

Flavonoids from the Leaves of *Bridelia stipularis* with *in Vitro* Antioxidant and Cytotoxicity Activity

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Abstract

Methanolic extract of the leaves of *Bridelia stipularis* was studied. From this study, we isolated three known flavonoids. They were identified as 7-O-methyl luteolin, apigenin and 5, 7, 2', 5' tetrahydroxyflavone by NMR spectroscopic studies. All of them are first time documented for this plant. Different solvent fractions were subjected to *in vitro* antioxidant and cytotoxicity studies. Both apigenin and ethyl acetate soluble fraction of *Bridelia stipularis* showed strong antioxidant activity having IC₅₀ value of 8.005, 8.77 µg/mL respectively. Chloroform soluble fraction of *Bridelia stipularis* exerted the highest toxicity to brine shrimp and petroleum ether soluble fraction showed moderate toxicity having LC₅₀ value of 1.05, 1.71 µg/mL respectively.

Keywords

Bridelia stipularis, Flavonoid, Antioxidant, Cytotoxicity

1. Introduction

Currently in the developing world, about 25% of ingredients in the prescribed modern medicine are derived from the extracts of medicinal plant [1]. A wide range of drugs are derived from natural origins e.g. atropine from *Atropa belladonna*, camphor from *Cumanmomum camphora*, digoxin from *Digitalis purpurea*, and quinine from *Cinchona succirubra*, etc. [2]. So phytochemical research is achieving crucial significance especially for third world countries.

Bridelia is a plant genus of the family Phyllanthaceae first described as a genus in 1806 [3]. Approximately it includes 60 - 70 species [4]. *Bridelia stipularis* (L) Blume is a climbing shrub, which grows in shady, moist forest floors (**Figure 1**)



Figure 1. *Bridelia stipularis* with fruits.

[5]. The plant is distributed in Tropical Africa, Madagascar, Yemen and in different areas of Asia [5]. In Bangladesh, it is known as Harinhara, Pat Khowi [5] and Bangari gach [6]. Bioactive steroid and triterpenoids have been identified from the methanolic extract of stem bark of *Bridelia stipularis* [4]. Bridelyl alcohol and a phlobatanin were isolated from leaves while taraxenone was isolated from the hexane extract of roots of *Bridelia stipularis* [5]. A wide range of biological activities have been also reported by *Bridelia stipularis* e.g. antibacterial and antifungal activity [7], anti-candidal [8], antioxidant [9], anti-diabetic [10], cytotoxicity [7] and thrombolytic [11].

Due to diversified biological activity and less extensive research work, it is necessary to further study this plant to identify and depict bioactive principles. Our focus is to isolate and identify secondary metabolites from polar methanolic fraction of the leaves of *Bridelia stipularis*. In this study, we mention isolation of three flavonoids from the leaves of *Bridelia stipularis* along with its *in vitro* antioxidant and cytotoxicity activity. All of them are very first time reported for this plant.

2. Materials and Method

2.1. Collection and Preparation

The leaves of *Bridelia stipularis* was collected in October 2018 from Narsingdi district. Later it was identified by an expert from Bangladesh National Herbarium (BNH). After cleaning and shade drying for two weeks, they were crushed into coarse powder using high capacity grinding machine.

2.2. Extraction

About 1500 gm of powdered plant material was taken in an amber-colored bottle and soaked with distilled methanol for 15 days with occasional shaking and stirring. The mixture was therefore filtered using a fresh cotton plug. The solvent of the mixture was evaporated using Buchii Rotavapour rotary evaporator at 40°C temperature and low pressure and the extract was prepared.

2.3. Chromatographic Separation

Subsequently adding dichloromethane (DCM) to the extract, dichloromethane (DCM) soluble fraction was removed and the remaining portion was methanolic fraction of the extract. The methanolic fraction was subjected to column chromatography to separate their constituents based on adsorption using wet packing method [12]. Later these column fractions were analyzed by thin layer chromatography [13] and compounds of interest were isolated using preparative layer chromatography (PLC) [13].

