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Phytochemical Analysis and *In vitro* Bioactivity Study of Methanol Extract of *Byttneria pilosa* (Family: Malvaceae)

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ABSTRACT: Despite numerous promising traditional medicinal applications of *Byttneria pilosa* (Malvaceae), it has largely been unexplored. In this study, the extensive biological and chemical investigation on the aerial part of *Byttenria pilosa* led to the isolation of saturated aliphatic hydrocarbon having two hydroxyl groups. The antioxidant properties of methanol (MeOH) extract of *Byttneria pilosa* (MEBP) were determined with regard to DPPH free radical scavenging capacity (quantitative and qualitative antioxidant properties), Total Phenolic Content (TPC), Total Flavonoid Content (TFC), and Total Tannin Content (TTC). Disc diffusion technique was used to assess the antibacterial activity of methanol extract of *B. pilosa*. *In vitro* blood coagulation activity of MEBP was measured by widely used prothrombin time test (also called the pro test or PT test). Isolation of pure compounds was done through Thin Layer Chromatography (TLC) and Column Chromatography (CC) followed by some detection methods such as NMR and Mass spectroscopy. Phytochemical analysis showed that phenolic hydroxylated compounds were present in *B. pilosa* which were also responsible for observed bioactivities (i.e. antioxidant, antibacterial and blood coagulation activity). The possible structure of the isolated compound was illuminated by meticulous interpretation of spectroscopic data, comprising MS and NMR data. The isolated compound was 2,4,4,7-tetramethyloctane-3,5-diol (Molecular formula $C_{12}H_{24}O_2$ and Molecular mass 202) or 2,2,4,6,6-pentamethylheptane-3,5-diol (Molecular formula- $C_{12}H_{24}O_2$ and MW-202). The present findings show that *B. pilosa* possesses significant antioxidant and blood coagulation activity. It was confirmed that the isolated compound could be an aliphatic hydroxylated saturated compound having a molecular formula of $C_{12}H_{26}O_2$ (Molecular mass= 202).

KEYWORDS: *Bytteneria pilosa*; Saturated aliphatic hydrocarbon; Antioxidant activity; Antibacterial activity; Blood coagulation activity

1. INTRODUCTION

Drug exploration and development process is potentially contributed by the natural sources. Traditional medication has been widely used for the management of many ailments throughout human history [1]. There are plethora of reasons for growing interest in edible plants, for example, chemical diversity in screening programs, low cost and availability [1-2]. Screening of the plant is a prerequisite to assess the therapeutic effect which leads to the isolation of new bioactive compounds and thus helps in the development of new drugs [1-3]. Many phytochemicals have, for example, been reported to possess promising bioactivities that might be potentially useful in the treatment of inflammatory ailments, including rheumatoid arthritis, asthma, hepatitis, enteritis, as well as metabolic maladies, neurodegenerative disorders and a wide range of infectious diseases [4, 5]. *Byttneria pilosa* (Family: Malvaceae), commonly known as Harjora, Shaha, Salam Vra, Chaala ludhi is a woody and climbing lianas [6, 7]. The branchlets of this plant are dark brown when dry, smooth and with stellate hairs. The leaf blade is cordate or orbicular, usually having (3-5) lobes, both the surfaces are yellowish brown stellate puberulent and covered with hairs; the hairs are denser abaxially with seven basal veins; the base is cordate; the margin is serrulate; the apex can be obtuse or acute. The petals are four-lobed and the lobes are obtuse. The capsule is globose, yellowish pink, ~2 cm in diameter, whereas the yellow ovoid seed has brown mottling (~5 mm) [5-6]. *B. pilosa* can be found

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in regions spanning the globe. Its native range is China (S. Yunnan) to Peninsula Malaysia. It is also found in largest quantities in Myanmar (Burma), India (Darjeeling, Assam, Meghalaya, Manipur), Sikkim, Bangladesh, Laos, Thailand, Vietnam, Java. It is found at Chittagong, Chittagong Hill Tracts, Cox's Bazar, Sylhet, Srimongal, Sherpur and Habiganj in Bangladesh [6].
The plant is very popular in tribal community of Bangladesh for instance, Chakma, Marma, Khumi for its various medicinal benefits. The root of this plant is masticated; juice is topically used as antidote. Fractured bones can be treated with the paste of the tendrillar stem. Moreover, the crushed stems are used for the management of boils and elephantiasis. Scabies can be treated with an infusion of the leaves used in baths [7]. Regardless of several promising traditional usages of *B. pilosa*, it has been basically underexplored. As a part of our current research on medicinal plants [8-9], here we isolated pure compound from the methanol extract of *Byttneria pilosa* (MEBP) along with different pharmacological activities like antioxidant, antibacterial, and blood coagulation activity.

