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GAS CHROMATOGRAPHY-MASS SPECTROMETRY ANALYSIS OF AQUEOUS FRACTION AND HEAVY METAL CONTENT IN STEM-BARK EXTRACT OF *BLIGHIA SAPIDA*

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Abstract:

The study determined the bioaccumulation of heavy metals (Co, Cr, Cd, Pb, Ni, and Mn) in the ethanol extract of *B. sapida* and identified the bioactive compounds present in the aqueous fraction of the plant using the gas chromatography-mass spectrometry (GC-MS) technique. The fresh samples were dried under a shed, pulverized, and subjected to 70 % ethanol extract followed by fractionation. The plant extract was digested with a dilute acid solution (mixture of nitric and perchloric acid in a ratio of 2:1, at 90 °C), filtered, and analyzed for metal contents using atomic absorption spectrophotometry. The metal contents are as follows; Co, Cr, Cd, Pb, Ni, and Mn are 0.041±0.006, 0.617±0.058, 0.021±0.006, 0.810±0.058, 0.933±0.058, and 0.080±0.006 Mg/kg respectively. Nineteen prominent compounds were identified in the aqueous fraction of the extract by GC-MS. The results of the study revealed the presence of the metals investigated in the extract of *B. sapida;* however, these metal levels were lower than the stated values by (WHO/FDA). The aqueous fraction of *B. sapida* possessed bioactive compounds with biological activities, such as antifungal, antibacterial, anti-inflammatory, pharmaceutical, antioxidant, herbicidal, and anticancer properties with various applications in the places of synthetic chemicals.

Keywords: Bioaccumulation, bioactive compounds, *Blighia sapida*, heavy metal, plant extract.

Introduction

Interest in herbal treatments may have resulted from wrong intuition regarding the low documented occurrence of harmful impacts or the absence of accurate information on the complications of medicinal plants amid other factors. But the safety of herbal medicines depends on the natural toxic metabolites in the medicinal plants and the general property of the plants (Hu *et al*., 2013; Vaikosen and Alade, 2017). Consumption of natural products contaminated with heavy metals has elicited adverse effects on living organisms (Sethy and Ghosh, 2013). *Blighia sapida* K.D. Koenig is a medicinal plant with several uses; it belongs to the family Sapindaceae which naturally grows in most West African countries; its fruits and oil are essential as food supplements for humans and animals (Aloko *et al*., 2017). Usually, plants are drastically responsive to environmental conditions and can accumulate heavy metals in their roots, stem bark, leaves, and fruits (Tangahu *et al*., 2011). As long as metals do not undergo biodegradation by microorganisms, they persist in the soil for an extended period (Briffa *et al*., 2020). The introduction of these metals into plants can be through root uptake, foliar absorption, and deposition of specific elements in leaves. The level of their uptake can change the entire elemental formation of the metabolites in plants, which affects their growth and development as well as the efficiency of the natural products obtained from medicinal plant species. In addition to the undesirable functions of these heavy metals in plants, they may be consumed by humans and animals through the food chain. Moreover, the constant utilization of plants obtained from ranges of polluted or contaminated areas is of critical interest in herbal treatment. Studies have reported differences in metal bioaccumulation of several plant species between sampling locations and distribution in various parts of the same plant species. Investigations have revealed the degree to which metals bioaccumulate in different plant species, sampling locations, and distribution in other parts of plants (Guala *et al*., 2010; Vaikosen and Alade, 2017).

Heavy metals are of different categories in terms of their roles. Metals such as Fe, Cu, Zn, Mn, and Ni play essential functions in physiological and biochemical processes even in their trace amounts. However, at levels above recommendation, they could be deleterious to the system, while metals such as Pb, Cd, As, and Cr have no specific role in humans (Fasani *et al*., 2018; Yan *et al*., 2020). Heavy metals such as Pb and Cd, which gain access to the human body through different means, exhibit a deleterious effect on human health (Baghaie and Fereydoni, 2019). Because of the envisaged harmful impact on end-users, there is a need for the quantitative determination of these heavy metals in plants before use.

