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GC-MS Analysis of Chemical Constituents and *in vitro* Antioxidant Activity of the Organic Extracts from the Stem of *Bridelia stipularis*

(Analisis GC-MS Juzuk Kimia dan Aktiviti Antioksidan Ekstrak Organik secara *in vitro* daripada Batang *Bridelia stipulari*)

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ABSTRACT

In the present study the stems of the Bridelia stipularis *(L.) Blume, which is traditionally used by ethnic communities in Sabah, Malaysia, has been investigated for its chemical composition, total flavonoid content (TFC) and total phenolic content (TPC)* via *Gas-Chromatography-Mass Spectroscopy (GC-MS) analysis consuming hexane, chloroform and ethyl acetate as extraction solvents and gallic acid and quercetin as internal standards.* In vitro *antioxidant activity (AA) was determined by the application of 1,1-diphenyl-2-picryl hydrazine (DPPH) radical scavenging assay using tert-butyl-1-hydroxytoluene (BHT) as comparative drug. The GC-MS profiling showed the presence of 1-dodecanol (40.917%), oxalic acid, cyclobutyl octadecyl ester (24.985%), 1-octanol,2-nitro (12.424%), benzaldehyde, 2,4-dimethyl- (9.583%), 4-tridecanol (6.359%) and nitric acid, nonyl ester (5.616%) as major constituents. The TPC (224.62 ± 0.08 mg QE/g) and TFC (160.48 ± 0.08 mg GAE/g) was reported highest for the most polar solvent i.e. ethyl acetate. The* in vitro *antioxidant study disclosed highest IC_{50} value for ethyl acetate (2.15 mg/mL), queued by chloroform (1.19 mg/mL) and hexane (0.89 mg/mL), displaying that polar solvents are good extraction solvents for the identification of free radical scavenging properties, TFC and TPC.*

Keywords: Antioxidant activity; GC-MS; medicinal plants; total flavonoid content; total phenolic content

ABSTRAK

Dalam kajian terbaharu, batang Bridelia stipularis *(L.), Blume secara tradisi digunakan oleh etnik pribumi di Sabah, Malaysia, telah dikaji kerana komposisi kimia, kandungan jumlah flavonoid (TFC) dan jumlah (kandungan fenolik KUHP) melalui analisis Gas Jisim Spektroskopi Kromatografi (GC-MS) menggunakan heksana, kloroform dan etil asetat sebagai pengekstrakan pelarut, asid galik dan Quercetin sebagai piawai dalaman. Aktiviti antipengoksida (AA)* in vitro *ditentukan oleh aplikasi hidrazina 1,1-difenil-2- pikril (DPPH) radikal menghapus-sisa cerakin menggunakan tert-butil-1-hidroksitoluen (BHT) sebagai perbandingan dadah. GC-MS pemprofilan mendedahkan kehadiran 1-dodekanol (40.917%), asid oksalik, ester oktadekil cyclobutyl (24.985%), 1-oktanol,2-nitro (12.424%), benzaldehid, 2,4-dimetil-(9.583%), 4-tridekanol (6.359%) dan asid nitrik, ester nonil (5.616%) sebagai juzuk utama. Dalam TPC (224.62 ± 0.08 mg QE/g) dan TFC (160.48 ± 0.08 mg GAE/g) dilaporkan tertinggi bagi pelarut paling terkutub iaitu etil asetat. Kajian antioksidan* in vitro *dinyatakan nilai$_{50}$ IC tertinggi untuk etil asetat (2.15 mg/mL), diikuti oleh kloroform (1.19 mg/mL) dan heksana (0.89 mg/mL), menunjukkan kutub pelarut yang baik pengekstrakan pelarut bagi pengenalpastian sifat radikal skaveng, TFC dan TPC.*

Kata kunci: Aktiviti antioksidan; GC-MS; jumlah kandungan fenolik; jumlah kandungan flavonoid; tumbuhan perubatan

Introduction

For thousands of years, people all over the world have used medicinal plants as base in making traditional medicines and had given great advantages to mankind to come up with new remedies (Krishnaiah et al. 2011). Medicinal plants contain bioactive compounds, for instance, saponins, tannins, flavonoids and essential oils (Sofowora 1993). These phytochemicals are produced as a result of normal metabolic activities of plants and are also known as secondary metabolites (Meskin et al. 2002). These are the originator of medicinal properties within plants, as exemplification, antimicrobial (Sachidananda et al. 2015), anti-aging (Fujita et al. 2012), antioxidant (Wu et al. 2004) and most importantly antidiabetic (Firdous 2014).

Traditional medicines are not only contributing to primary health care, but also in the development of modern drugs (Vedavathy 2003). Countries like India and China are popularly known when it comes to traditional medicines because they believe them to be safer, more effective and inexpensive (Katewa et al. 2004). In Malaysia, the earliest report on phytochemical screening of 205 medicinal plants was performed in the year 1954 by Arthur in Sabah (East Malaysia) (Arthur 1954), followed by the identification of alkaloids in 200 plant species in 1957 by Douglas and