2. RESULTS AND DISCUSSION

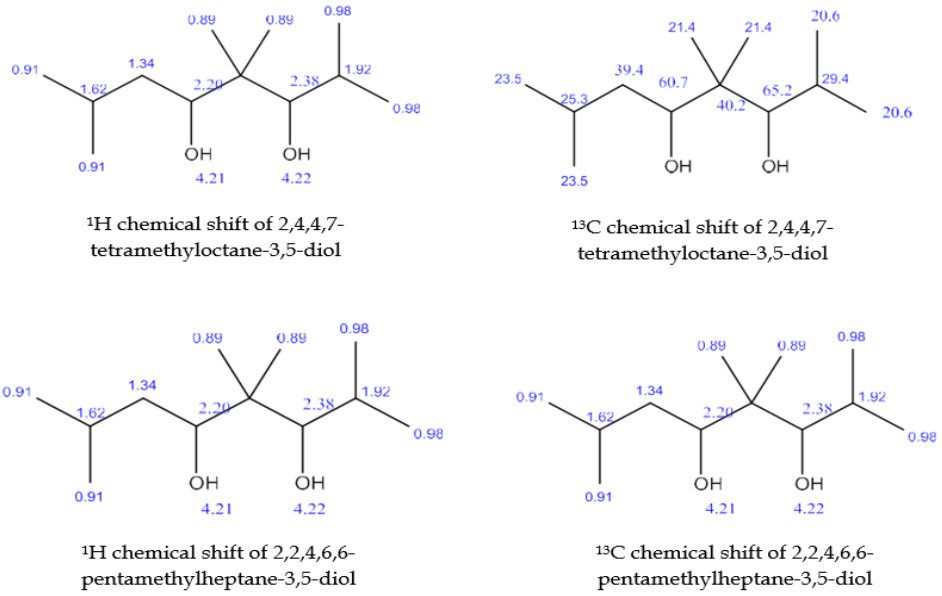
2.1. Results

2.1.1. Identification of phytochemical constituents

Initial phytochemical screening of MeOH extract of *B. pilosa* revealed the presence of reducing sugar, flavonoids, tannins, alkaloids, saponin, glycosides, terpenoids and steroids. The outcome of initial phytochemical screening triggered to undertake in depth analysis to isolate bioactive compounds along with the determination of biological activities.

2.1.2. Structure determination

The ^{1}H NMR of fraction 1D at chemical shift of 0.89 (3H, t, J=7.5) indicated a proton peak of a -CH_3 group attached to any methylene group. There might be the proton peaks of any saturated hydrocarbon containing –OH group at the chemical shift of 2.07 (1H, S and 2.12 (1H, S). The peak at the chemical shift of 4.14 (1H, q, J=7.2 Hz) indicated the proton peak of a saturated hydrocarbon attached to a CH_3 group and to a carbon containing electronegative atom. The ^{13}C NMR of fraction 1D provided various important information like carbon peak of any -CH_3 group at the chemical shift of about 14.12, 21, 22; peak of a carbon attached to a -OH containing carbon at the chemical shift of 38.16; peak of –OH containing carbons at the chemical shift of 59.54 and 60.42. In case of 2D NMR (COSY, HSQC, HMBC) the fraction 1D did not show spot in all chemical shift such at 2.07, 2.12, 4.14 etc. From proton and ^{13}C NMR data, it was found that the chemical shift for protons was 0.84 to 4.14 while the chemical shift for carbons was 14.12 to 60.42. It indicated that the compound must be aliphatic and saturated in nature having about 26 hydrogens, 12 carbons and two oxygen atoms. The molecular formula might be $C_{12}H_{26}O_2$ (MW 202). An ion peak was observed in 201 in case of negative scan in mass spectroscopy that means the molecular mass would be (201+1) or 202 which comply with the structure (Molecular formula- $C_{12}H_{24}O_2$) obtained from the proton and carbon NMR data. From the above data, it can be stated that the possible structure of the compound might be 2,4,4,7-tetramethyloctane-3,5-diol (Molecular formula $C_{12}H_{24}O_2$ and Molecular mass 202) or 2,2,4,6,6-pentamethylheptane-3,5-diol (Molecular formula- $C_{12}H_{24}O_2$ and MW-202) (Figure 1, Table 1, Table 2 and Table 3).



¹H chemical shift of 2,4,4,7-tetramethyloctane-3,5-diol

¹³C chemical shift of 2,4,4,7-tetramethyloctane-3,5-diol

¹H chemical shift of 2,2,4,6,6-pentamethylheptane-3,5-diol

¹³C chemical shift of 2,2,4,6,6-pentamethylheptane-3,5-diol

Figure 1. Possible structure and chemical shift of the isolated compound.

Table 1. Proton NMR values of fraction 1D.

