ISSN: 2469-9062 (print), 2467-9240(e)

*Journal of Institute of Science and Technology, 2015, **20(1):** 97-101, © IOST, Tribhuvan University*

Bioassay Guided Isolation of Free Radical Scavenging Agent from the Bark of *Bridelia retusa*

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ABSTRACT

Bioassay guided fractionation of ethyl acetate soluble fraction of methanolic extract of *Bridelia retusa* yielded tambulin (**1**), *β*-sitosterol (**2**), and *β*-sitosterol glucoside (**3**), and their structures were elucidated using modern spectroscopic techniques. Compounds **1-3** were evaluated for their antioxidant activity and compound **1** was found to be potent antioxidant. The DPPH assay showed that the compound **1** has potent antioxidant activity with IC_{50} 166.15±1.92 SEM [μM] and the radical scavenging activity was found to be 86.03% where as the standard butylated hydroxy toluene (BHT) has IC_{50} value 128.83±2.1 SEM [μM]. The radical scavenging activity of standard butylated hydroxy toluene was about 86% .The total phenolic content in ethyl acetate soluble fraction was found to be 147.20±1.5 mg GAE/gm and the total flavonoid content was found to be 16.64±0.00 mg QE/gm.

Keywords:- *Bridelia retusa*, tambulin, free radical scavenging, DPPH

INTRODUCTION

Bridelia retusa is a moderate sized tree belonging to Euphorbiaceae family found growing throughout Nepal. Traditionally the Paste of the stem bark is applied to wounds and bark juice taken internally in case of snake bite (Joshi *et al*. 2006, Anna *et al*. 2011). The roots and barks of *B. retusa* have been used for the treatment of headache, abnormal pain and indigestion (Mostafa *et al*. 2006, Thadhani *et al*. 2011). The bark of *B. retusa* has been traditionally used for the management of different ailments such as rheumatism, anti-inflammatory, immune enhancer, diarrhea and dysentery (Banarjee *et al*. 2009, Tatiya *et al*. 2011). Essential oils of leaf and fruit of *B. retusa* were active against *Staphylococcus aureus*, *Escherichia coli* and *Pseudomonas aeruginosa* (Duangsuree *et al*. 2012). Kumarihamy *et al*. (2005) reported sesquiterpenes (E)-4-(1, 5- dimethyl-3-oxo-1-hexenyl) benzoic acid, (E)- 4 - (1,5-dimethyl- 3-oxo-1, 4- hexadienyl) benzoic acid, (E)-4-(1,5- dimethyl-3-oxohexyl) benzoic acid (artodomatuic acid), 5-allyl-1, 2, 3 - trimethoxy-benzene (elemicin) and 4-isopropylbenzoic acid (cumic acid) as an antifungal constituents from the methanolic bark extracts of *B. retusa*.

In the study, 80% methanolic extract of bark of *B. retusa* was fractionated by solvent–solvent extraction and the ethyl acetate soluble fraction was subjected to determine polyphenol, flavonoid content and antioxidant activity. The result showed that the fraction has high polyphenol content, flavonoid content and possesses potent antioxidant activity. The ethyl acetate fraction was further subjected to repeated column chromatography which yielded compounds (**1**), (**2**) and (**3**). The compound tambulin (**1**) was found to be active against DPPH antioxidant activity.

MATERIALS AND METHODS

Plant materials

The bark of *B. retusa* (Euphorbiaceae) was collected in the January 2, 2013 from Syangja district, Chinnebash ward no. 6. The plant was identified by Rita Chhetry, Research Officer, National Herbarium and Plant Resources, Ministry of Forests and Soil Conservation, Godawari, Nepal.

Extraction

The plant sample was shade dried at room temperature and powdered material was then weighed (8.5 kg), soaked in methanol water (80%) for 72 h and filtered. The filtrate obtained was concentrated under reduced pressure in a rotatory evaporator to obtain the crude extract (500 g).The crude extract was dissolved in distilled water (4 L) and extracted by solvent-solvent extraction with increasing order of polarity. The crude extract was not soluble in hexane and dichloromethane due to which it was further extracted with ethyl acetate.