Kiang in the peninsular area (West Malaysia) (Douglas & Kiang 1957). Malaysia is the habitat of range of medicinal plants due to its tropical climatic conditions and some of the them are currently undergoing research such as *Eurycoma longifolia* Jack (tongkat ali), *Labisia pumila* (kacip fatimah) and *Orthosiphon stamineus* to mention a few (misai kucing) (Jamal 2006). In Sabah, there is an abundance of natural resources such as plants and therefore, the indigenous society are inseparable from the natural environment (Halim et al. 2013). The ethnic groups are utilizing their traditional knowledge and experience in inheriting them to their younger generations in order to treat ailments (Kodoh et al. 2017). Their daily lives are depended on nature and this has influenced and helped them in forming traditional knowledge (Halim et al. 2013). It is estimated that nearly 70 species of *Bridelia* are genus of Africa and Asia (Kathriarachchi et al. 2005). The most studied species is *Bridelia ferruginea* which is used traditionally to treat rheumatism pain and intestine disorders. Some of the chemical compounds found in this species are tannins and quercetin. Currently, approximately 119 subspecies are recognized in the genus *Bridelia*. *Bridelia stipularis* (L.) Blume is a woody evergreen climber shrub, which belongs to the family *Euphorbiaceae* and preferably grows in shady and moist forest floors (Figure 1) (Anjum et al. 2011). It is valuable as a medicine in treating several diseases (Ngueyem 2009). For example, it is traditionally used in amoebic dysentery, chest pain, constipation, diarrhea, leucoderma and strangury (Nasir 2006). Moreover, the leaves are also for curing jaundice (Krishnan 1992). Furthermore, several compounds have been isolated from this plant which are pharmaceutically important, as an example, phytochemicals, proteins, carbohydrate, vitamins, alkaloids, flavonoids, steroids and phenolic substances (Mall et al. 2017; Ngueyem 2009). According to a recent survey done by Kulip (2003), it is found to be believed by the native communities of Sabah that are Kadazandusun and Murut, that its roots and fruits cure diabetes mellitus.

FIGURE 1. Plant of *Bridelia stipularis*

Polyphenols existing in medicinal plants have proven to have oxidizing effects which in turn affects a wide range of biological activities (Xu et al. 2010), and have shown potential antioxidant properties (Ho 1992), which showed that it can act as an effective antioxidant (Kahkonen et al. 1999). Therefore, it can fight against diseases caused by oxidation of low density lipoproteins such as atherosclerosis and coronary heart disease in addition to being able to prevent cancerous cell formation (Peterson et al. 2001). Flavonoid on the other hand, is an important part of polyphenol, which possess tannin and lignin precursors, which are proven to be antioxidants, and which acts as reactive oxygen species scavenging components within human body (Malencic et al. 2007). For better future, flavonoids are being researched more thoroughly, because of their immense bioactivities which includes anti-inflammatory, anti-genotoxic, anti-cancer, neuro-protective, anti-Alzheimer and antiglycative properties (Murthy et al. 2012).

Gas chromatography-mass spectrometry (GC-MS) is used as a technique that serves a broad range of applications aimed at sample identification, quantitative determination or both. The sample identification (qualitative analysis) needs a high degree of selectivity whereas quantitative analysis requires high accuracy (the precision and trueness) (Fialkov et al. 2006). GC-MS is one of the valuable tool for the identification of phytochemical compounds. The first step in investigating the presence of metabolites in any medicinal plant is by phytochemical screening that gives a broad idea on the nature of chemical constituent (Konig et al. 2004). To identify the compound, processing data from GC-MS must fulfil two criteria which includes: correct determination of mass spectrum of individual compounds; and accurate calculation of the abundance of chromatographic peaks corresponding to those compounds in each sample (Johnsen et al. 2001). Moreover, for sample introduction into GC-MS, there are three considerations. Firstly, the constituent of the sample must be volatile and secondly the analytes must be present at concentration which is appropriate to it. Thirdly, while injecting the sample, the sample must not degrade the separation (Fialkov et al. 2006).

Bridelia stipularis is a plant with strong medicinal properties but so far there is no research performed on determination of the chemical composition, TPC, TFC and antioxidant activity of its stem. Hence, in continuation to our ongoing project on the survey of medicinal plants, we herein report, the GC-MS based chemical profiling, TPC, TFC and antioxidant activity of the crude extracts from the stem of *Bridelia stipularis*.

MATERIALS AND METHODS

CHEMICALS, REAGENTS AND INSTRUMENTS

Folin-Ciocalteau reagent, Aluminium chloride, sodium carbonate, potassium acetate, BHT, gallic acid, quercetin and DPPH were obtained from Sigma-Aldrich. Hexane, chloroform, ethyl acetate and methanol were purchased from Merck. All chemicals were of analytical grade and used without any further purification. The GC-MS analysis was conducted on a Perkin Elmer Clarus 500, GC-MS spectrometer equipped with VF-5 MS fused silica capillary

column of 30 m × 0.25 i.d. and 0.25 μm film thickness. Perkin Elmer Lamda 20 UV-Vis spectrophotometer was used to measure the absorbance for the determination of AA, TPC and TFC of all the crude extracts.

PLANT MATERIAL AND SAMPLE COLLECTION

The fresh stems of *Bridelia stipularis* were collected near Keningau (specimen number: BOHR 4439) and stored in polyethylene bags at 4°C until required. The leaves were later separated and the stems were cut and ground to form powder. This contributes to a better extraction because of small surface area (Azmir et al. 2013). The samples were stored at 4°C covered with aluminum foil to avoid exposure to light, heat and moisture until further use.