Gas chromatography-coupled mass spectrometry (GC-MS) has become an established technique for identifying bioactive compounds existing in both plant and non-plant species (Jayapriya and Shoba, 2015). Thus, because of the roles of plants in food and medicinal treatment, and due to the rate at which soil is being polluted by heavy metals from both natural and anthropogenic sources, there is a need for continuous evaluation of metal levels in medicinal plants before usage. *B. sapida* stem-bark extract has been reported for therapeutic values, but up to date, based on findings, has no single investigation on metal content. The present study determined the level of selected heavy metals (Co, Cr, Cd, Pb, Ni, and Mn) in the extract, using Atomic Absorption Spectrophotometer (AAS), and the bioactive compounds in the aqueous fraction of the plant were also identified using gas chromatography-mass spectrometry (GC-MS) technique. It is expected that this study will provide information on the need to evaluate metal levels in medicinal plants and compare the result with recommended values.

Materials and methods

Plant Collection and Authentication

Fresh *B. sapida* stem bark was collected from Sekona-Ede Road, Osun State, Nigeria. The plant material was identified and authenticated with specimen voucher

number 17623 at IFE Herbarium, Department of Botany, Obafemi Awolowo University, Ile-Ife, Nigeria.

Plant sample preparation

The powdered material (1 kg) of the prepared stem bark of *B. sapida* was macerated in (70 %, V/V) of ethanol/water for 72 h at room temperature, as reported by (Adekola *et al*., 2020). The resulting suspension was filtered, and the filtrate was sieved using white cotton gauze, followed by using filter paper and concentrated with a rotary evaporator at 40ºC to yield ethanol extract (EE). The extract was weighed, labeled, and stored in the desiccator until needed for further analysis.

Fractionation of ethanol extract

The EE was partitioned with solvents of varying polarities as described by (Apalowo *et al*., 2019). The extract (30 g) was suspended in distilled water (200 mL) allowed until totally dissolved, shaken thoroughly, and filtered with filter paper. The filtrate was partitioned sequentially with 400 mL of each solvent (ethyl acetate and butanol) using a separating funnel. The mixture was thoroughly shaken, allowed to separate into layers, and separated. The fractions of the different solvents were concentrated in a rotary evaporator separately, while the volume of the aqueous fraction was only reduced before lyophilization. The ethyl acetate fraction (EAF), butanol fraction (BF), and aqueous fraction (AQF) were obtained and kept in desiccators until needed.

Plant extract digestion

The plant extract (5g) was measured into a 50 mL beaker. An acid mixture of nitric and perchloric acid in a ratio of 2:1 was added, and the beaker content was placed on a hotplate under a fume cupboard and allowed to undergo heating at 90ºC for 30 min. The beaker content was removed from the hotplate, allowed to cool, and the digested sample was then filtered and made up to 25 mL. The solution was then analyzed on Atomic Absorption Spectrophotometer (AAS) model 210/211 VGP to determine Lead, Cadmium, Cobalt, Chromium, Nickel, and Manganese.

Instrumentation and measurements

Heavy metal measurements were carried out with a Varian Atomic Absorption Spectrophotometer (AAS), model Spectra AA 600 (Varian, California, USA) with a flame system inter-phased to a computer and printer. The instrument was calibrated before use.

Quantitative analysis of phytochemical compounds by using GCMS

The GC–MS analysis of bioactive compounds in the aqueous extract of the stem bark of *B. sapida* was carried out using Agilent Technologies GC systems with GC-1909IJ/MS-5975C model (Agilent Technologies, Santa Clara, CA, USA) equipped with HP-5MS column (30 m in length × 250 mm in diameter × 0.25 mm in thickness of film). Spectroscopic detection by GC–MS involved an electron ionization system that utilized high-energy electrons (70 eV). Pure helium gas (99.995%) was used as the carrier gas with a flow rate of 1.504 mL/min. The initial temperature was set at 50 –150°C with an increasing rate of 3°C/min and a holding time of about 10 min. Finally, the temperature was increased to 300°C at 10°C/min. One microliter of the prepared extract dissolved in respective solvents was injected in a splitless mode. The relative quantity of the chemical compounds present in the extract of *B. sapida* was expressed as a percentage based on the peak area produced in the chromatogram. The compounds were identified by comparing the mass spectra (peak) obtained with those of the standard mass spectra obtained from the National Institute of Standards and Technology (NIST) 11, (NIST) library or database.

Data analysis

The data obtained were analyzed using the software Graph Pad Prism (version 5). Values were represented as mean ± standard error mean.

Results

Metal concentration in the plant extract

The metal content analyzed and detected in the stem-bark extract of *Blighia sapida* is as presented in Table 1. The concentration of the metals are in the following order; Ni > Pb > Cr > Mn > Co > Cd.