Total polyphenol content determination in ethyl acetate fraction

The total phenolic content was determined using the Folin–Ciocalteu phenol reagent. 0.1 mL of each extract (2.5 mg/mL) was separately mixed with the 1 mL of Folin–Ciocalteu phenol reagent and 0.8 mL of aqueous 1 M Na_2CO_3 solution. The reaction mixture was allowed to stand for about 15 minutes and the absorbance of the reactants was measured at 765 nm using the UV- visible spectrophotometer. The calibration curve was obtained using the solution of gallic acid as standard in methanol using the concentration ranging from 25-250 µg/mL. Based on this standard graph, the concentration of the individual samples were calculated. The total polyphenol content was expressed in terms of the milligrams of the gallic acid equivalent per gram of the dry mass mg GAE g^{-1} (Velioglu *et al*. 1998).

Total flavonoid content determination in ethyl acetate fraction

The total flavonoid content was estimated by using the Aluminium chloride ($AlCl_3$) colorimetric method. 0.25 mL of extract (10 mg/mL) was separately mixed with the 0.75 mL of methanol, 0.05 mL of the 10% aluminum chloride, 0.05 mL of the 1 M potassium acetate (CH_3COOK) and 1.4 mL of the distilled water. The reaction mixture was allowed to stand for about 30 minutes in room temperature. The absorbance of the mixture was measured at 415 nm using the UV visible spectrophotometer. The calibration curve was constructed with the help of standard quercetin solutions in methanol with the concentration ranging from the 10-100 µg /mL. The total flavonoid content was expressed in terms of the milligram of quercetin equivalent per gram of the dry mass mg QE/gm (Sirappuselv *et al*. 2012).

DPPH radical scavenging assay of ethyl acetate fraction

DPPH solution (95µL, 300µM) in ethanol is mixed with test solution (5µL, 500µM). The reaction is allowed to progress for 30 min at 37 ^oC and absorbance was monitored by multiplate reader, Spectra Max340 at 517 nm. Upon reduction, the color of the solution fades (Violet to pale yellow). Percent Radical Scavenging Activity (% RSA) was determined by comparison with a DMSO containing control. The concentration that causes a decrease in the initial DPPH concentration by 50% is defined as IC_{50} value. The IC_{50} values of compounds were calculated by using the EZ-Fit Enzyme kinetics software program (Perrella Scientific Inc. Amherst, MA, USA). N-acetylcysteine, ascorbic acid and BHA are used as the reference compounds. The inhibition curve was plotted for the triplicate experiments and represented as percentage of mean inhibition ± standard deviation and the IC_{50} values were obtained.

Antimicrobial activity of ethyl acetate fraction

The antibacterial activity of ethyl acetate fraction of bark of *B. retusa* against the test organisms was performed by agar well diffusion method. Sterile Muller Hinton Agar (MHA) plates of approximately 4 mm thickness were prepared and dried under hot air oven at appropriate temperature. A sterile cotton swab was dipped into the prepared inoculums and swabbed carefully all over the plate. The inoculated plates were left to dry for few minutes at room temperature with the lid closed. Then with the help of sterile cork borer no 5, wells were made in the inoculated media plates and labeled properly. So, the diameter of a well was 6 mm. Then 50 µL of the plant extract was introduced into respective well. In one well pure methanol was filled as control. The plates were then left for half an hour with the lid closed so that the extract diffused into media. The plate was incubated overnight at 37 ^0C. After proper incubation (18-24 hours) the plates were observed for the zone of inhibition around well.

Isolation of pure compounds

The shade dried bark of *B. retusa* (8.5 kg) was extracted with 80% methanol water (30 L) for three times. The concentrated methanolic extract (500 g) after evaporation of solvent was then dissolved distilled water (5 L). The aqueous layer was then subjected to solvent-solvent extraction. First the aqueous layer was extracted with n-hexanes (each 2 L volume of aqueous layer three times with 2 L of n-hexanes) and the aqueous layer was then extracted with CH_2Cl_2. The extract was insoluble in both the hexanes and dichloromethane solvents. The aqueous layer was then extracted with ethyl acetate and 15 g of crude ethyl extract fraction was obtained. The ethyl acetate fraction (15 g) was subjected to column chromatography in order of increasing polarity of ethyl acetate in hexane, which yielded many sub fractions. Out of them sub fraction **B** (200 mg) obtained by 25% EtOAc/hexane was further subjected to silica gel column chromatography using 15% EtOAc/hexane as an eluting agent which yielded compound **1** (20 mg). The sub fraction **A** (300 mg) obtained by 10% EtOAc/hexanes was further subjected to silica gel column chromatography using 5% EtOAc/hexanes as an eluting agent which yielded compound **2** (65 mg). The sub fraction **C** (200 mg) obtained by 40% EtOAc/hexanes was further subjected to silica gel column chromatography using 30% EtOAc/hexanes as an eluting agent which yielded compound **3** (10 mg).