2.4. Structure Elucidation

Finally their structures were elucidated using ^1H NMR spectroscopy (400 MHz, CD_3OD).

2.5. Determination of DPPH Scavenging Activity

The free radical scavenging activities of the plant extracts on 1,1-diphenyl-2-picrylhydrazyl (DPPH), a stable radical, were estimated [14]. 2.0 mL of a methanol solution of the extract at different concentration from 400.0 to 1.5625 $\mu\text{g}/\text{mL}$ were mixed with 2.0 mL of a DPPH methanol solution (20 $\mu\text{g}/\text{mL}$). After 30 minutes reaction period at room temperature in dark place the absorbance was measured at 517 nm against methanol as blank by UV spectrophotometer. The antioxidant potential was assayed from the bleaching of purple colored methanol solution of DPPH radical by the plant extract as compared to that of tert-butyl-1-hydroxytoluene (BHT) by UV spectrophotometer.

Inhibition of free radical DPPH in percent (I %) was calculated as follows:

$$I\% = \left(1 - \frac{\text{Absorbance of sample}}{\text{Absorbance of blank}} \right) \times 100\%$$

Where, Absorbance of blank is the absorbance of control reaction (containing all reagents except the test material).

Extract concentration providing 50% inhibition (IC_{50}) was calculated from the graph plotted inhibition percentage against extract concentration.

2.6. Brine Shrimp Lethality Bioassay

Brine shrimp eggs were hatched in simulated sea water to get nauplii. By the addition of calculated amount of dimethylsulphoxide (DMSO), desired concentration of the test samples were prepared. The nauplii were counted by visual inspection and were taken in vials containing 5 ml of simulated sea water. Then samples of different concentrations were added to the pre-marked vials through micropipette. The vials were then left for 24 hours. Survivors are counted after 24 hours [15]. The median lethal concentration (LC_{50}) value was calculated from the graph plotted percentage mortality rate against extract concentration.

3. Results and Discussion

Three known flavonoids have been isolated from the methanolic fraction of the

leaves of *Bridelia stipularis*. They were identified as 7-O-methyl luteolin (**1**), apigenin (**2**) and 5, 7, 2', 5' tetrahydroxyflavone (**3**) by ^1H NMR spectroscopic studies and comparing with the published data (**Figure 2**).

Ethyl acetate soluble fraction of *Bridelia stipularis* and apigenin isolated from the plant showed strong antioxidant activity having IC_{50} value of 8.77, 8.005 $\mu\text{g}/\text{mL}$ respectively against BHT with IC_{50} value 5.64 $\mu\text{g}/\text{mL}$. Chloroform soluble fraction of *Bridelia stipularis* was found to be most toxic to brine shrimp and petroleum ether soluble fraction showed moderate toxicity having LC_{50} value of 1.05, 1.71 $\mu\text{g}/\text{mL}$ respectively with compared to vincristine sulphate with LC_{50} value 0.9258 $\mu\text{g}/\text{mL}$.

3.1. Characterization of Compound 1

Compound **1** was obtained from the test tubes 80 - 83 of column chromatography by PLC as colorless liquid and molecular formula of **1** was determined as $\text{C}_{16}\text{H}_{13}\text{O}_7$. ^1H NMR spectrum (400 MHz, CD_3OD) of **1** (**Table 1**) showed a proton singlet at δ 6.60 proton was assigned to H-3. Three broad singlets at 6.18, δ 6.42 and δ 7.49 was comparable to that of three *meta* coupled aromatic doublets which were assigned to H-6, H-8 and H-2'. A proton signal at δ 6.93 (d, $J = 8.4$ Hz) proton was assigned to H-5' and another proton signal at 7.51 (d, $J = 8.4$ Hz) was comparable to that of an *ortho* and *meta* coupled aromatic proton assigned to H-6'. Finally three protons singlet at δ 3.97 was characteristic for the methyl proton located at 7 position of the benzene ring. So **1** was identified as 7-O methyl luteolin [16].