Chemical shift (ppm)	Proton number	Multiplicity	J value (Hz)	Comments
0.89	3	Triplet (t)	7.5	Proton peak of a –CH_3 group
4.14	1	Quartet (q)	7.2	Saturated hydrocarbon attached to a -CH_3 group
2.07	1	Singlet (s)	N/A	Proton peaks of any saturated hydrocarbon with –OH group
2.12	1	Singlet (s)	N/A	Proton peaks of any saturated hydrocarbon with –OH group

Table 2. Carbon NMR values of fraction 1D.

Type of Carbon	Chemical shift (ppm)	Comments
—— CH_3	14.12	Carbon peak of any -CH_3 group
—— CH_3	21	Carbon peak of any -CH_3 group
—— CH_3	22	Carbon peak of any -CH_3 group

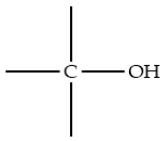
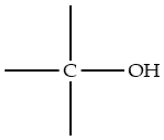
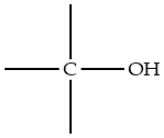
	38.16	Peak of carbon attached to a –OH group containing carbon
	59.54	Peak of –OH containing carbons
	60.42	Peak of –OH containing carbons

Table 3. Ion peaks in negative scan found in mass spectroscopy of fraction 1D.

m/z	Absolute intensity	m/z	Absolute intensity
100.95	603170	311.15	807395
115.85	1066660	325.20	1485623
116.90	1050493	339.15	2022244
130.95	895255	713.40	612202
136.80	3835751		
145.00	2537480		
150.90	1389804		
162.90	593934		
166.90	9143165		
168.85	3407204		
193.85	1107772		
200.80	2152347		
234.80	992748		

2.1.3. Assessment of Antioxidant activity

The appearance of strong yellow spot on a purple background on the TLC plate of *B. pilosa* extract is an indication of free radical scavenger (Figure 2). Furthermore, in 2,2-Diphenyl-1-picrylhydrazyl (DPPH) quantitative analysis, *B. pilosa* extract revealed free radical scavenging activity with approximate IC_{50} value of 45.71 µg/mL in comparison to ascorbic acid (~22.64 µg/mL) (Table 4). In polyphenolic compound determination, the TPC and TTC was found to be ~ 80 mg GAE/g (Table 5) and ~ 217 mg GAE/g (Table 5) of dried extract respectively, using Gallic acid calibration curve. In addition, the TFC was determined as ~156 mg QE/g of dried extract where Quercetin calibration curve was used (Table 5).

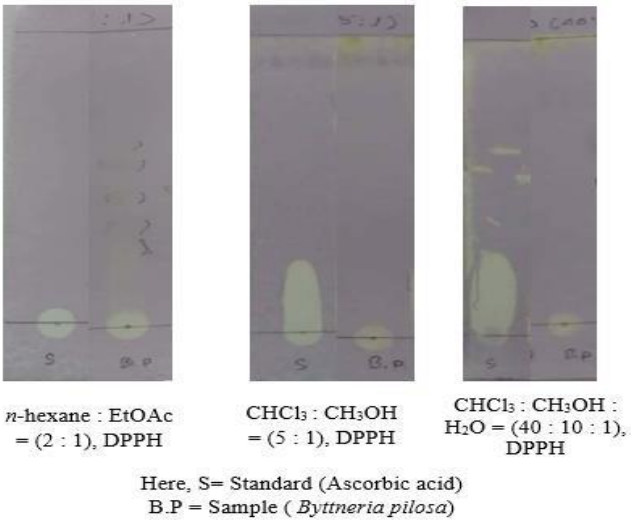


Figure 2. Comparison of TLC plate for *B. pilosa* with standard after applying DPPH.

Table 4. Percentage Inhibition of standard and sample at different concentration.

Conc. (µg/mL)	%inhibition		IC_{50} (µg/mL)	
	Standard	Sample	Standard	Sample
1	9.41	6.65		
2	14.08	11.8		
4	21.93	19.59		
8	32.53	27.75		
16	43.08	39.48	**22.64**	**45.71**
32	55.06	49.37		
64	69.62	58.18		
128	80.26	68.84		
256	88.2	73.46		
512	93.93	78.07		

Table 5. Determination of phenolic content of *B. pilosa* extract.

Phenolic group	Content
Total phenolic content (mg GAE/gm)	80.37 (mg GAE/gm)
Total flavonoid content (mg QE/gm)	217.19 (mg QE/gm)
Total tannin content (mg GAE/gm)	156.14 (mg GAE/gm)

2.1.4. Assessment of Antibacterial activity

In this investigation, we used the crude methanol extract of aerial part of *B. pilosa* and the extract did not show antibacterial activity against all the tested bacterial strains (*Staphylococcus aureus*, *Escherichia coli*, *Salmonella enteritidis*, *Bacillus subtilis*) at the doses of 250 and 500 µg/disc (Table 6).