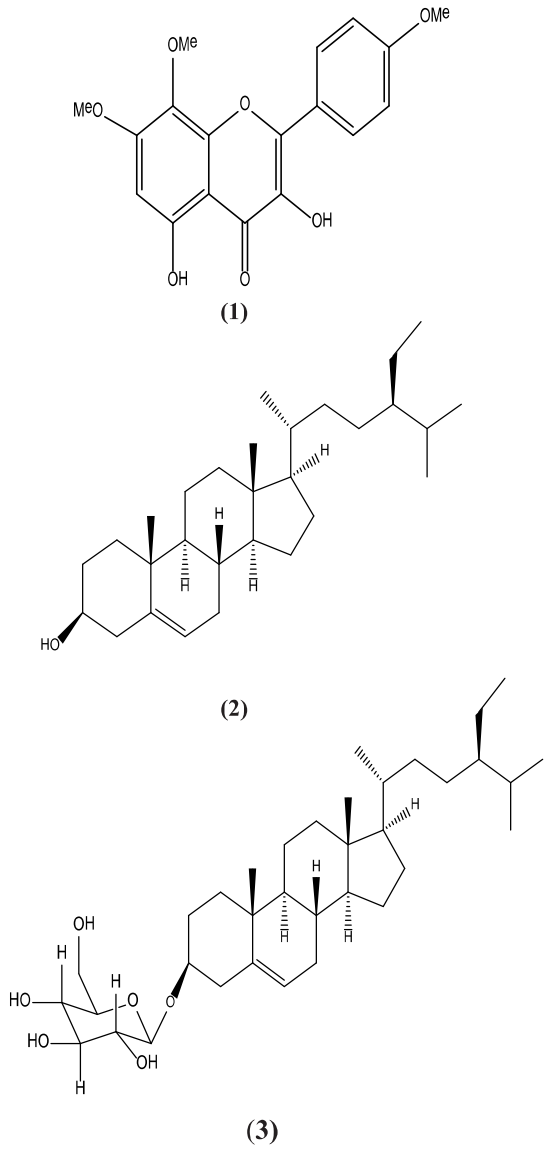

Fig. 1. Structure of isolated compounds

Toxicity Assay

Cytotoxicity activity of compound **1** was evaluated on 3T3 cell line by using the standard MTT (3-[4,5-dimethylthiazole-2-yl]-2,5-diphenyl-tetrazolium bromide) colorimetric assay according to Mosmann T (1983). In this assay compound **1** showed no toxic effect (IC_{50} = 200 µM).

RESULTS AND DISCUSSION

Compound **1** was obtained as yellow powder. The EI-MS spectrum of compound **1** showed molecular ion [M^+] at *m/z* 344 and base peak at *m/z* 329 which was corresponding to molecular formula $C_{18}H_{16}O_7$. The IR spectrum displayed absorptions at 3327 (OH), 1651 (aromatic), and 1556 (olefinic) cm^{-1}. The UV spectrum showed absorptions at 367, 325 and 273 nm.