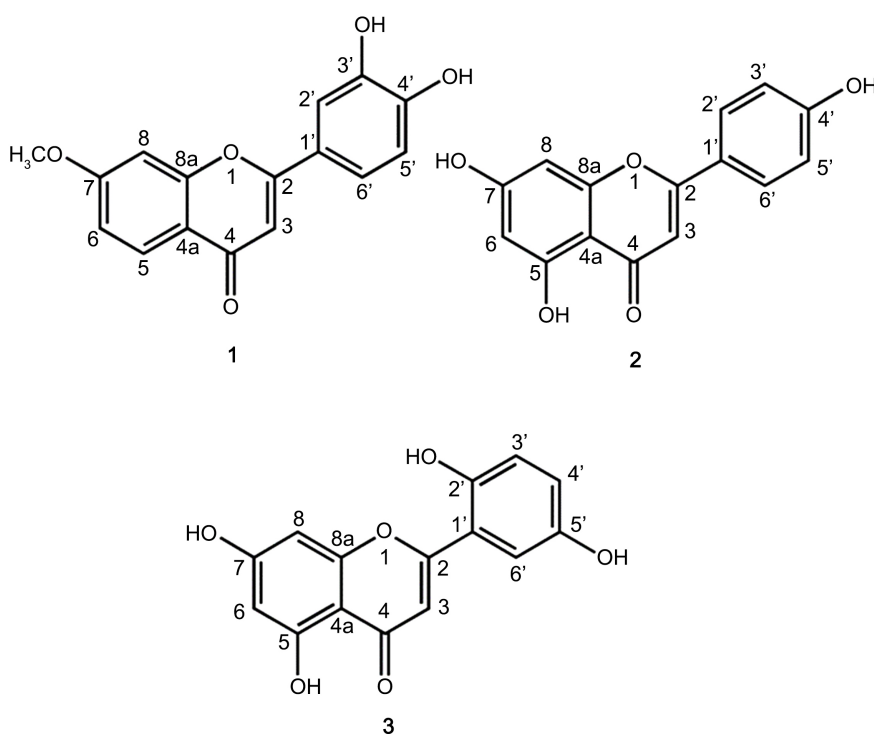


Figure 2. Chemical structure of compound **1**, **2** and **3**.

Table 1. ¹H NMR (400 MHz, CD₃OD) spectroscopic data of compound **1**, **2** and **3**.

Position	δ_{H} , J in Hz		
	1	2	3
H-3	6.60 (s)	6.58 (s)	6.53 (s)
H-6	6.18 (bs)	6.18 (d, $J = 2.0$ Hz)	6.20 (d, $J = 2.0$ Hz)
H-8	6.42 (bs)	6.46 (d, $J = 2.0$ Hz)	6.43 (d, $J = 2.0$ Hz)
H-2'	7.49 (bs)	7.85 (d, $J = 8.8$ Hz)	-
H-3'	-	6.93 (d, $J = 8.8$ Hz)	6.89 (d, $J = 8.4$ Hz)
H-4'	-	-	7.38 (dd, $J = 2.4$ Hz, 8.4 Hz)
H-5'	6.93 (d, $J = 8.4$ Hz)	6.93 (d, $J = 8.8$ Hz)	-
H-6'	7.51 (d, $J = 8.4$ Hz)	7.85 (d, $J = 8.8$ Hz)	7.37 (d, $J = 2.4$ Hz)
OCH ₃ -7	3.97 (s)	-	-

3.2. Characterization of Compound 2

Test tubes 230 - 234 of column chromatography yielded compound **2** by PLC as colorless liquid and its molecular formula was found to be C₁₅H₁₀O₅. In ¹H NMR spectrum (400 MHz, CD₃OD) of **2** (Table 1), a proton singlet at δ 6.58 proton was assigned to H-3. Two protons signals at δ 6.18 (d, $J = 2.0$ Hz) and δ 6.46 (d, $J = 2.0$ Hz) protons were assigned to H-6 and H-8 respectively. H-6 and H-8 showed *meta* coupling ($J = 2.0$ Hz) to each other. Two protons signals at δ 6.93 (d, $J = 8.8$ Hz) were assigned to aromatic protons H-3', H-5'. Another two protons signals at δ 7.85 (d, $J = 8.8$ Hz) protons were assigned to H-2', H-6' of B ring respectively. H-2', H-6' and H-3', H-5' showed *ortho* coupling ($J = 8.8$ Hz) to each other. These data were characteristic for a 5, 7, 4' trisubstituted flavone. By analyzing the spectral data, compound **2** was identified as apigenin [16].

