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Cinnamic Acid from *Barringtonia asiatica* Stem-Bark Extract. Its Cytotoxicity, Antioxidant and Bioactive Potentials on Some Selected Pathogens

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ABSTRACT

Barringtonia asiatica (L.) Kurtz is used as decoction in Malaysian and Nigerian traditional medication with applications against back-aches, stomach ache, tumours, epilepsy, constipation and yaws. The present study reported the extraction pattern of the isolated compound, as well as the free radical scavenging capacity of the isolated compound. Antimicrobial analyses of these substances against the bacteria Staphylococcus aureus, Exiguobacterium aquaticum, Escherichia coli, Acinetobacter baumanni,and pathogenic fungi Aspergillus niger, Aspergillus flavin, Candida tropicalis. And Fusarium oxysporium were also tested, as well as cytotoxicity using brine shrimp (Artemia salina). The secondary metabolites in Barringtonia asiatica stem-bark extracts is isolated by conventional phytochemical methods as cinnamic acid with a significant antioxidant activity in reactive oxygen species (ROS). The isolated compound showed significant toxicity LC_{50} of 3.48 µg/mL when compared to the control LC_{50} of 1.16 µg/mL. The IC_{50} of the cinnamic acid was 17.27 µg/mL higher than that of the control of 12.22 µg/mL. To our knowledge, this is the first comprehensive report of the chemical characterization of Barringtonia asiatica stem-bark isolate and also of its activity towards reactive oxygen species and bacterial, fungi and to understand its safety for medicinal agents.

Keywords: *Cinnamic acid, Barringtonia asiatica, Extract, Cytotoxicity, Antioxidant, Pathogens.*

INTRODUCTION

Barringtonia asiatica (L.) Kurtz belong to a Family of Lecythidaceae is a species native to mangrove habitats in the tropical region of Malaysia and some part of Asia. Easily available in Kuching Wetlands Sarawak and Bako National Park. It is also found in tropical Africa especially in Nigeria and Madagascar. In Sarawak this plant is popularly known as putat, inhabitants of several West African countries, Nigeria and the Polynesian Islands use liquid from the crushed bark of *Barringtonia asiatica* to treat chest pains and heart problems (Umaru et al., 2018a).

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The plant is generally refered as Barringtonia asiatica, it has a long history of traditional efficacy in Malaysia. Most of the plant parts are used for decoction in traditional medicine with application against stomach-aches in Papua New Guinea, Stem-bark is used to treat back-aches, stomach ache, tumours, epilepsy, constipation and yaws (Ravikumar et al., 2015; Govindam et al., 2011)

However, the chemical and biological assay related to the traditional uses and safe recommendation of this plant are meagre. Due to the importance of *Barringtonia asiatica*, it was selected for this study that aims to extract, isolate, characterize and evaluate the biological potential of the phytochemical. As well as to include the Malaysian medicinal flora in the Malaysian Herbal Pharmacopoeia. Accordingly, the purpose of this research is to investigate the major secondary metabolites contained in the stem-bark extracts of *Barringtonia asiatica*, and to quantify the compound by column chromatography, GCMS, NMR, and FTIR and characterize some efficacy parameters such as the free radical scavenging capacity, anti-bacterial and anti-fungal activity of the pure compound and also to analyze the safety of the isolate. To our knowledge, cinnamic acid is isolated in stem bark of *Barringtonia asiatica* for the first time.