PREPARATION OF CRUDE EXTRACTS FOR CHEMICAL PROFILING, TPC, TFC AND *in vitro* ANTIOXIDANT ACTIVITY

The dried stem powder was divided in three portions of 100 g and extracted with 400 mL of methanol using Soxhlet extraction method at room temperature (Figure 2). Prior to extraction the samples were soaked in methanol and left for 48 h. The methanol from the extract was recovered under reduced pressure. The obtained crudes were further extracted with 1:1 v/v ratio of water:hexane, water:chloroform and water:ethyl acetate within a separating funnel in order to receive their respective residual fractions. The water layer was discarded and the organic fractions were filtered using Whatman's filter paper. The extraction was repeated in triplicate and the final volume of all the three extracts, were then evaporated under reduced pressure via rotary evaporator in order to obtain the crude residues. All the crude residues were weighed and stored at 4°C until further use (Khan et al. 2018).

FIGURE 2. Sample soaked in methanol

GC-MS ANALYSIS OF CRUDE EXTRACTS

The samples of *Bridelia stipularis* stems were prepared in hexane, chloroform and ethyl acetate by diluting them using methanol as diluting solvent (1:100, v/v). 2 mL each of crude samples were suctioned using syringe and filtered by using Whatman's syringe filter (0.2 μm TF) and transferred into glass vials. Then, 1 μL of diluted sample was analyzed by injecting into GC MS with a split injector at 300°C. The VF-5 MS fused silica capillary column (30 m × 0.25 mm × 0.25 μm) was employed. The temperature programme was 50°C, held for 10 min, increased at 3°C/min to 250°C and finally hold for 10 min. Inert helium gas was employed as a carrier gas at a constant flow rate of 1.0 mL/min. The compounds were identified by comparison of their retention indices (RI) with those provided in National Institute of Standards and Technology (NIST) library. Identification was assumed when a good match of RI was achieved (Khan et al. 2016).

DETERMINATION OF TPC OF CRUDE EXTRACTS

The TPC was determined using spectroscopic method by Ainsworth et al. (2007). The reaction mixture was prepared by mixing 1 mL plant extracts (1 mg/mL), 1 mL of 10% Folin-Ciocalteu's reagent dissolved in 13 mL of deionized water followed by the addition of 5 mL of 7% Na_2CO_3 solution. The mixture was mixed thoroughly and kept in the dark at room temperature for 2 h. The blank solution was also prepared. The absorbance was recorded using UV-Vis spectrometer at 760 nm. All the analysis was repeated three times and the mean value of absorbance was obtained. TPC was determined by extrapolating calibration line which was construed by gallic acid solution. The TPC was expressed as gallic acid equivalent (mg GAE) per gram of the dried sample.

DETERMINATION OF TFC OF CRUDE EXTRACTS

The TFC was determined by using aluminium chloride calorimetric method based on the methods reported by Afify et al. (2012), in which 0.5 mL of sample (1 mg/mL) was mixed with 1 mL of 10% aluminium chloride, 1 mL of potassium acetate (1 M) and 2.5 mL of distilled water. Quercetin was used as the internal standard. The absorbance of the mixtures was measured at 415 nm by using UV-spectrophotometer. The TFC was expressed in terms of quercetin equivalent (mg QE/g of sample). All the analyses were repeated three times and the mean value of absorbance was obtained.

In vitro ANTIOXIDANT ACTIVITY OF CRUDE EXTRACTS

The AA of all the three extracts were quantitatively assessed on the basis of free radical scavenging activity of stable DPPH radical according to the reported method by Brand-Williams et al. (1995), in which 0.1 mM stock solution of DPPH was prepared by dissolving 2.0 mg of DPPH in 50 mL of methanol and kept in dark covered with aluminum foil to prevent light degradation. BHT used as the positive control was prepared by diluting 10 mg standard BHT with 10 mL methanol (ratio 1:1, v/v) to obtain the concentration of 1 mg/mL. Control or blank was prepared by mixing methanol to DPPH-methanolic solution. Furthermore, standard solution of BHT was diluted by methanol to get a series of concentrations viz. 0.01, 0.05, 0.1, 0.25 and 0.50 mg/mL followed by addition of 3 mL of 0.01 mM

DPPH solution to each of the five concentrations. Exactly similarly, the concentrations of crude extracts hexane, chloroform and ethyl acetate ranging from 4, 2, 1, 0.5 and 0.25 mg/mL were prepared by adding 3 mL of 0.01 mM DPPH. Incubation of the resulting solutions were carried out for 60 min in the dark at 37°C. The absorbance was measured calorimetrically at 517 nm. The experiments were carried out in triplicate. The percentage inhibition (I%) was calculated using formula 1, where Ao is the absorbance of the control (no sample, DPPH solution only); and As is the absorbance in the presence of the sample. IC_{50} of the samples was calculated from the plot of inhibition percentage (I%) against extract concentration by using formula 2. All the data were analyzed through linear regression using Microsoft Office Excel.

$$\text{\% of radical sca enging activity} = 100 \times \frac{A_{control} - A_{sample}}{A_{control}} \quad \textbf{-1}$$

$$\mathbf{IC_{50}}\text{: } Y = mx + C \quad \textbf{-2}$$

STATISTICAL ANALYSIS

To properly determine the relationship between the total phenolic content, total flavonoid content and the antioxidant activity, Spearman's correlation test was done. Data were analyzed in three replicates for each treatment. With the help of Microsoft Excel, average values, means of variables and the standard deviations (SD) were calculated to authenticate the significant differences. All statistical testing was done by using IBM SPSS 23.0, a standard statistical software program for windows.