Table 1. Metal levels bioaccumulation in stem-bark extract of *blighia sapida*

The study		Regulatory limits (ppm)		
Metal	Concentration (Mg/kg)	WHO/FDA	EDQM	CFDA
Co	0.041±0.006	0.14-0.48	-	-
Cr	0.617±0.058	2.00	-	-
Cd	0.021±0.006	0.30	1.00	0.30
Pb	0.810±0.058	10.00	5.00	5.00
Ni	0.933±0.058	1.63	-	-
Mn	0.080±0.006	44.6-339	-	-

The result showed that the levels of Co, Cr, Cd, Pb, Ni, and Mn in the stem bark of *B. sapida* are Co (0.041 ppm), Cr (0.617 ppm), Cd (0.021 ppm), Pb (0.81 ppm), Ni (0.933 ppm), and Mn (0.08 ppm) respectively. The result showed that nickel (0.933 ± 0.058 ppm) has the highest concentrations among the six elements analyzed, followed by lead (0.81 ± 0.058 ppm), and the lowest concentration of cadmium (0.021 ± 0.006 ppm) was detected.

Chemical composition of the fraction by GC-MS analysis

The GC-MS spectral studies revealed the presence of 19 compounds from the aqueous fraction (AQF) of *B. sapida* based on their retention time. The GC-MS chromatogram of an aqueous fraction (AQF) of *B. sapida* is as shown in Figure 1. The prominent compounds were 4-Ethylbenzoic acid (8.01), Benzeneacetaldehyde (5.40), 2-Methoxy-Phenol (11.42), Benzenemethanol (10.99), 2-Methoxyl-4-vinyl phenol (12.31), and 1, 2-Propanediol (5.42) among others, while 2-Methoxyl-4-vinyl phenol is the most prominent compound with a peak value of (12.31). The retention time (RT), peak area, name of the compound, molecular formula, molecular weight, nature of the compound, and structural formula were presented in Table 2. Table 3 showed the bioactive compounds and their reported biological activities.

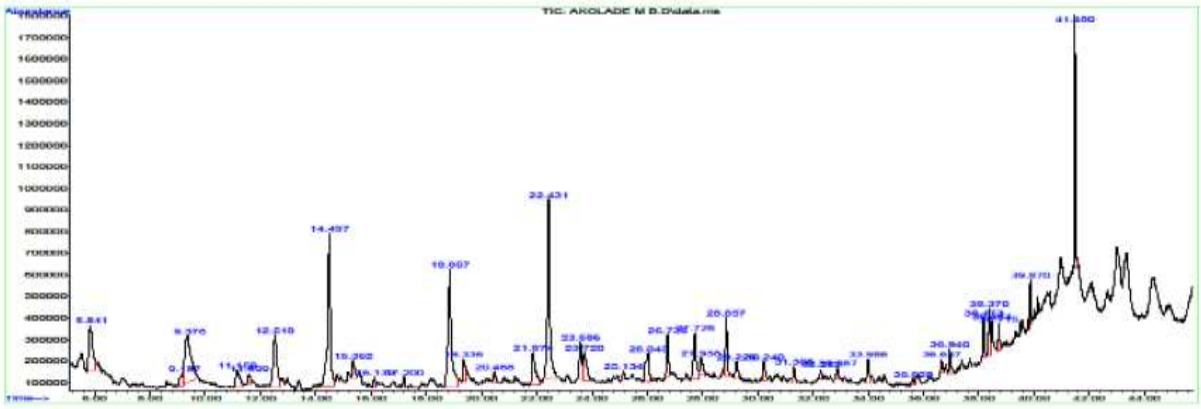
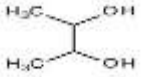
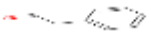
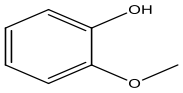
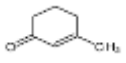
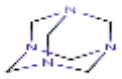
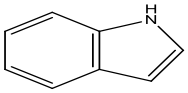
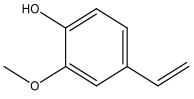


Figure 1. The GC - MS Chromatogram of aqueous fraction (AQF) from the stem-bark extract *B. sapida*

Table 2. List of bioactive compounds identified by the GC-MS analysis in aqueous fraction of *Blighia sapida*