X

Table 6. *In vitro* antibacterial activity of crude extract compared to ciprofloxacin.

Bacterial strains	Diameter of zone of inhibition in mm ± SD			
	Blank	Extract (250 µg/disc)	Extract (500 µg/disc)	Ciprofloxacin (30 µg/disc)
Staphylococcus aureus	0	5.5 ± 0.7	5.8 ± 0.7	28 ± 0.7
Bacillus subtilis	0	5.4 ± 0.1	5.7 ± 0.0	35 ± 0.1
Escherichia coli	0	5.8 ± 0.7	6.0 ± 0.0	29 ± 0.0
Salmonella enteritidis	0	5.9 ± 0.5	6.0 ± 0.7	31 ± 0.7

2.1.5. Evaluation of in vitro Blood Coagulation activity

In blood coagulation activity test, the clotting times for the *B. pilosa* extract were 2 min 44 s, 2 min 56 s, 3 min 21 s, and 4 min 34 s at the concentration of 200, 100, 50, 25 mg/mL respectively, whereas the clotting times for the phytomenadione (standard) were 2 min 13 s, 4 min 40 s, 5 min 36 s, and 7 min 27 s at the concentration of 10, 5, 2.5, 1.25 mg/mL respectively, (Table 7). All of these values were compared with the negative control group where the recorded clotting time was 8 min 40 s.

Table 7. Blood clotting time of *B. pilosa* compared to phytomenadione.

Treatment Group	Conc. Of Sample (mg/mL)	Average coagulation time (min) ±SD	SEM
Negative control		8.67	--
Positive control	10	2.21 ± 0.06	0.04
	5	4.67 ± 0.12	0.085
	2.5	5.6 ± 0.09	0.07
	1.25	7.45 ± 0.07	0.05
Sample	200	2.74 ± 0.01	0.01
	100	2.94 ± 0.02	0.015
	50	3.35 ± 0.03	0.02
	25	4.56 ± 0.04	0.025

p value: $p < 0.001$

3. DISCUSSION

Secondary metabolites for example, flavonoids, tannins, alkaloids, saponin, glycosides are considered as vital for therapeutic activity including antioxidant, antimicrobial, blood coagulation activity [10-13]. In the last few years, multiple biological activities of these compounds toward different disorders have gained special attention [14]. From numerous studies, it has been found that the secondary metabolites from natural origin shows health benefits for the prevention and treatment of age-related diseases, cancers, heart diseases ans so forth [14, 15]. The outcome of initial phytochemical screening triggered to undertake in depth analysis to isolate bioactive compounds along with the determination of biological activities. The isolated saturated aliphatic hydroxylated compound found in *Byttneria pilosa* might be responsible for performing multiple functions in plants, for instance, serve as weapons counter to other plants, micro-organisms and animals (e.g. fungi, bacteria, insects); symbiosis agent between microbes and plants as well as differentiation effector [12, 13].

The search for natural antioxidants has acknowledged ample consideration and efforts have been given to isolate compounds from natural resources that scavenge free radicals which ultimately help to preclude oxidative damage related disorders. The antioxidant potential of plants is primarily related with the existence of polyphenols as phenolic acids and flavonoids that show their antioxidant activity through the neutralisation of free radicals by the donation of electron or hydrogen atoms [12-13]. This activity has engrossed significant concern in recent years due to its associated bioactivities, for example, anti-inflammatory, anti-cancer, anti-aging effect [12, 13, 16]. Flavonoids are responsible for several biological activities comprising antimicrobial, antiulcer, antiarthritic, antiangiogenic, anticancer, protein kinase inhibition [17]. Moreover, flavonoids are predominantly beneficial for anti-aging component whereas

tannins have been reported to possess anticarcinogenic and antimutagenic potentials as well as coagulating and anti-parasitic properties [17, 18]. *B. pilosa* might be a source good of antioxidant as indicated by the present study.

According to one estimate, medicinal plants have been recognized to contain an array of phytochemicals with antibacterial activity belonging to several chemical classes like tannins, flavonoids, alkaloids, and phenolic compounds [19]. Antimicrobial activity of tannins has been confirmed in numerous fields providing significant result including anticarcinogenic and antimutagenic activity. Flavonoids typically exert antimicrobial activity by several ways like inhibiting the synthesis of the nucleic acids, tampering with the integrity of the cytoplasmic membrane function and the energy metabolism process [20]. The antimicrobial potential of alkaloids are found through intercalating into cell wall and DNA of parasites. In case of polyphenols, a range of mechanisms (enzyme inhibition, membrane disruption, metal ion complexation) has been established [20]. According to tribes, the crushed stem of *B. pilosa* is used for the treatment of boils. In this study, the reasons of not observing any antibacterial activity against the tested bacterial strains might be duue to multiple factors, namely, the selection of aerial part of the plant, the use of methanol as extraction solvent, the absence of significant amount of flavonoids, tannins and phenolic compounds needed to be effective against the experimental microorganisms, inhibition of bioactivity of one bioactive metabolite by another bioactive metabolite present in the plant. İn addtion, after 18 hours, under incubation temperature of 37°C, some volatile compounds could have evaporated, thus, affecting the measurements recorded [20]. So, further investigation should be performed to investigate whether the other part of the plant extracted by another solvent along with MeOH show antibacterial activity against the bacterial strains used in this investigation as well as against other microbes or not.