RESULTS AND DISCUSSION

DISCUSSION ON GC-MS, COMPOUND ISOLATION AND IDENTIFICATION

In the present study, liquid-liquid extraction technique was used to separate (partition) the components of a mixture into two immiscible solvents of different densities, which depends on parameters such as solvent types, solvent strength, extraction time, agitation speed, sample-solvent ratio as well as temperature (Altemimi et al. 2017). To mention, solubility of natural products and the choice of solvent could also determine the yield of extracts. For instance, lipophilic compounds, such as terpenoids and alkaloids should be extracted via non polar solvent for instance n-hexane. Whereas alkaloids, flavonoids and terpenoids should be extracted preferably via ethyl acetate. Moreover, the extraction of flavones, alkaloids, polyphenols and saponins should be done by polar solvents such as methanol, ethanol and acetone as these are relatively polar solvents (Monte et al. 2014).

EXTRACTION OF *Bridelia stipularis*

Stems of *B. stipularis* were grounded to powder to maximize contact with the extraction solvent. The solvent used varied in polarity. According to Zuo et al. (2002), polar substances tend to dissolve in polar solvents whereas non-polar substances tend to dissolve in non-polar solvents. In this study, the solvents used were hexane, chloroform and ethyl acetate and they were subjected for extraction in order of their increasing polarity to help achieve better extraction and enhance separation of a wide range of components present in the samples. The percentage yields of all the extracts are displayed in Table 1, which was calculated by formula 3 and by putting the initial weight of the powdered sample as 300 g.

$$\textit{Percentage yield (\%)} = \frac{\textit{Weight of crude extract (g)}}{\textit{Initial weight of sample (g)}} \times 100\% \quad \textbf{-3}$$

TABLE 1. Percentage yield of *Bridelia stipularis* stem extracts

Extracts	Weight of crude extract (g)	Percentage yield (%)
Hexane	5.14	1.71
Chloroform	1.88	0.63
Ethyl acetate	1.03	0.34

Extraction using hexane displayed highest percentage yield. The extraction yield is strongly dependent on the solvent. Thus, the selection of extraction solvents is critical for complex plant samples (Tan et al. 2013). According to Rafat et al. (2010) the extraction solvent is generally selected based on the purpose of, polarity of the interested compounds, polarity of undesirable components, overall cost, safety and environmental concern.

CHEMICAL PROFILING BY GAS CHROMATOGRAPHY-MASS SPECTROMETRY ANALYSIS

Qualitative determination of chemical profile and the presence of different types of low and high molecular weight polar and non-polar chemical entities with varying quantities in each of the stem extract viz. hexane, chloroform and ethyl acetate of *Bridelia stipularis* were identified by implementing them to GC-MS analysis. The concentration of each compound was given as percentage of compound abundance over total abundance which was relative chromatogram area (%). Compound with the highest peak area were chosen. The identification of components was done by interpretation of mass spectra of GC-MS and comparing them with the database of NIST library (Ravisankar 2014). The data obtained included retention time, molecular formula, molecular weight and peak area percentage. The mass spectrums of chosen compounds are shown in supplementary material.

CHEMICAL COMPOSITION OF HEXANE STEM EXTRACT

The chromatogram of hexane stem extract (Figure 3) showed the presence of total 13 compounds of different classes such as alcohol, fatty acids, ester, straight chain alkanes and others, which are listed in Table 2. The major component with the highest percentage peak area was oxalic acid, cyclobutyl octadecyl ester (24.985%)