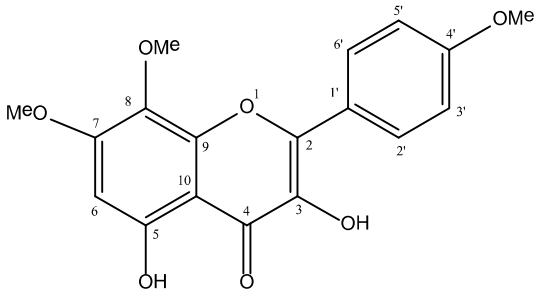

Fig. 2. Structure of compound 1

The ^{1}H-NMR spectrum exhibited resonances for three signals at δ 3.88 s, 3.92 s, and 3.94 s were attributed for protons of methoxy group attached to C-4', C-7 and C-8 respectively. A downfield singlet resonated at δ 6.43 was ascribed to H-6, similarly two downfield ortho coupled doublets at δ 7.02 d (J = 9.0 Hz) and 8.22 d (J = 9.0 Hz), were assigned to H-3'/5' and H-2'/6' respectively. A down field signal at δ 11.58 s was assigned to intramolecular hydrogen bonded C_5-OH proton and a broad singlet at δ= 6.55 was assigned to C_3- OH proton. The ^{13}C-NMR spectra (broad band and DEPT) displayed the resonances for all eighteen carbons including three methyl, five methine and ten quaternary carbons. Structure of compound was further confirmed from 2D-NMR spectra (COSY, HSQC, HMBC and NOESY). Position of hydroxyl and methoxy groups was assigned with the help of the HMBC correlation. The HMBC correlation between methoxyl protons at δ 3.88 showed long range correlation with (C-4') δ 160.5 and 3.92 showed correlation with (C-8) δ 129.4, 3.94 and 159.1 (C-7) clearly indicated the position of methoxy groups in compound. The key HMBC correlations in compound **1** are shown in Fig.3.

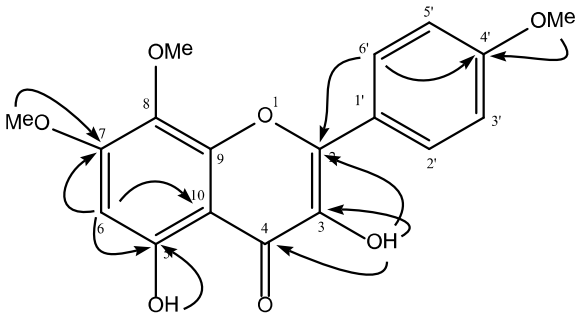

Fig. 3. Key HMBC correlations of compound 1.

All the spectral data of compound **1** were unambiguously matched with reported data of tambulin (Babu *et al.* 2007).

Table 1. ^{1}H-NMR and ^{13}C-NMR chemical shift of compound 1

Position	δH (ppm)	δC (ppm)
C_1	-	-
C_2	-	145.84
C_3 (-OH)	6.55	135.38
C_4	-	175.51
C_5 (-OH)	11.58	156.3
C_6	6.43	95.15
C_7	-	159.1
C_8	-	129.49
C_9	-	161.23
C_{10}	-	109.33
$C_{1'}$	-	123.35
$C_{2'}$	7.71	129.49
$C_{3'}$	6.98	114.7
$C_{4'}$		160.5
$C_{5'}$	6.55	114.18
$C_{6'}$	7.68	129.95
C_7 (-OCH_3)	3.92	55.93
C_4' (-OCH_3)	3.88	55.41
C_8 (-OCH_3)	3.94	61.59

Anti- microbial, antioxidant, total phenolic and flavonoid content of ethyl acetate fraction

The ethyl acetate fraction showed potent antimicrobial activity against *S. aureus*, *S. typhi, E. coli* and *B. subtilic* in which the diameter of zone of inhibition was compared with standard drugs Ciprofloxacin, Erythromycin and Chloramphenicol.

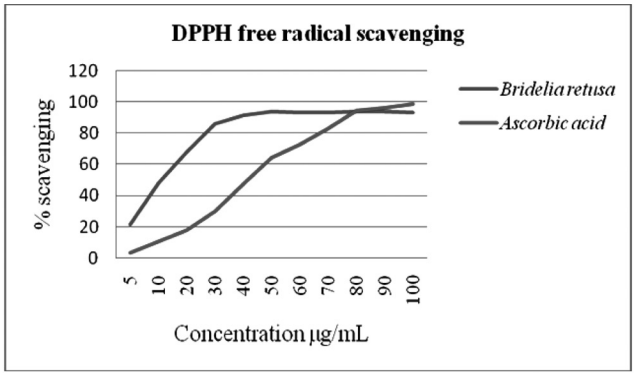

Fig. 4. Free radical scavenging of ethyl acetate fraction with ascorbic acid

Table 2. Anti-microbial activity of ethyl acetate fraction

Diameter of Zone of Inhibition (ZOI) well diameter 6 mm					
Concentration	Control	*Staphylococcus aureus*	*Salmonella typhi*	*Escherichia coli*	*Bacillus subtilic*
10 mg/mL	6 mm	13 mm	12 mm	16 mm	11 mm
15 mg/mL	6 mm	14 mm	13 mm	17 mm	16 mm
20 mg/mL	6 mm	19 mm	16 mm	19 mm	18 mm