Generally, the coagulation process is regulated by numerous inhibitors which limit the formation of clot that in turns prevent the thrombus propagation. The thrombohaemmorhagic balance is well-regulated in the body by the intricate interactions between coagulation and the fibrinolytic system along with platelets and vessel wall. The haemostatic system is always vital that may be hampered by surgery, trauma, chronic jaundice, hemophilia or infectious agents [21, 22]. To restore this balance, coagulant or anti-coagulant agent is taken depending on the pathological condition. The leaves paste of this plant is used in cuts to stop bleeding traditionally that can be correlated with our finding as the methanol extract of *B. pilosa* showed a very potent blood coagulation activity. Phytochemical groups namely flavonoids, tannins may be responsible for the blood coagulation activity [23]. In our study, the isolated hydroxylated saturated compound might be responsible for blood coagulation activity. To confirm this finding, in-depth analysis is required using the isolated compound.

Broadly speaking, simple phelolics, phenolic acids, flavonoids, alkaloids, tannins, saponins etc. isolated from natural origin specifically from plants are the predominant compounds which extert biological activities [24-26]. This study demonstrated the identification, isolation and structure determination of hydrocarbon containing two hydroxyl groups from the methanol extract of *Byttneria pilosa*. The *in vitro* bioactivity data obtained from this study reveals the potential bioactivity which are quite unusal and uncommon for hydrocarbons particularly hydrocarbon with hydroxyl groups. However, having limited study on *Byttneria pilosa* along with the potential bioactivities found in this study, *B. pilosa* seems an interesting and promising plant that warrants further in-depth exploration.

4. CONCLUSION

The present outcomes show that *Byttneria pilosa* possess potential antioxidant and blood coagulation activities which are attributed to the phytoconstituents present in the plant. The saturated aliphatic compound (along with/without other compounds) which has been isolated and identified in this study might be accountable for the observed activities.

5. MATERIALS AND METHODS

5.1. Chemicals and Reagents

Analytical-grade chemicals such as methanol, ethanol, *n*-hexane, ethyl acetate, chloroform, DPPH, and ascorbic acid were procured from Sigma-Aldrich, Germany. Ciprofloxacin antibiotic disc was also obtained from Sigma-Aldrich, Germany. All other reagents like DMSO, H_2SO_4, and distilled water were also used.

5.2. Collection of Plant material and Extraction

Byttneria pilosa was collected from Komolgonj, Moulobibazar, Sylhet, Bangladesh in October, 2017 at the daytime (11:00 am) avoiding any type of adulteration. It was acknowledged by the specialists of Bangladesh National Herbarium, Dhaka, Bangladesh (Accession No.: 42286 DACB) and as an in-built process, a voucher sample was stored in the Herbarium (Bangladesh National Herbarium). The plant was shade-dried at room temperature for about 15 days to confirm the active ingredients free from decomposition and also to evade any degradation. Then, the shade-dried plant was powdered and 700 g powder was soaked in 3 L methanol in a suitable container for 15 days associated with consistent shaking and stirring. After 15 days, filtration was done to remove the plant debris followed by the evaporation of the solvent. Finally, the obtained crude extract (% yield= 3.29%) was stored in refrigerator.

5.3. Microorganisms

Antibacterial activity of MEBP was measured against both gram positive and gram negative bacterial strains. These bacteria were sourced from the Microbiology Laboratory of Pharmacy Discipline, Khulna University, Khulna, Bangladesh.

5.4. Drugs

The standard drug ciprofloxacin disc was obtained from the Microbiology Laboratory of Pharmacy Discipline, Khulna University, Bangladesh. Vitamin K1 injection was purchased from local pharmacy in Khulna, Bangladesh.

5.5. Identification of phytochemical constituents

Initial phytochemical screening was performed to detect the principal functional groups according to the established methods [27].