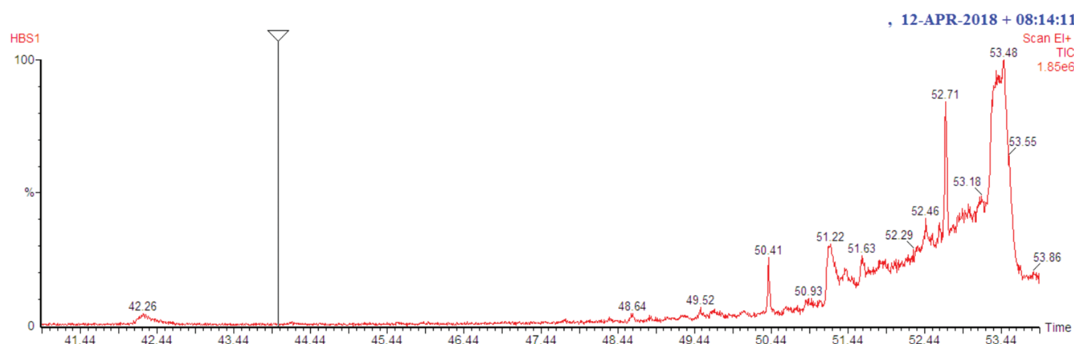


FIGURE 3. Chromatogram of hexane stem extract

TABLE 2. Chemical profile of hexane extract

No.	RT (min)	Name of compound	MF	Mw (g/mol)	Peak Area (%)	PM (%)
1	2.114	Methane-d, trichloro-	$CC_{13}D$	119	5.267	98.5
2	42.260	2,2' – Bioxirane	$C_4H_6O_2$	86	2.135	50.6
3	49.971	2-Propenoic acid,2-propenyl	$C_7H_{10}O_2$	122	2.528	41.6
4	50.407	Decanoic acid, 2-methyl	$C_{11}H_{22}O_2$	186	2.272	16.4
5	51.219	(2S,3S)-(-)-3-Propyloxirane methanol	$C_6H_{12}O_2$	116	3.321	29.7
6	51.929	Methyl 2-hydroxydodecanoate	$C_{13}H_{26}O_3$	230	4.718	12.6
7	52.231	1-Octanol, 2-nitro	$C_8H_{17}NO_3$	175	2.746	14.9
8	52.459	4-Tridecanol	$C_{13}H_{28}O$	200	6.359	21.0
9	52.714	Oxirane, [(tetradecyloxy)methyl]-	$C_{17}H_{34}O_2$	270	4.178	4.20
10	52.935	Nitric acid, nonyl ester	$C_9H_{19}NO_3$	189	5.616	7.87
11	53.016	1-Octanol, 2-nitro	$C_8H_{17}NO_3$	175	4.884	7.87
12	53.183	1-Octanol, 2-nitro	$C_8H_{17}NO_3$	175	3.979	7.13
13	53.478	Oxalic acid, cyclobutyl octadecyl ester	$C_{24}H_{44}O_4$	396	24.985	8.37

RT= Retention time, MF= Molecular formula, Mw= Molecular weight, PM= Probability Match

at retention time 53.478 min, followed by 4-Tridecanol and nitric acid, nonyl ester with percentage peak area of 6.359% and 5.616%, respectively. Biological activity has been performed on the compound oxalic acid, cyclobutyl octadecyl ester (Chowdury et al. 2017), but in the report published by Chandrakasan and Neelamegam (2011), there is no activity performed on the hydroxyl compound i.e. methyl 2-hydroxydodecanoate and the rest of the compounds.

CHEMICAL COMPOSITION OF CHLOROFORM STEM EXTRACT

A total of 8 compounds were identified from the chloroform extract of the stems *B. stipularis* (Figure 4), which are displayed in Table 3. The major components were 1-hexene, 3,5-dimethyl- at retention time 32.316 followed by 1-dodecyn-4-ol at retention time 50.850, nitric acid, nonyl ester at retention time 52.949, methyl-2-hydroxydodecanoate at retention time 53.043 and

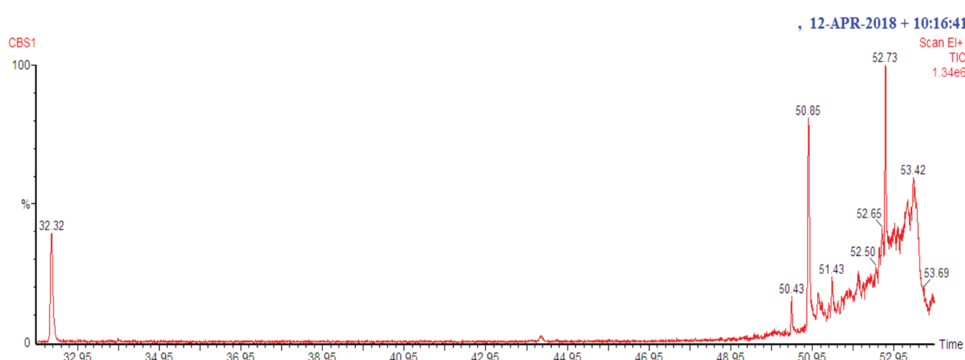


FIGURE 4. Chromatogram of chloroform extract

TABLE 3. Chemical profile of chloroform extract

No.	RT (min)	Name of compound	MF	Mw (g/mol)	Peak Area (%)	PM (%)
1	2.154	Methylene chloride	CH_2Cl_2	84	12.980	65.6
2	32.316	1-Hexene, 3,5-dimethyl-	C_8H_{16}	112	3.772	7.06
3	33.956	Pyrrolidine,1-methyl-3,2'-spiro-benzo-1,3-dioxolane	$C_{11}H_{13}NO_2$	191	2.315	95.2
4	50.850	1-Dodecyn-4-ol	$C_{12}H_{22}O$	182	4.627	8.27
5	51.427	4-nonene, 5- nitro	$C_9H_{17}NO_2$	171	2.298	26.8
6	52.372	1-Octanol, 2-nitro-	$C_8H_{17}NO_3$	175	2.692	15.8
7	52.654	1-Octanol, 2-nitro-	$C_8H_{17}NO_3$	175	2.585	19.8
8	52.734	1-Octanol, 2-nitro-	$C_8H_{17}NO_3$	175	6.868	15.6
9	52.949	Nitric acid, nonyl ester	$C_9H_{19}NO_3$	189	5.637	35.6
10	53.043	Methyl-2-hydroxydodecanoate	$C_{13}H_{26}O_3$	230	2.985	21.0
11	53.277	1-Octanol, 2-nitro-	$C_8H_{17}NO_3$	175	10.593	25.2
12	53.418	1-Octanol, 2-nitro-	$C_8H_{17}NO_3$	175	12.424	14.5

RT= Retention time, MF= Molecular formula, Mw= Molecular weight, PM= Probability Match

1-octanol, 2-nitro- at retention time 53.418. Based on the result, compound 1-octanol, 2-nitro is the most abundant component of chloroform extract and it also possesses antimicrobial properties as reported by Priya et al. (2011). However, there is no reported data or record, giving details about the bioactivity of the rest of the compounds.