Table 3. Anti-microbial activity standard drugs

Diameter of Zone of Inhibition (ZOI)					
Standard drugs	Control	*Staphylococcus aureus*	*Salmonella typhi*	*Escherichia coli*	*Bacillus subtilic*
Ciprofloxacin	6 mm	32 mm	30 mm	46 mm	16 mm
Erythromycin	6 mm	22 mm	-	30 mm	20 mm
Chloramphenicol	6 mm	-	25 mm	-	-

DPPH scavenging activity of compound (1)

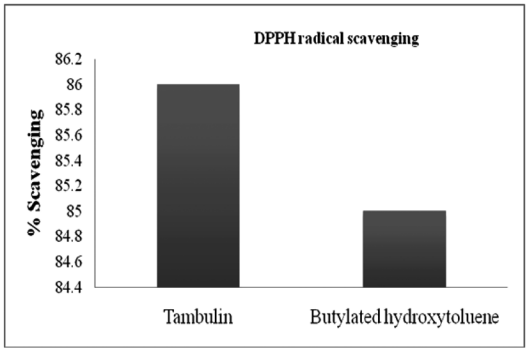

Fig. 5. Radical scavenging of tambulin and standard BHT

Total phenolic content in ethyl acetate fraction of bark of *B. retusa* was found to be 147.20±1.5 mg GAE/gm and the total flavonoid content was found to be 16.64±0.0 mg QE/gm. The DPPH antioxidant activity (IC_{50} value) of ethyl acetate fraction of *B. retusa* was found to be (15.07±0.0 μg/mL).

The total phenolics content of the plant extracts is compared to the extracts of some previously studied plants (Subedi *et al*. 2014). Total phenolics of some previously studied plant extracts was found as *Origanum dictamnus* (8.2±0.3 mg GA/gm), *Eucalyptus globules* (10.5±0.3), *Sideritis cretica* (8.6±0.2), *Thymus vulgaris* (8.0±0.1), *Satureja thymbra* (9.2±0.1), *Lavandula vera*

(4.9±0.1), *Lippa triphylla* (7.7±0.1) and *Matricaria chamomilla* (6.1±0.1) (Procestos *et al*. 2013). The result showed that the ethyl acetate soluble fraction studied in this work showed the potent sources of secondary metabolites and could be used as the sources to isolate the active ingredient.

The radical scavenging activity showed that the compound **1** has potent antioxidant activity with IC_{50} 166.15±1.92 SEM [μM] and the radical scavenging activity 86.03% where as the standard butylated hydroxytoluene has IC_{50} 128.83±2.1 SEM [μM] and 85.87% radical scavenging activity.

CONCLUSIONS

Ethyl acetate soluble fraction of methanolic bark extract of *Bridelia retusa* showed potent antibacterial activity against gram negative *E. coli* and *Salmonella Typhi* and gram positive bacteria such as *Staphylococcus aureus* and *Bacillus subtilic*. In this way the plant extract could be used for isolation of active ingredient against gram positive and gram negative bacteria. DPPH radical scavenging assay showed that the ethyl acetate soluble fraction showed potent antioxidant activity. Three compounds tambulin, *β*-sitosterol and *β*-sitosterol glucoside were isolated from ethyl acetate soluble fraction. The structures of the compounds were confirmed by modern spectroscopic technique. The compound tambulin was subjected for antioxidant activity. The compound showed potent antioxidant activity in DPPH radical scavenging activity.

ACKNOWLEDGEMENTS

We are thankful to the Nepal Academy of Science and Technology, Nepal, for providing Ph. D. fellowship to Mr. Khaga Raj Sharma. Rita Chhetry, Ministry of Forests and Soil Conservation, Godawari, Nepal is thankful for identification of plant.

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