5.6. Structure determination

The Ethyl acetate fraction of methanol extract of *B. pilosa* was subjected to repeated column chromatography to yield new compound (hydrocarbon). Among the 24 fractions obtained from column chromatography, one fraction (named as 1D) was taken into consideration for further analysis by NMR and Mass spectroscopy. The selection was made on the basis of visualisation of the fractions at short and long wavelength after developing on TLC plate with *n*-hexane:ethyl acetate (8:2) or (9:1) ratio could be the as mobile phase. Moreover, the developed TLC plate was also derivatised with different reagents (i.e. 10% H_2SO_4, Vanillin-H_2SO_4, Dragendorff's reagent) to enhance the visualisation of the compound after reacting with the reagents. Proton NMR (^{1}H NMR) at 400 MHz and carbon ^{13}C (^{13}C NMR) at 100 MHz spectra of fraction 1D were recorded by using Tetramethylsilane (TMS) as the internal standard. Two dimensional NMR was done by Correlation Spectroscopy (COSY) (^{1}H-^{1}H correlation), Heteronuclear single quantum correlation (HSQC) (^{1}H-^{13}C correlation) and Heteronuclear Multiple Bond Correlation HMBC (correlations between carbons and protons separated by two, three, and, occasionally in conjugated systems, four bonds). The solvents were $CDCl_3$. LC-ESI-MS was performed to determine the mass spectrum of isolated fraction (1D).

5.7. Assessment of Antioxidant activity

As most of natural antioxidants have reactive hydrogen atoms that serve as the reductants, the DPPH assay is a smart measure of the standard antioxidant profile. Basically, DPPH free radical produces a violet solution in ethanol which demonstrates a characteristic spectrum having a maximum absorbance close to 517 nm. The stable (room temperature) free radical is reduced to a light yellow or colourless ethanol solution through electron transfer reaction in the occurrence of an antioxidant molecule by electron transfer [28].

5.7.1. Qualitative Analysis

The DPPH assay implemented in this test is followed by the procedure described by Sadhu et al. [29] with slight adjustments. DPPH based qualitative analysis was performed based on their scavenging potential of DPPH free radical by TLC technique [29]. In brief, the extract and the standard ascorbic acid were spotted on TLC plate followed by the development by ascending system in three solvent systems i.e. non-polar (*n*-hexane: Ethyl acetate=2:1), medium polar ($CHCl_3$:CH_3OH=5:1) and polar ($CHCl_3$:CH_3OH:H_2O=40:10:1) solvent system. After drying the TLC plates, they were viewed in UV detector both in short wavelength (254 nm) and long (366 nm) wavelength. The plates were then sprayed with 0.02 % ethanol solution of DPPH by spray gun. Finally, the plates were dried with a current of air.

5.7.2. Quantitative Analysis

DPPH based quantitative analysis of *B. pilosa* was accomplished according to the technique described by Lu et al. [30]. Briefly, the sample and ascorbic acid (positive control) were dissolved in DMSO to get the desired concentrations by dilution method. Then 10 µL of various concentrations (1, 2, 4, 8, 16, 32, 64, 128, 256, and 512 µg/mL) of the extract and ascorbic acid were taken in each well of the microplate and 190 µL DPPH in methanol was added to each well. After mixing, the plate was retained at ambient temperature for 30 min, and finally the absorbance was taken at 517 nm using micro plate reader.

5.8. Determination of Total Phenolic Content (TPC)

The TPC assay was executed based on the procedure defined by Wolfe et al. with slight adjustments [31]. In this assay, Gallic acid is usually used as reference standard and the result is usually stated as Gallic acid equivalence [32]. For the determination of total phenolic content, 0.5 mL of various concentrations (0.15, 0.1, 0.08, 0.06, 0.04, 0.02 mg/mL) of standard and extract solution were taken separately into different test tubes. Then 5 mL FC reagent (1/10) and 7% Na_2CO_3 were added to each test tube. Then, they were remained for 30 min at 40°C temperature. The absorbance was measured at 765 nm alongside blank for each concentration after 30 min.

5.9. Determination of Total Tannin Content (TTC)

The TTC was measured by Folin-Ciocalteu Colorimetric method where Gallic acid was used as standard and the result was stated as Gallic acid equivalence [31]. In Total Tannin Content determination, 0.1 mL of various concentrations (0.5, 0.4, 0.3, 0.2, 0.1 mg/mL) of standard and extract sample solution was taken in different test tube. Then 7.5 mL of distilled water and 0.5 mL of FC reagent was added to the test tube followed by the addition of 35% Na_2CO_3 (1 mL). The test tubes were kept at room temperature for 30 min. The absorbance was measured at 725 nm against blank for each concentration after 30 min.

5.10. Assessment of Antibacterial activity

The evaluation of antibacterial potential of MEBP was carried out by disc diffusion method against both gram positive and gram negative bacteria with ciprofloxacin antibiotic disc as standard [33, 34]. Sample impregnated discs (250 and 500 µg/disc), standard antibiotic discs (30 µg/disc) and negative control discs (10 µL 50% ethanol/disc) were located softly on the seeded agar plates with the sterile forceps to reassure full contact with the surface of the medium. Then, the plates were incubated at 37°C for 18 h for observation. In the following day, the antibacterial activity of the sample and positive control was obtained by taking the diameter of zone of inhibition in terms of millimeter.