CHEMICAL COMPOSITION OF ETHYL ACETATE STEM EXTRACT

The chromatogram of ethyl acetate stem extract (Figure 5) showed the presence of total 14 compounds of different classes which are listed in Table 4. The major component with the highest percentage peak area was 1-dodecanol (40.917%) at the retention time 32.289 followed by benzaldehyde, 2,4-dimethyl- (9.583%) at the retention time 21.097. Based on the study by Togashi et al. (2007), it was reported that the long chain fatty acids such as 1-dodecanol possess potential antibacterial activity against *Staphylococcus aureus*. Most of the fatty acids are known to display good antibacterial, antifungal and anti-inflammatory properties (Aparna et al. 2012), whereas no pharmacological or biological activity has been reported for the rest of compounds present in ethyl acetate extract. It is worth mentioning, that there are no records based on the biological activity for some of the major components found in the GC-MS analysis of the stem of *B. stipularis* such as (2S,3S)-(-)-3 propyloxiranemethanol, methyl 2-hydroxydodecanoate, 4-tridecanol, oxirane, [(tetradecyloxy)methyl]-, pentanoic acid, 5-hydroxy-, 2,4-di-t-butylphenyl esters, 1-tridecyn-4-ol, pyrrolidine, 1-methyl-3,3'-spiro-benzo-1,3-dioxolane and 1-dodecyn-4-ol. Hence, we subjected all the three extracts to *in-vitro* antioxidant assay in order to check their potential as free radical scavengers.

DETERMINATION OF TPC AND TFC

The result of TPC was estimated by using Folin-Ciocalteu reagent. The phenolic content in plant extracts is expressed as milligram of Gallic acid equivalent (GAE) per gram of dry weight of extract, which is solvent dependent. The higher the polarity of solvent the more will be the TPC and TFC in the extract and as the TFC is component of TPC, its quantity is usually less when compared to TPC. But as both possess unimpeachable biological profile, especially as antioxidants, they are both indispensable. Therefore, three solvents were chosen in order to obtain the TPC and TFC i.e. hexane, chloroform and ethyl acetate. The flavonoid concentration expressed as quercetin equivalent

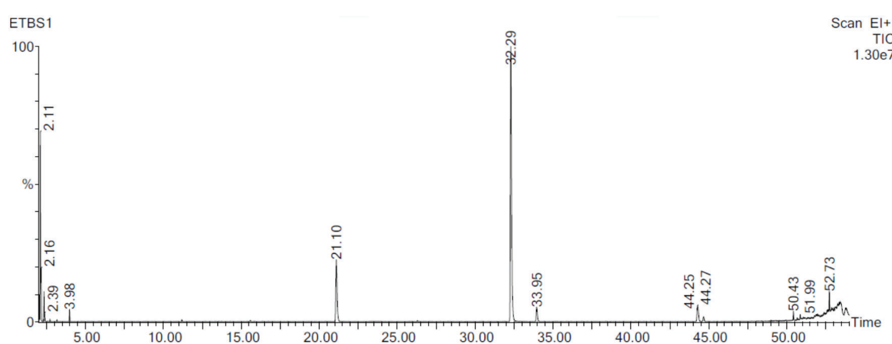


FIGURE 5. Chromatogram of ethyl acetate extract

TABLE 4. Chemical profile of ethyl acetate extract

No	RT (min)	Name of compound	MF	Mw (g/mol)	Peak Area (%)	PM (%)
1	2.114	Methylene Chloride	CH_2C_{12}	84	8.103	91.8
2	2.161	Methane-d, trichloro	$CC_{13}D$	119	2.398	55.6
3	21.097	Benzaldehyde, 2,4-dimethyl-	$C_9H_{10}O$	134	9.583	29.7
4	32.289	1-Dodecanol	$C_{12}H_{26}O$	186	40.917	14.2
5	33.952	Pentanoic acid, 5-hydroxy-, 2,4-di-t-butylphenyl esters	$C_{19}H_{30}O_3$	306	2.269	37.6
6	44.292	1-hexanol, 3-methyl-	$C_7H_{16}O$	116	2.683	37.3
7	44.667	Oxalic acid, allyl heptylester	$C_{12}H_{20}O_4$	228	1.653	24.9
8	50.430	Octanoic acid, methyl ester	$C_9H_{18}O_2$	158	1.714	30.8
9	50.676	Oxalic acid, cyclobutyl heptylester	$C_{13}H_{22}O_4$	242	1.683	70.0
10	51.876	Nitric acid, heptyl ester	$C_7H_{15}NO_3$	161	1.947	23.3
11	52.875	Nitric acid, nonyl ester	$C_9H_{19}NO_3$	189	2.393	30.4
12	53.271	1-Tridecyn-4-ol	$C_{13}H_{24}O$	196	2.079	13.2
13	53.425	1-Octanol, 2-nitro-	$C_8H_{17}NO_3$	175	3.293	9.75
14	53.780	Nitric acid, nonyl ester	$C_9H_{19}NO_3$	189	1.872	16.9

RT= Retention time, MF= Molecular formula, Mw= Molecular weight, PM= Probability Match

was determined by using aluminum chloride method. The results obtained are displayed in Table 5, giving comprehensible and relative values of TPC and TFC.

ANTIOXIDANT ACTIVITY

DPPH free radical scavenging assay was used to identify the *in-vitro* antioxidant potential of the organic extracts bearing different bioactive components. Tert-butyl-1-hydroxytoluene (BHT) was used as an internal standard for comparing the results.