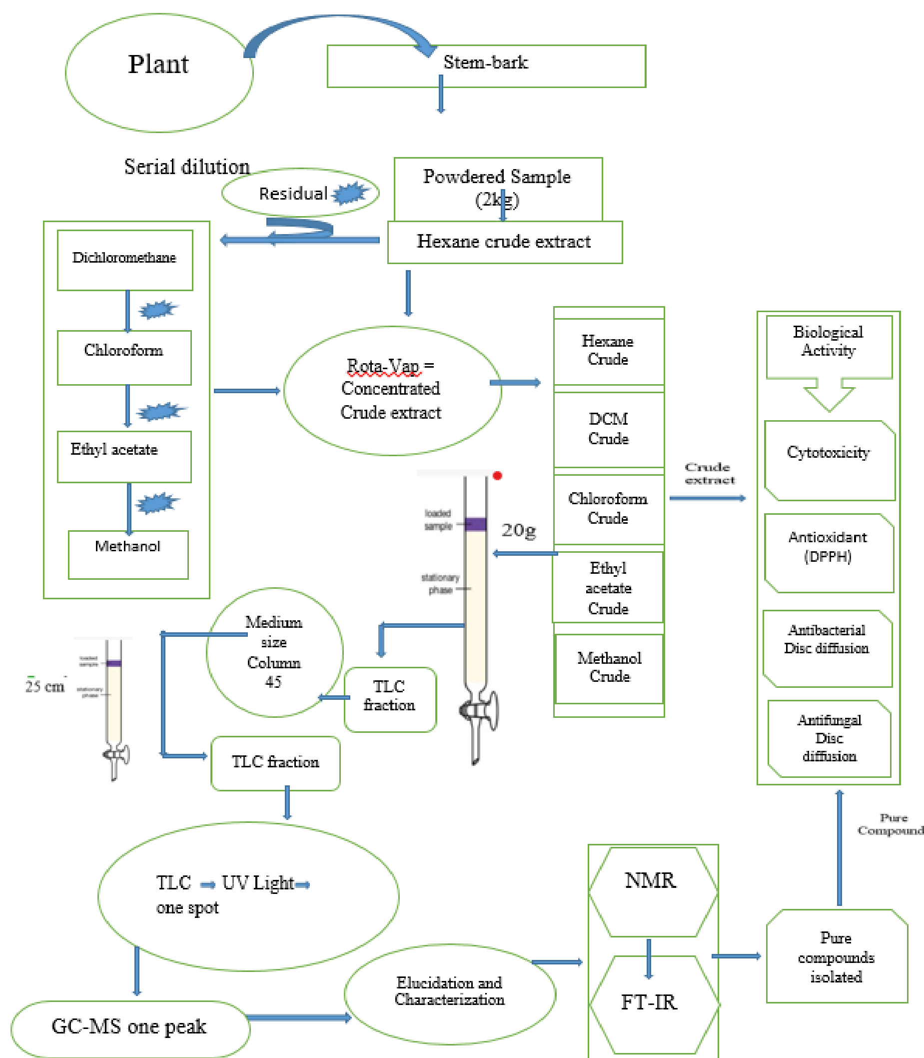

Fig. 1: schematic diagram of the study isolation and biological activity

MATERIALS AND METHODS

Sample Preparation

Dried stem-bark was ground into fine powder form using laboratory pestle and mortar and electric grinder. The finely ground powdered samples was packed into clean, dry sample containers and labelled appropriately and kept for further use. Extraction was carried out by the conventional solvent extraction method described by Umaru et al., (2019a).

Isolation, Purification and Identification of Secondary Metabolites

Column Chromatography

The basic principle of column chromatography is to separate a mixture of metabolites based on their molecular weight and polarity. A glass column of size 40/34 (large) was used for chromatography, and the sorbent used was silica gel 60 (Merck 70-230 Mesh ASTM 0.063 0.200 mm). Silica gel slurry was prepared by dissolving silica gel (150 g) with suitable solvent, usually hexane. The column was prepared by pouring a slurry mixture of silica gel and solvent, into a glass column and allow it to settle down (Fasihuddin *et al*., 2010). Fraction with single component (one spot) that appeared in TLC plate was treated as possible pure secondary metabolite. The combined fractions which contained the same single component was then allowed to air-dried or evaporated to dryness to obtain a pure secondary metabolite.

Thin Layer Chromatography (TLC)

The eluents collected from column chromatography, was subjected to thin layer chromatography (TLC) analysis. TLC was carried out using the method described by Umaru et al. (2019a).

Gas Chromatography – Mass spectrometry (GC-MS)

Gas chromatography (GC) analysis of fractions that were obtained from TLC as single spot was performed using a Shimadzu GC-Mass Spectrometry model QP2010 plus, equipped with a BPX-5 column (5% phenyl polysylphenlenesiloxane) of 30 m in length, film thickness of 0.25 μm and internal diameter of 0.25 mm. The operating method was based on the method described by Kalaiselvan et al. (2012).

Fourier Transform Infra-Red Spectrometry (FT-IR)

Fourier Transform Infra-Red (FT-IR) was performed using FTIR spectroscopy (Thermos Scientific, Nicolet iS10 SMART iTR) to detect the chemical bonds (functional groups) of the compounds. The operating system was based on the method described by Shalini and Sampathkumar (2012).

Nuclear Magnetic Resonance (NMR)

Nuclear Magnetic Resonance (NMR) spectrometry was performed using JEOL JNM-ECA 500 Spectrometer. The operating system was based on the method described by Efdi et al. (2010).

Brine shrimp (*Artemia salina*) Lethality Test

The LC_{50} of the plant extracts was determined using brine shrimp lethality test. The test was conducted using larvae of *Artemia salina* based on method developed by McLaughlin et al. (1998) as reported by Wakawa et al. (2017).