S/NO	Retention time	Peak area (%)	Name of compound	Molecular formula	Molecular weight	Nature of compound	Structural formula
1.	5.84	4.75	2,3-Butanediol	$C_4H_{10}O_2$	90.12	Glycol, Secondary Alcohol	
2.	9.38	8.01	4-Ethylbenzoic acid	$C_9H_{10}O_2$	150.17	Organic Compound, Benzoic acid	
3.	12.52	5.40	Benzeneacetaldehyde	C_8H_8O	120.15	Organic Compound	
4.	14.50	11.42	2-Methoxy-Phenol	$C_7H_8O_2$	124.14	Phenol	
5.	15.36	1.17	Tetrasiloxane	$H_{10}O_3Si_4$	170.42	Unbranched Siloxane	
6.	18.86	10.99	Benzenemethanol	$C_7H_{10}O$	110.15	Aromatic Alcohol	
7.	19.34	1.52	Methenamine	$C_6H_{12}N_4$	140.19	Heterocyclic organic compound	
8.	21.87	3.04	Indole	C_8H_7N	117.15	Aromatic heterocyclic organic compound	
9.	22.43	12.31	2-Methoxyl-4-vinyl phenol	$C_9H_{10}O_2$	150.17	Conjugated phenol	

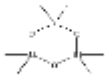
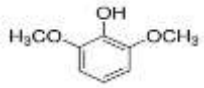
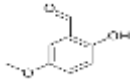
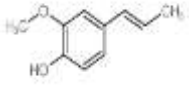
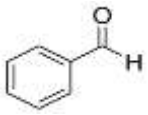
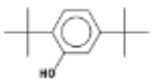
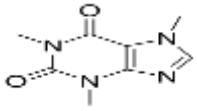
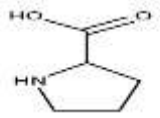
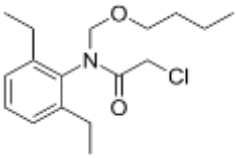
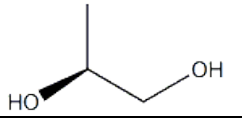
10.	23.59	2.49	Hexamethylcyclotrisiloxane	$C_6H_{18}O_3Si_3$	222.46	Highly flammable	
11.	23.72	2.20	2, 6-Dimethoxy-Phenol	$C_8H_{10}O_3$	154.16	Phenol	
12.	26.05	2.72	2-Hydroxy-5-Methoxy-Benzaldehyde	$C_8H_8O_3$	152.15	Organic compound	
13.	26.74	2.16	Trans-Isoeugenol	$C_{10}H_{12}O_2$	164.20	Polyphenolic, Allergen, and Sensitiser	
14.	27.73	3.62	Benzaldehyde	C_7H_6O	106.12	Aromatic aldehyde	
15.	28.86	2.92	2, 5-Bis (1, 1-dimethyl ethyl) Phenol	$C_{14}H_{22}O$	206.32	Phenol	
16.	33.99	1.43	Caffeine	$C_8H_{10}N_4O_2$	194.19	Stimulant, Xanthine	
17.	36.94	1.29	L-Proline	$C5H_9NO_2$	115.13	Natural Amino acid, Cryoprotectant	
18.	39.87	0.83	Butachlor	$C_{17}H_{26}ClNO_2$	311.85	Hydrophobic	
19.	41.48	5.42	1, 2-Propanediol	$C_3H_8O_2$	76.09	Glycerol	

Table 3. Reported bioactivity of phytocomponents identified in the aqueous fraction of *B. sapida* by GC-MS