3.3. Characterization of Compound 3

Compound **3** was obtained from the test tubes 240-246 of column chromatography as colorless liquid by PLC. Molecular formula of **3** was determined as C₁₅H₁₀O₆. In ¹H NMR spectrum (400 MHz, CD₃OD) of **3**, (Table 1) showed a proton singlet at δ 6.53 which was assigned to H-3. Two proton signals at δ 6.20 (d, $J = 2.0$ Hz) and 6.43 (d, $J = 2.0$ Hz) protons were assigned to H-6 and H-8 respectively. H-6 and H-8 showed *meta* coupling ($J = 2.0$ Hz) to each other. Three proton signals at δ 7.38 (dd, $J = 8.4$ Hz, 2.4 Hz) and 6.89 (d, $J = 8.4$ Hz), 7.37 (d, $J = 2.4$ Hz) indicated the ABC type aromatic ring where they were assigned to H-4', H-3' and H-6' of the B ring. Based on above features, compound **3** was confirmed as 5, 7, 2', 5' tetrahydroxyflavone [17].

3.4. Free Radical Scavenging Activity

By measuring free radical scavenging activity, antioxidant activity of plant extracts can be measured. Table 2, Figure 3 showed inhibition rates of different fractions of *Bridelia stipularis*. Table 3, Figure 4 showed different de-

gree of antioxidant activity exerted by different solvent fractions of the plant as well as apigenin. Here apigenin as well as ethyl acetate soluble fraction showed strong antioxidant activity. Antioxidant activity of *Bridelia stipularis* has also been reported previously [4] [9] [11] [18] which can be explained from the presence of flavone derivatives. The chemical structure of flavone derivatives has impact on free radical scavenging activity. Their antioxidant activity is directly proportional to the number of hydroxyl moieties present in their structure [19].

3.5. Brine Shrimp Lethality Bioassay

Brine shrimp lethality bioassay has been utilized as a primary screening method of lethality of different plant extracts. It is a simple, rapid, inexpensive, in house screening method. All the samples having LC₅₀ value < 1000 µg/mL are considered for further pharmacological analysis [15]. **Table 4, Figure 5** demonstrated mortality rate of different fractions of *Bridelia stipularis* while **Table 5, Figure 6** showed different degree of lethality of plant extracts of *Bridelia stipularis* to Brine shrimp. Among the fractions, chloroform soluble fraction was found to be

Table 2. % Inhibition value of different fractions of *Bridelia stipularis*.

Conc. (µg/mL)	BHT	BSA	BSE	BSC	BSP	Apigenin
400	96.36%	78.46%	96.36%	80.45%	70.51%	93.04%
200	95.03%	74.16%	95.03%	75.81%	67.19%	88.73%
100	91.39%	68.2%	94.37%	67.86%	60.90%	80.45%
50	87.08%	65.21%	92.71%	49.30%	57.26%	67.53%
25	77.47%	59.24%	76.47%	32.73%	49.97%	65.21%
12.5	64.21%	48.64%	53.94%	27.43%	41.35%	58.91%
6.25	51.95%	41.02%	41.02%	23.46%	38.37%	47.32%
3.125	37.71%	35.06%	30.42%	17.83%	36.71%	41.02%
1.5625	26.77%	26.77%	17.83%	11.53%	28.42%	26.77%

BHT = Tert-butyl-1-hydroxytoluene, BSA = Aqueous soluble fraction, BSE = Ethyl acetate soluble fraction, BSC = Chloroform soluble fraction, BSP = Petroleum ether soluble fraction, BS = *Bridelia stipularis*; Absorbance of blank = 0.3018.

Table 3. Antioxidant activity of different fractions of *Bridelia stipularis*.