CHEMISTRY OF DPPH ASSAY

The basic principle of DPPH method is based on the reduction of DPPH and a visible color change from purple to yellow in the presence of a hydrogen donating antioxidant. The degree of discoloration indicates the scavenging potential of antioxidant compounds of the extracts. The absorption strength decreases in the presence of a free radical scavenging antioxidant at 517 nm (Huang et al. 2005). This demonstrates that the antioxidants present in the sample mixtures have interacted with the free radicals (Figure 6) (Kedare et al. 2011; Rhashid et al. 2000). BHT appears to be the best standard antioxidant because 1 mole of BHT reduces about 3 mole of DPPH•, even though it reacts very slowly (Bondet et al. 1997). Brand-Williams et al. (1995) and Cuvelier et al. (1992) suggested three possible pathways for BHT/DPPH• reaction as shown in Figure 7. DPPH is the easiest *in-vitro* assay as it requires simple procedure and low cost and hence it has been used mostly for the antioxidant activity evaluation (Ahmad et al. 2010).

EVALUATION OF ANTIOXIDANT ACTIVITY OF VARIOUS ORGANIC EXTRACTS OF *Bridelia stipularis*

In the present study, the AA results obtained showed an increase in the percentage inhibition as the concentration increases. The highest percentage inhibition exhibited by

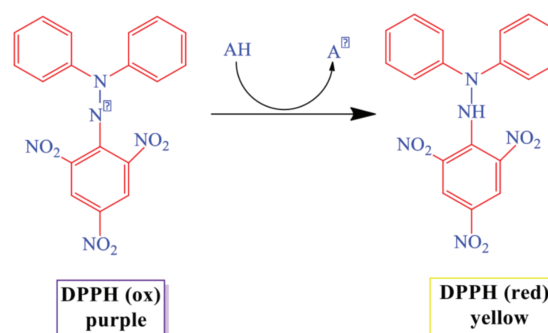


FIGURE 6. Reduction of DPPH free radical (purple to yellow) (Rashid et al. 2000)

TABLE 5. TPC and TFC in ethyl acetate, chloroform and hexane extracts

Plant extracts	Total phenolic contents (mg GAE/g)	Total flavonoid contents (mg QE/g)
Ethyl acetate	224.62 ± 0.08	160.48 ± 0.08
Chloroform	170.40 ± 0.18	56.84 ± 0.06
Hexane	111.82 ± 0.96	32.06 ± 0.08

[a] Data are expressed as mean ± S.D [b] Results are the mean of triplicate values (*n*=3)

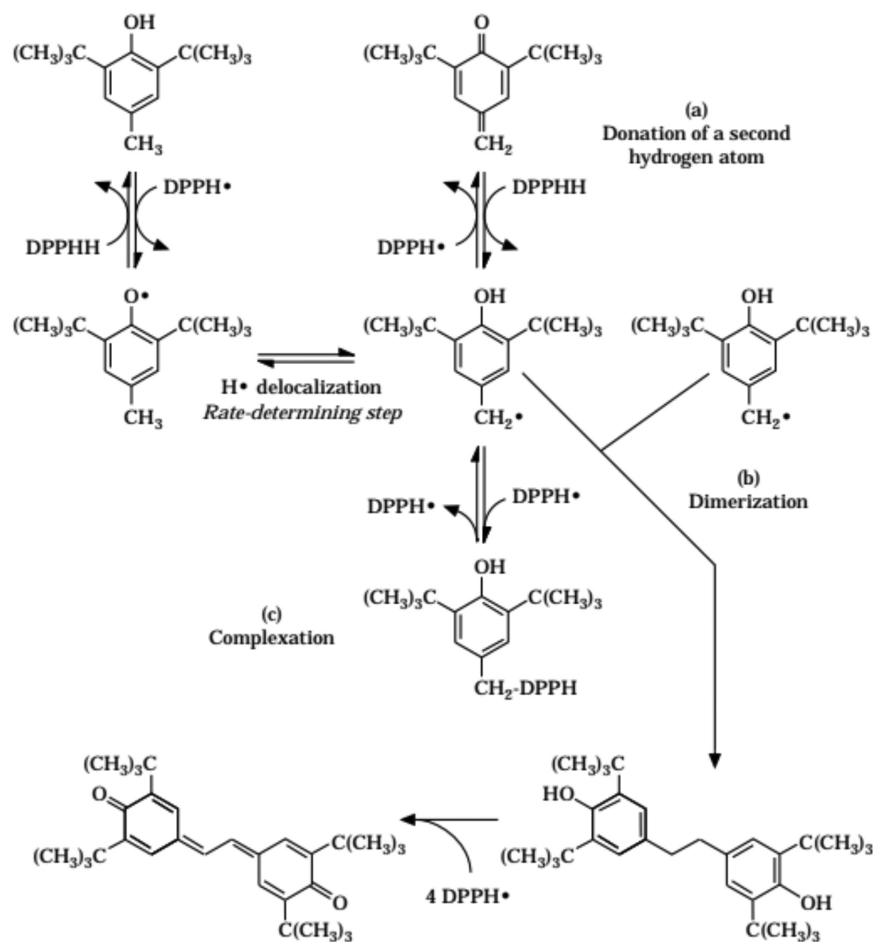
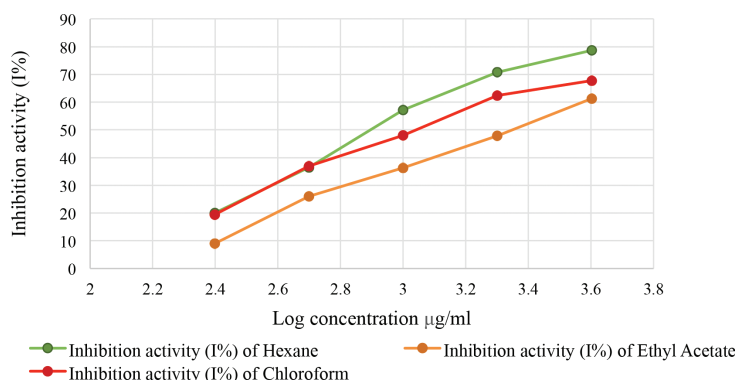


