to prove its utility as a natural antibacterial chemical against pathogenic bacteria in domesticated animals and humans.

3.5 Total Phenolic Content Analysis

Phenolic compounds are useful due to their ability to scavenge free radicals. Hydroxyl groups are responsible for scavenging those free radicals[33]. The total phenoliccontents of different extracts are represented in Table 4. The total phenolic content of various fractions of *M. nudiflorus* bark varied from (11.27±0.64) mg to (133.67±0.99) mg of GAE (gallic acid equivalent)/g of dry fractions. EA fraction exhibited the maximum phenolic content of all the fractions (133.67±0.99 mg of GAE/g of dry fractions). The least quantity of phenolic content (11.270.64 mg GAE/g of dry fraction) was found in the HEX extract. A considerable amount of phenolic content was detected in the DCM, ME, and $CHCl_3$ extracts. The findings confer that *M.nudiflorus* can serve as a dietary resource of antioxidants.

3.6 Measurement of Free Radical Scavenging Activity

The scavenging of DPPH radicals is the basis for performing the DPPH antioxidant test[34]. Antioxidants can scavenge DPPH free radicals because of their capacity to donate hydrogen[35]. Oxidation in dietary and biological systems is closely associated with the production of free radicals[36]. Superoxide anion and hydroxyl radicals are examples of oxygen-derived freeradicals that are cytotoxic and cause tissue damage. To prevent the damage done by free radicals, antioxidants serve as a key line of defence against toxicity produced by radicals[37]. Significant activity of the extracts was demonstrated in this investigation by the EA fraction having found the value of IC_{50} is (12.08±0.12) µg/ml, while Standard BHT had a value of IC_{50} is 28.08±0.81 µg/ml. The increasing order of IC_{50} values measured in

Table 2. Analysis of Cytotoxicity for different fractions of *M. nudiflorus* soluble in 2.5% of DMSO

Sample	Observation		Remarks
	Vero	HeLa	Cell cytotoxicity was detected for the sample. CHCl3 and EA on both the Vero and HeLa celllines.
Solvent (-)	100%	100%	
DMSO (solvent) (+)	>95%	>95%	
CHCl3	5%-10%	5%-10%	
EA	<5%	5%-10%	

The Vero and HeLa cell lines employed in the cytotoxicity experiment revealed that $CHCl_3$ and EA portions are hazardous, indicating that the study plant cannot be used for nutritional purposes.

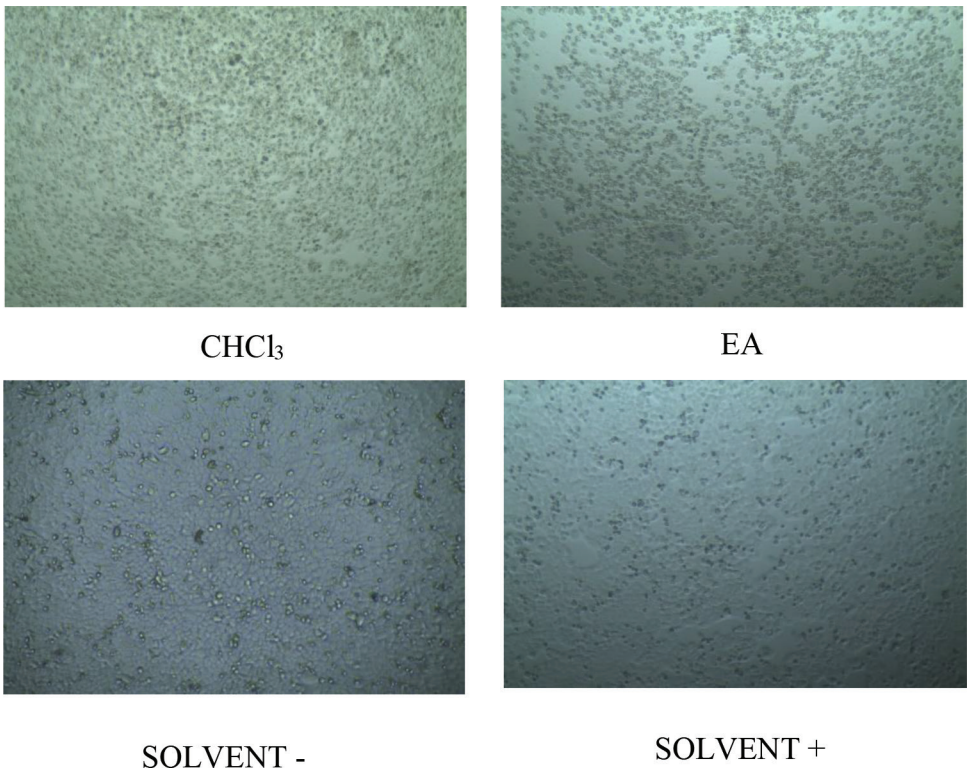


Figure 5. Analysis of Cytotoxicity for $CHCl_3$ and EA fractions of *M. nudiflorus* soluble in 2.5%of DMSO (SOLVENT +) against HeLa cell line.

µg/ml remaining fractions is as follows: ME (32.33±0.63) < AQ (38.5±20.40) < DCM (173.10±0.83) < HEX (222.53±1.65). Since the higher the IC_{50} value, the lower the antioxidant capacity. Both Hexane and DCM extract showed the minimum antioxidant activity. Extracts with, respectively, showed antioxidant activities (Table 5). The degree of cellular antioxidant influences the likelihood of oxidative damage, and it often changes in response to oxidative stress[38]. The antioxidative system defends the body against oxidative damage brought on by ROS. According to the study's findings, *M. nudiflorus* has strong antioxidant activity. Considering this, the various extracts might be a possible source of antioxidants that may be employed in medicinal and culinary preparations because any use of synthetic antioxidants, including BHT, is restricted, which may cause cancer[39].