Different fractions	IC ₅₀ value	Regression Equation	R ²
BHT	5.64	$y = 0.1327 \ln(x) + 0.2705$	0.9412
BSA	36.65	$y = 0.0622 \ln(x) + 0.276$	0.2227
BSE	8.77	$y = 0.1571 \ln(x) + 0.1589$	0.9186
BSC	42.26	$y = 0.1374 \ln(x) + 0.0043$	0.953
BSP	24.78	$y = 0.0777 \ln(x) + 0.2513$	0.9861
Apigenin	8.005	$y = 0.1162 \ln(x) + 0.2583$	0.9842

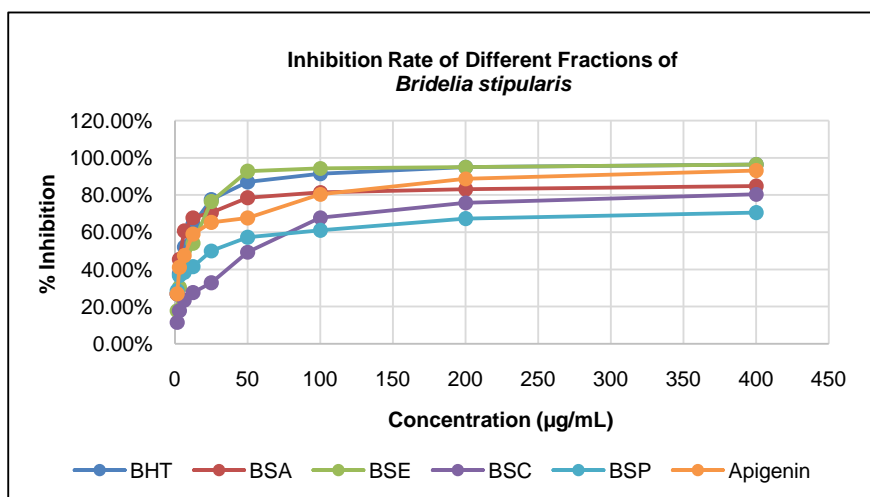


Figure 3. Inhibition rate of different fractions of *Bridelia stipularis*.

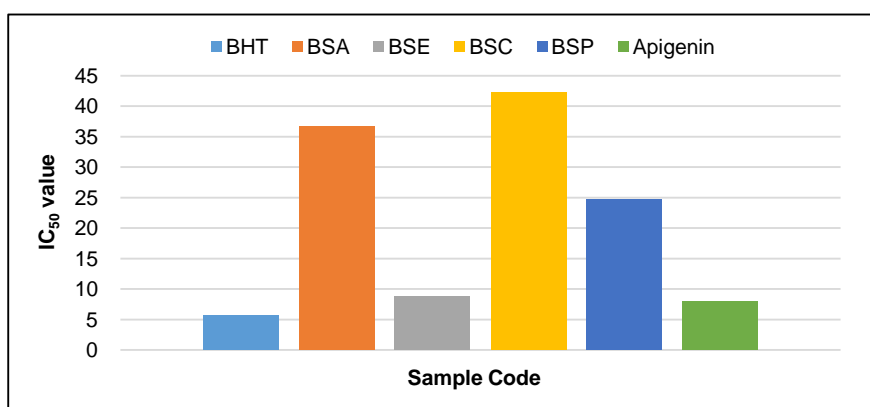


Figure 4. IC₅₀ value of different fractions of *B. stipularis* and apigenin.