5.11. Evaluation of *in vitro* Blood Coagulation activity

Coagulation test was performed to diagnose the hemostasis system and to assess the blood clotting function in patients [35]. The purpose of this test is to evaluate the *in vitro* blood coagulation activity of MEBP by Prothrombin Time (PT) or Thrombin Time test using Vitamin K1 (phytomenadione) injection as standard. PT test directly determines a possible imperfection in stage II of the clotting system through the investigation of the clotting capability of plasma coagulation factors (i.e. fibrinogen, prothrombin, factor V,

factor VII, and factor X). Additionally, the PT can be used to assess dysfibrinogenemia, the heparin effect, liver failure, and vitamin K deficiency [36]. In this test, the blood sample and the plant samples (200, 100, 50, and 25 mg/mL) and/or standard (10, 5, 2.5, and 1.25 mg/mL) were gently mixed in a test tube. Then the test tubes were put in a water bath of 37°C. In every 15 s, each test tube was tilted to observe whether clotting was formed or not. The clotting was taken into account when the tube could be upturned without substantial movement of blood in the tube [37].

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REFERENCES

[1] Bibi Y, Nisa S, Chaudhary FM, Zia, M. Antibacterial activity of some selected medicinal plants of Pakistan. BMC Complementary Med. Ther. 2011; 11, 52. [CrossRef]

[2] Bilal M, Rasheed T, Iqbal HMN, Hu H, Wang W, Zhang X. Macromolecular agents with antimicrobial potentialities: A drive to combat antimicrobial resistance. Int. J. Biol. Macromol. 2017; 103, 554–574. [CrossRef]

[3] Altemimi AW, Watson DG, Kinsel M, Lightfoot DA. Simultaneous extraction, optimization, and analysis of flavonoids and polyphenols from peach and pumpkin extracts using a TLC-densitometric method. Chem. Cent. J. 2015; 9, 1–15. [CrossRef]

[4] Wang Y, Zeng KW. Natural products as a crucial source of anti-inflammatory drugs: recent trends and advancements. Tradit. Med. Res. 2019; 4 (5), 257-268. [CrossRef]

[5] Henty EE, Handbooks of the flora of Papua New Guinea. Melbourne University Press, Melbourne 1981.

[6] Lim TK. Edible Medicinal and Non-medicinal Plants: *Byttneria pilosa*. Springer Press, Natherlands 2011.

[7] Ghani A. Medicinal Plants of Bangladesh: Chemical Constituents and Uses. The Asiatic Society of Bangladesh, Dhaka 2003.

[8] Karmakar UK, Sultana S, Nishi N, Biswas NN, Hossain L, Sheikh S. Antioxidant, Analgesic, Antimicrobial, and Anthelmintic Activity of the Dried Seeds of *Bixa orellana* (L). Int. J. Pharm. 2018; 8 (1), 150-163.

[9] Aliza, Sultana S, Hossain ML. *In vivo* Assessment of Neuropharmacological Activity of Methanol Leaves Extract of *Callicarpa arborea* (Family: Verbenaceae) in Swiss albino Mice. Sch. Int. J. Tradit. Complement. Med. 2019; 2(6), 85-89.

[10] Jayaprakasha GK, Rao JML, Sakariah KK. Chemistry and biological activities of *C. longa*. Trends Food Sci. Technol. 2005; 16, 533-548. [CrossRef]

[11] Soobrattee MA, Neergheen VS, Luximon-Ramma AOI, Aruoma TB. Phenolics as potential antioxidant therapeutic agents: mechanism and actions. Mutat. Res. 2005; 579, 200-213. [CrossRef]

[12] Ramos A, Visozo A, Piloto J, García A, Rodríguez CA, Rivero R. Screening of antimutagenicity via antioxidant activity in Cuban medicinal plants. J. Ethnopharmacol. 2003; 87:241–246. [CrossRef]

[13] Duthie GG, Duthie SJ, Kyle JA. Plant polyphenols in cancer and heart disease: implications as nutritional antioxidants. Nutr. Res. Rev. 2000; 13, 79-106. [CrossRef]

[14] Zhang Y, Cai P, Cheng G, Zhang Y. A Brief Review of Phenolic Compounds Identified from Plants: Their Extraction, Analysis, and Biological Activity. Nat. Prod. Commun. 2022; 17(1): 1–14. [CrossRef]

[15] Vaher M, Ehala S, Kaljurand M. On-column capillary electrophoretic monitoring of rapid reaction kinetics for determination of the antioxidative potential of various bioactive phenols. Electrophoresis. 2005; 26:990–1000. [CrossRef]

[16] Tuba AK, Gulcin I. Antioxidant and radical scavenging properties of curcumin. Chem. Biol. Interact. 2008; 174, 27-37. [CrossRef]