S/NO	Name of compound	Reported activity
1	2,3-Butanediol	Softening agents, drugs, plasticizers, polyester, and cosmetics (Lee and Seo, 2019). Antifreeze, chemical, pharmaceutical, and cosmetic (Mozzi, 2016).
2	4-Ethylbenzoic acid	Environmental Contaminant (Wishart *et al*., 2018). Antifungal (Perez-Castillo *et al*., 2020).
3	Benzeneacetaldehyde	Antibacterial (Manyi-Loh *et al*., 2011).
4	2-Methoxy-Phenol	Antioxidant, Anti-lipid peroxidation (Fujisawa *et al*., 2007). Anticancer (Adekola *et al*., 2020).
5	Tetrasiloxane	Antifungal and Antibacterial (Moustafa *et al*., 2013).
6	Benzenemethanol	Anaesthesia (Rodan and Rothenfluh, 2010).
7	Methenamine	Antibacterial (Lo *et al*., 2014).
8	Indole	Anti-inflammatory and Antinociceptive (Guerra *et al*., 2011).
9	2-Methoxyl-4-vinyl phenol	Antioxidant, Cytotoxic, and Antibacterial (Adekola *et al*., 2020; Rubab *et al*., 2020).
10	Hexamethylcyclotrisiloxane	Antibacterial (Rahdary, 2012).
11	2, 6-Dimethoxy-Phenol	Antioxidant, Antibacterial (Yang *et al*., 2016).
12	2-Hydroxy-5-Methoxy-Benzaldehyde	Antibacterial (Yong-Ming *et al*., 2011).
13	Trans-Isoeugenol	Antioxidant (Renzo *et al*., 2010).
14	Benzaldehyde	Bactericidal (Zhao *et al*., 2007).
15	2, 5-Bis (1, 1-dimethyl ethyl) Phenol	Antioxidant, Anti-inflammatory, and Cytotoxic (Zhao *et al*., 2020).
16	Caffeine	Antimicrobial (Omkar *et al*., 2016). Memory enhancer (Morava *et al*., 2019).
17	L-Proline	Antibacterial (Lemia *et al*., 2016).
18	Butachlor	Herbicide (Tripthi *et al*., 2020).
19	1, 2-Propanediol	Antimycotic (Faergemann and Fredriksson, 2009).

Discussion

Plants can bioaccumulate heavy metals from soil (DalCorso *et al*., 2019). This property is employed in phytoremediation; however, it proves to be hazardous when the plants are considered as food or for therapeutic purposes in traditional medicine (Cao *et al*., 2014). Similarly, medicinal plants are obtained from different locations, including areas contaminated with different heavy metals. The concentrations of all the metals determined in the *B. sapida* extract were below the permissible limit. This implies that the concentration of heavy metals in plants is dependent on the location of the plants and the activities in the area. The cleaner the location in terms of any activities that could lead to the release of metals into the environment, the lower the metal content in the plant samples. The quality of a medicinal plant and its toxicity can be partly assessed by determining the heavy metal contents of the plant (Jan *et al*., 2015).

Some metals such as (Co, Cr, Cu, Fe, Mn, Mo, Se, and Zn) play a crucial role in a living organism. However, high level of metals generally can be harmful to the organism; therefore, estimation of heavy metals in medicinal plants, is necessary due to their effect on chemical processes (Paula *et al*., 2020). The influence of industrial activities on the metal contents of plant materials has been reported by Tabande and Taheri (2016). Therefore, low levels of metals (Co, Cr, Cd, Pb, Ni, and Mn) evaluated in the study plant, below the permissible limits as stated by regulatory bodies, could be related to the absence of any industrial activity in the location where the plant sample was obtained. Heavy metals and their ions have been implicated in DNA impairment, apoptosis, and carcinogenesis, which resulted from their interaction with DNA and nuclear protein, causing site-specific damage (Tchounwou *et al*., 2012). The damage caused by these metals may directly affect the structural orientation of biomolecules, or indirectly produce free radical species and activation of biochemical pathways. Free radicals generated by metal toxicity have been reported as the main reason for changes in the basic structure of DNA, alteration in metal-mediated calcium and sulphydryl homeostasis, and lipid peroxidation (Gautam *et al*., 2016; Valko *et al*., 2005). Metal-mediated formation of free radicals leads to the mutagenicity of altered DNA bases, displaying the association between the formation of cancerous cells and oxidative damage (Valko *et al*., 2005). High level of metals and their deleterious effects rely upon conditions such as exposure to metals, distribution in the tissues and organs, the amount in the system, and the rate at which the metals are eliminated. Mechanisms of metal toxicity include inhibition of enzyme action, protein synthesis, changes in nucleic acid function, and loss of cell membrane integrity. Metals that are toxic and carcinogenic interact with nuclear materials, including protein and DNA leading to the deterioration of proteins, lipids, carbohydrates, and nucleic acid by oxidation (Briffa *et al*., 2020). Heavy metals, most especially lead at minute quantity, should be controlled in consumable materials because they tend to bioaccumulate in different body tissues (Gilbert and Adedoyin, 2017). A long period of exposure to lead has been associated with a high level of blood pressure, hormonal imbalance, and neuronal systems disorganization, thus affecting the role in maintaining the heartbeat, vascular resistance, and cardiac output. The binding of lead to different proteins occurs in renal cells and some of the complexes formed have been implicated in lead toxicity. The presence of lead has been reported to disrupt the process by which blood cells are produced in the body, causing high-level porphyrins in the urine and anemia (ATSDR, 2007). Conditions such as programmed cell death, an imbalance between free radicals and antioxidants in the body, and DNA methylation and damage have been reported to be promoted by cadmium. Cadmium induces certain response genes, causing its effect on target gene expressions, such as genes for cell growth, division, proliferation, and cell differentiation. The toxic effect of cadmium is mainly through deactivation of an important DNA repair process, which includes the inactivation of the mismatch repair (MMR) system when interrupted by alteration of genetic information, a noticeable cause of the high level of genome disorganization and different forms of cancers (Valko *et al*., 2005). Heavy metals such as cobalt, chromium, nickel, and manganese exhibit different toxicological processes and effects, which are detrimental to the biological system. The complexity of toxic and carcinogenic effects of chromium is mediated by intermediates produced when chromium IV is reduced to chromium III along with oxidative reaction (Briffa *et al*., 2020). The inhibitory action of cobalt on essential enzymes has been attributed to its high affinity for the sulphydryl groups, which consequentially lead to inhibition of calcium ions in the calcium channel, and competition with the calcium ion for binding sites, among other effects (Simonsen *et al*., 2012). The nickel acts as an activator of the transcription factors such as NF- κB and ATF-1 during the inflammation process mediated by nickel (Valko *et al*., 2005). Manganese disrupts the synthesis of ATP by inhibiting the ATP synthase or NADH dehydrogenase, reducing the availability of the ATP within the cell. *In vivo* interruption of the dopaminergic system was observed to result from oxidation of dopamine-mediated by manganese (Engwa *et al*., 2019). The metal concentrations in the studied extract were compared with permissible limits of different regulatory bodies globally (WHO/FDA, EDQM, and CFDA) to assess the level of contamination in the extract. However, the heavy metals estimated in the study plant were below the regulatory limits, when compared with the values stated by different regulatory bodies.