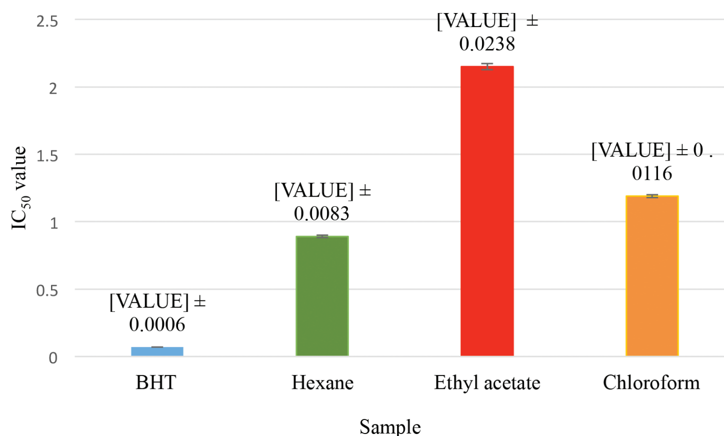
FIGURE 7. Proposed mechanism for BHT/DPPH• reaction (Brand-Williams et al. 1995; Cuvelier et al. 1992)

BHT was 83.99%. Meanwhile, for hexane, chloroform and ethyl acetate the highest percentage inhibition were 78.74%, 67.80% and 61.27%, respectively (Figure 8). The higher is the percentage inhibition, the higher is the AA, which indicates an increased ability to donate hydrogen ions in a lighter solution which is proportional to the number of electrons gained (Villano et al. 2007). Further, the IC_{50} value was calculated. IC_{50} value is the concentration of sample at which the inhibition percentage reaches 50%. In the case of hexane extract, the IC_{50} was reported to be 0.8873 ± 0.0083 mg/mL, 1.1924 ± 0.0116 mg/mL for chloroform extract and 2.1451 ± 0.0238 mg/mL for ethyl acetate extract. BHT was used as standard had IC_{50} value of 0.0678 ± 0.0006 mg/mL (Figure 9). The difference in their IC_{50} value is due to the presence of different phytochemicals in different crude extracts. The lower the IC_{50} value, the higher the free radical scavenging activity (Li et al. 2009). Therefore, ethyl acetate extract



[a]Results are the mean of triplicate values (*n*=3)

FIGURE 8. Inhibition activity (I%) of various organic extracts against log concentration (μg/mL)



[a]Experiments were run in triplicate (*n*=3)
[b]Results are mean of ± SD value

FIGURE 9. The IC_{50} value of various organic extracts and BHT

displayed the strongest free radical scavenging properties over the rest three when compared to BHT. All the data related to GC-MS and statistical calculations are provided in supplementary information section.

It is important to mention the use of different solvents for better extraction. Hexane is usually chosen to extract terpenoid-derived molecules (Harman et al. 2016). However, ethyl acetate for extracting phenolic and nitrogenous compounds (Zlotek et al. 2016), which are known to scavenge free radicals and reactive oxygen species (ROS) and most importantly superoxide anion, hydroxyl radicals and singlet oxygen (Shanta et al. 2013). Chloroform results in the least extraction of phytochemicals, which it attributed to poor solubility of phytochemicals in chloroform, therefore it should not be the solvent of choice for the extraction of phytochemicals (Dhawan et al. 2017).

Conclusion

The outcome of the study, thus, provides strong evidence in establishing the radical scavenging properties of *Bridelia stipularis* stem. The bioactivity of antioxidant of the plant can be attributed to the presence of phytochemicals. In the present work, the stems of *Bridelia stipularis* were extracted with various organic solvents such as hexane, chloroform and ethyl acetate. The chemical profiling via GC-MS showed 1-dodecanol (40.917%), oxalic acid, cyclobutyl octadecyl ester (24.985%), 1-octanol,2-nitro (12.424%), benzaldehyde, 2,4-dimethyl- (9.583%), 4-tridecanol (6.359%) and nitric acid, nonyl ester (5.616%) as major components. The antioxidant activity was carried out via DPPH radical scavenging assay using BHT as internal standard. The IC_{50} value of BHT was reported to be 0.07 mg/mL with maximum percentage inhibition of 83.99%. The IC_{50} value of hexane crude extract was found to be lowest among the three (0.89 mg/mL) with percentage inhibition of 78.74%. Chloroform ext6ract displayed IC_{50} value of 1.19 mg/mL with highest percentage inhibition of 67.80%. The lowest percentage inhibition was reported for ethyl acetate (61.27%) with highest IC_{50} value (2.15 mg/mL). Hence, it is reasonable to postulate on the basis of presented data that the stem of *Bridelia stipularis* has potential antioxidant properties.

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