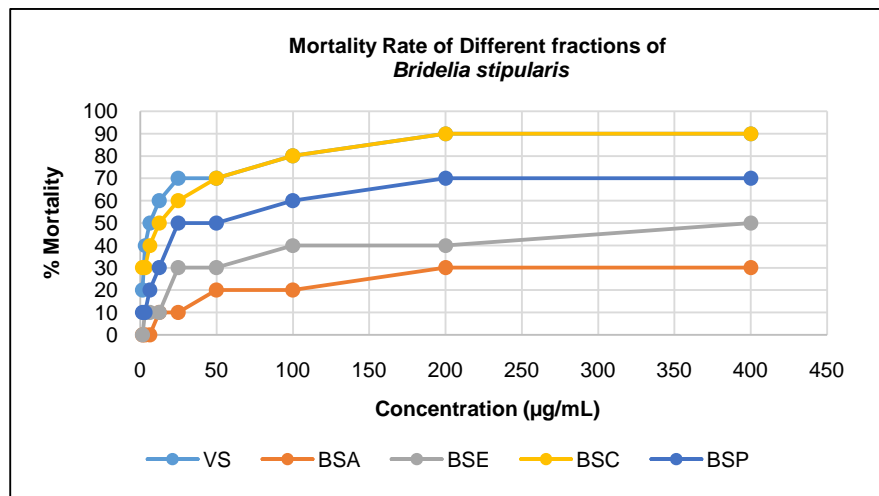
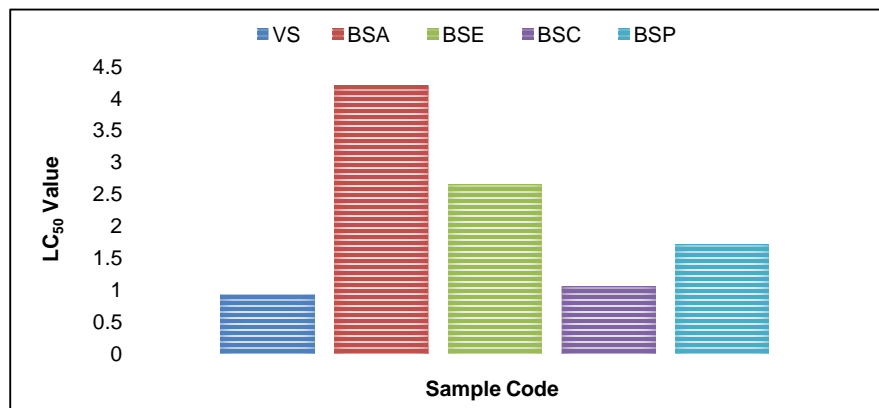
Table 4. % Mortality rate of different fractions of *Bridelia stipularis*.

Conc. (µg/mL)	VS	BSA	BSE	BSC	BSP
400	90	30	50	90	70
200	90	30	40	90	70
100	80	20	40	80	60
50	70	20	30	70	50
25	70	10	30	60	50
12.5	60	10	10	50	30
6.25	50	0	10	40	20
3.125	40	0	10	30	10
1.5625	20	0	0	30	10

VS = Vincristine sulphate.

Table 5. Cytotoxicity of different fractions of *Bridelia stipularis*.

Different fractions	LC ₅₀ value	Regression equation	R ²
VS	0.9258	$y = 27.985x + 24.091$	0.9614
BSA	4.2	$y = 12.885x - 4.0732$	0.9127
BSE	2.65	$y = 20.485x - 4.1928$	0.942
BSC	1.05	$y = 28.79x + 19.753$	0.9797
BSP	1.71	$y = 28.79x + 0.8645$	0.9611

**Figure 5.** Mortality rate of different fractions of *Bridelia stipularis*.**Figure 6.** LC₅₀ value of different fractions of *Bridelia stipularis*.

most toxic to brine shrimp and petroleum ether soluble fraction showed moderate toxicity compared to anticancer drug vincristine sulphate. Cytotoxic activity of this plant extracts has also been previously reported [7]. As there was no available data on Acute Oral Toxicity Assay using this plant extract, further *in vivo* study is required to ensure its activity without toxicity in susceptible biological systems.

4. Conclusion

Three flavonoids, named as 7-O-methyl luteolin, apigenin and 5, 7, 2', 5' tetra-hydroxyflavone have been isolated from the methanolic extracts of leaves of *Bridelia stipularis*. Isolation of these compounds from this plant is documented for the first time. Some of the fractions showed antioxidant activity as well as lethality. So the investigation revealed the phytochemical importance of this plant.

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Conflicts of Interest

The authors have no conflict of interest.

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