A total of nineteen bioactive compounds belonging to hydrocarbons, glycol, alcohol, phenol, Aromatic heterocyclic, and Glycerol were identified in the aqueous fraction of *B. sapida* stem-bark extract through GC-MS analysis. Many of these identified compounds and several others have been previously isolated from different plant species, and their defense activities have been reported (Mooza *et al*., 2014).

The study by Emmanuel *et al*. (2014) identified n-hexadecanoic acid and other fatty acids in the oil obtained from the seed of *B. sapida* using GC-MS. Furthermore, (Adekola *et al*. 2020), using the same technique, also reported n-hexadecanoic acid with other bioactive compounds in ethyl acetate fraction of stem-bark extract of the same plant. Many of these compounds have displayed different activities such as anti-inflammatory, antibacterial, antihistaminic, insecticidal, and antitumor (Pramod *et al*., 2011; Hussein *et al*., 2016; Wei *et al*., 2011). The presence of 2-Methoxy-4-vinyl phenol in *Brassica oleracea* exhibited oxidative and microbiological stability in beef meat (Rubab *et al*., 2020). Isoeugenol and its antioxidant properties in radical

scavenging activity were observed in a study by Bortolomeazzi *et al*. (2010); this can be explored in the prevention of angiogenesis by increasing antioxidant enzymes and inhibiting reactive oxygen species production and oxidative stress, which plays an important role in the treatment of cancer (Casuga *et al*., 2016).
Other studies revealed pharmacological and biological activities of bioactive compounds such as bisphenol, caffeine, and proline. The investigation carried out by Zhao *et al*. (2020) reported the bioactivities of bisphenol and its analogs. Caffeine was assessed and observed for its biological activities by Lele *et al*. (2016). The azole derivatives of l-proline have antibacterial activity (Amarouche *et al*., 2016).

Conclusions
The present finding revealed that the extract of *B. sapida* has a low level of the evaluated metals. The concentrations of Co, Cr, Cd, Pb, Ni, and Mn assessed in the extract were below the permissible limits as defined by regulatory bodies such as WHO or FDA. It, therefore, means that the plant extract of *B. sapida* may not elicit any metal-based toxic effect in the cause of its usage for medicinal purposes. The GC-MS identified phytochemical compounds that established various biological activities such as drugs, plasticizer, antifungal, antioxidant, antibacterial, anesthesia, anti-inflammatory, antinociceptive, and many other activities, which justified the medicinal values of the plant.

Recommendations
1. Plants for medicinal purposes should always be obtained from residential or farm areas but not from industrial and traffic-congested sites.
2. The medicinal plant should be examined for the level of heavy metals and other contaminants before its usage for medication.

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