[17] Mathew N, Bhattacharya MS, Perumal V, Muthuswamy K. Antifilarial Lead Molecules Isolated from *Trachyspermum ammi*. Molecules. 2008; 13(9), 2156-2168. [CrossRef]

[18] Liu H, Orjala J, Sticher O, Rali T. Acylated flavonol glycosides from leaves of *Stenochlaena palustris*. J. Nat. Prod. 1999; 62, 70-75. [CrossRef]

[19] Saxena M, Saxena J, Nema R, Singh D, Gupta A. Phytochemistry of Medicinal Plants. J. Pharmacogn. Phytochem. 2013; 1(6), 168-182. [CrossRef]

[20] Cushnie TT, Lamb AJ. Antimicrobial activity of flavonoids. Int. J. Antimicrob. Agents. 2005; 26(5), 343-356. [CrossRef]

[21] Naganuma M, Shiga T, Nagao T, Maruyama K, Suzuki A, Murasaki K, Hagiwara N. Renal Function and Treatment Persistence with Non-Vitamin K Antagonist Oral Anticoagulants in Japanese Patients with Atrial Fibrillation: A Single- Center Experience. Jpn. J. Clin. Pharmacol. Ther. 2006; 47(3), 115-122. [CrossRef]

[22] Marri PR, Kirmani S, Rodriguez VJ. Hereditary Thrombocytosis in 3 Kuwaiti Siblings with Homozygous MPL Pro106Leu Mutation and Abnormal Platelet Aggregation. J. Genet Disor. Genet. Rep. 2006; 2(2), 1-3. [CrossRef]

[23] Cao P, Xie P, Wang X, Wang J, Wei J, Kang W. Chemical constituents and coagulation activity of *Agastache rugose*. BMC Complementary Med. Ther. 2017; 17(1), 93. [CrossRef]

[24] Li AN, Li S, Zhang YJ, Xu XR, Chen YM, Li HB. Resources and biological activities of natural polyphenols. Nutrients. 2014; 6(12):6020-6047. [CrossRef]

[25] Pandey KB, Rizvi SI. Plant polyphenols as dietary antioxidants in human health and disease. Oxid. Med. Cell. Longevity. 2009; 2(5):270-278. [CrossRef]

[26] Vita JA. Polyphenols and cardiovascular disease: effects on endothelial and platelet function. Am. J. Clin. Nutr. 2005; 81:292–297. [CrossRef]

[27] Trease G, Evans W. Textbook of pharmacognosy. Tindal Limited. London 1989.

[28] Huang D, Ou B, Prior RL. The chemistry behind antioxidant capacity assays. J. Agric. Food Chem. 2005; 53(6), 1841-1856. [CrossRef]

[29] Sadhu SK, Okuyama E, Fujimoto H, Ishibashi M. Separation of Leucas aspera, a medicinal plant of Bangladesh, guided by prostaglandin inhibitory and antioxidant activities. Chem. Pharm. bull. 2003; 51, 595-598. [CrossRef]

[30] Lu Y, Shipton FN, Khoo TJ, Wiart C. Antioxidant activity determination of citronellal and crude extracts of *Cymbopogon citratus* by 3 different methods. Pharmacol. Pharm. 2014; 5:395–400. [CrossRef]

[31] Wolfe K, Wu X, Liu RH. Antioxidant activity of apple peels. J. Agric. Food Chem. 2003; 51(3), 609-614. [CrossRef]

[32] Singleton VL, Orthofer R, Lamuela-Raventos RM. [14] Analysis of total phenols and other oxidation substrates and antioxidants by means of folin-ciocalteu reagent. Methods Enzymol. 1999; 299, 152-178. [CrossRef]

[33] Bauer AW, Kirby WM, Sherris JC, Turck M. Antibiotic susceptibility testing by a standardized single disk method. Am. J. Clin. Pathol. 1966; 45, 493-496.

[34] Ahmed F, Das PK, Islam MA, Rahman KM, Rahman MM, Selim MST. Antibacterial Activity of *Cordyline terminalis* Kunth. Leaves. J. Med. Sci. 2003; 3, 418-422. [CrossRef]

[35] Mielke CH, Kaneshiro MM, Maher IA, Weiner JM, Rapaport SI. The standardized normal Ivy bleeding time and its prolongation by aspirin. Blood. 1969; 34(2), 204-215. [CrossRef]

[36] Saxena R, Kannan M, Choudhry P. Laboratory studies in coagulation disorders. Indian J. Pediatr. 2007; 74(7), 649-655. [CrossRef]

[37] Ikese CO, Okoye ZC, Kukwa DT, Adoga SO, Lenka JL. Effect of aqueous leaf extract of *Tridax procumbens* on blood coagulation. Int. J. Pharm. Sci. Res. 2015; 6(8), 3391. [CrossRef]