3.7 Bioassay for Brine Shrimp Toxicity

The brine shrimp lethality assay which is an essential technique for the early cytotoxicity measurement of plant extracts is based on their capacity to kill lab-cultured larvae (nauplii). Active cytotoxic and antitumor drugs are determined using the brine

Table 3. Measurement of Inhibition Zone produced by various test Microorganism

Test organism	Diameter of the inhibition zone (mm)			
	DCM /mm	$CHCl_3$ /mm	EA /mm	STD (Ciprofloxacin) /mm
Bacillus cereus	11±0.08	13±0.13	10±0.21	40±0.21
B. megaterium	10±0.08	14±0.08	10±0.13	38±0.16
B. subtilis	9±0.34	12±0.09	9±0.08	38±0.29
Staphylococcus aureus	13±0.12	17±0.21	13±0.12	42±0.08
Sarcina lutea	10±0.16	10±0.16	8±0.08	36±0.12
Escherichia coli Pseudomonas	9±0.25	12±0.26	9±0.04	40±0.13
aeruginosa Salmonella	10±0.22	11±0.07	9±0.09	38±0.29
paratyphi	10±0.08	0	7±0.16	39±0.09
S. Typhi	9±0.08	12±0.12	9±0.12	40±0.16
Shigella boydii	9±0.13	13±0.16	9±0.11	40±0.12
Sh. Dysenteriae	9±0.21	14±0.12	9±0.08	35±0.16
Vibrio mimicus	10±0.12	11±0.20	9±0.17	38±0.20
V. parahemolyticus	9±0.13	11±0.08	8±0.26	38±0.17

Average results have been shown as mean ± SD (Standard deviation).



Figure 6. Several petri dishes for bacterial growth.

Table 4. The raw extract's total phenolic content and its various fractions of *M. nudiflorus*

Sample part	Fraction code	The Number of Total Phenols (mg of GAE/g of Dryfractions) ±SD
	ME	40.28±0.50
	HEX	11.27±0.64
Bark of *M. nudiflorus*	DCM	42.99±0.43
	CHCl3	70.27±1.21
	EA	133.67±0.99
	AQ	25.65±0.98

shrimp lethality test[40]. In the brine shrimp toxicity test, HEX and DCM extract showed comparable LC_{50} values (0.12 µg/ml and 0.36 µg/ml respectively) in comparison with Vincristine Sulfate (VS) (9.02 µg/ml). It reveals that both HEX and DCM extract have high mortality rates and consequently these fractions are highly toxic. Meanwhile, ME and $CHCl_3$ had moderate lethality, but EA and AQ had minimal activity (Table 6). The current work supports the use of *M. nudiflorus*

Table 5. *M. nudiflorus* bark's free radical scavenging activity

Sample Part	Fraction Code	IC50 (µg/ml) Value ±SD
	BHT	28.08±0.81
	ME	32.33±0.63
Bark of *M. nudiflorus*	HEX	222.53±1.65
	DCM	173.10±0.83
	CHCl3	67.91±0.74
	EA	12.08±0.15
	AQ	38.52±0.40

The data are provided as an average ± (SD= Standard deviation). BHT = tert-butyl-1-hydroxytoluene.

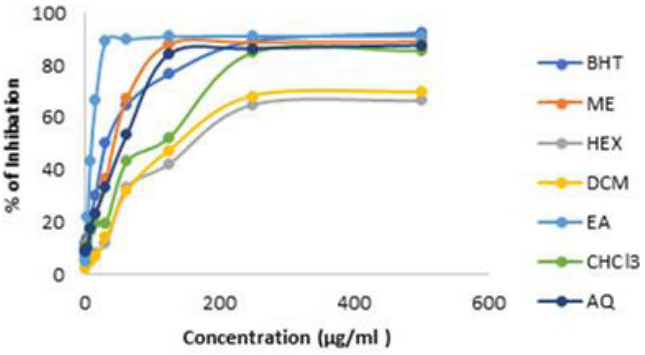


Figure 7. DPPH free radical scavenging activity of various extracts of *M. nudiflorus* bark.

Table 6. Bioassay of *M. nudiflorus* bark extract's mortality in brine shrimp

Sample Part	Fraction Code	LC_{50} (µg/ml) ±SD
	ME	18.82±1.64
	HEX	0.12±0.01
	DCM	0.36±0.02
Bark of *M. nudiflora*	CHCl3	100.44±0.88
	EA	129.19±0.57
	AQ	118.92±0.87
	VS	9.02±0.36

The data are provided as an average ± (SD= Standard deviation).

bark extracts in traditional medicine by demonstrating that brine shrimp bioassay is an easy, dependable, and practical approach for determining the bioactivity of the substance.

4. Conclusion

This research demonstrates that *M. nudiflorus* barks are abundant in phytochemicals, and their usage for health reasons should be highly advised. Numerous phytochemicals found in medicinal plants; including flavonoids, alkaloids, tannins, and terpenoids, have antibacterial and antioxidant activities. Because *M. nudiflorus* contains substantial phytochemical screening, cell cytotoxicity, fatty acids, antioxidant, phenolic, and brine shrimp lethality, it was identified as a viable plant for further chemical exploration. Some of the rural practitioner's traditional usage is justified by the reported biological activity.

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