Antioxidant (DPPH (2, 2-diphenyl-1-picryl-hydrazyl) Free Radical Scavenging Assay)

The free radical scavenging assay of compound 2, 2-diphenyl-1-pycryl-hydrazyl (DPPH) was used to evaluate the antioxidant properties of the crude extract. The measurement was based on the method described by Wang et al. (2008)

Antibacterial Assay

The antibacterial activity of the stem-bark of *Barringtonia asiatica* was determined using disk diffusion method as reported by various authors (Boyan et al., 2005; Prashanth et al., 2006).

Antifungal Assay

The antifungal activities of the stem-bark extract and the pure compounds isolated of *Barringtonia asiatica* at varying concentration (25, 50, 100, 250 and 500 μg/mL) prepared in methanol were performed against a standard drug fluconazole (positive control) (500 μg/mL) using standard paper dilution method as described by Aboh et al. (2014)

RESULT AND DISCUSSIONS

Purification and structural Elucidation of Compound 1

Purification

Compound 1 was obtained from chloroform crude stem-bark extract of *Barringtonia asiatica* Table 1. About 20 g of the extract was introduced into the column using a dry pack method, uploaded with silica gel in hexane 100 %. A suitable solvent ratio was used to elute the extract as shown in Table 2.

Table 1: Fractions collected from chloroform crude stem-bark extract of *Barringtonia asiatica*

Code of Fraction	Weight of fraction	Colour of fraction
BASBCF 1	24.0	Colourless
BASBCF 2	55.34	Dark brown
BASBCF 3	90.0	Dark brown
BASBCF 4	178.12	Brown
BASBCF 5	237.4	Brown
BASBCF 6	279.18	Dark brown
BASBCF 7	345.60	Dark brown
BASBCF 8	307.43	Dark brown
BASBCF 9	336.56	Light yellow
BASBCF 10	444.70	Light yellow

Compound 1 was isolated from the combined fraction BASBCF10 (*Barringtonia asiatica* stem-bark extract in chloroform) shown in Table 1. TLC analysis of the combined fraction BASBCF 10 was performed in different solvent system chloroform: ethyl acetate (3:7) and hexane: ethyl acetate (3:2) and the result is shown in Table 2.

Table 2: TLC and R_f value of BASBCF 10 in different solvent ratio system under UV light

Solvent system (v/v)	Number of spots	R_f value	Stained TLC colour
Chloroform: Ethyl acetate (3:7)	2	0.73 0.18	Yellow
Hexane: Ethyl acetate (3:2)	2	0.43 0.39	Yellow

Fractions containing a light yellowish spot was targeted combined and was labelled as BASBCF10-D. Combined fraction of BASBCF10-D was then further purified using chloroform: ethyl acetate (3:7) which gave a better separation. TLC of the fractions collected was performed and examined under UV light and vanillin staining. It showed two single clear spot as recorded in Table 3 and this fraction was labelled as BASBCF10-D1.

Table 3: TLC and R_f value of BASBCF10-D1 in different solvent ratio system under UV light

Solvent system (v/v)	Number of spots	R_f value	Stained TLC colour
Chloroform: Ethyl acetate (3:7)	2	0.73 0.18	Yellow

Combined fraction of BASBCF10-D1 was further purified using a smaller column with solvent ratio of chloroform: ethyl acetate (1:4) and a fraction containing the targeted spot from BASBCF10-D1 were then combined and labelled as BASBCF10-D2. TLC of the combined fraction was examined under UV light and vanillin staining. A single spot as observed as shown in Table 4.

Table 4: TLC and R_f value of BASBCF10-D2 in different solvent ratio system under UV light

Solvent system (v/v)	Number of spots	R_f value	Stained TLC colour
Chloroform: Ethyl acetate (1:4)	1	0.75	Yellow

Figure 2 shows the TLC profile for the combined fraction of BASBCL10-D2 in chloroform: ethyl acetate (1:4) as single spot.

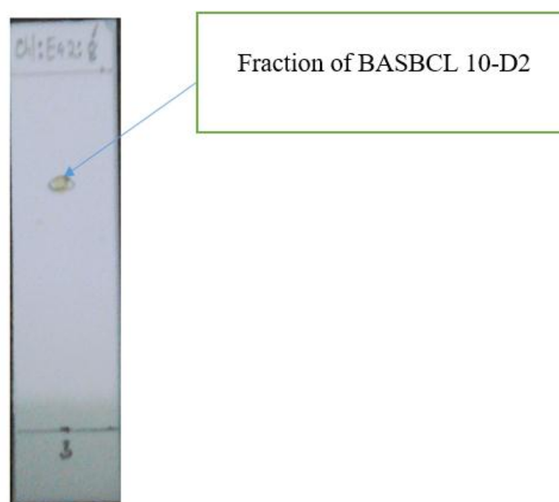

Fig. 2: TLC plate showing the spot in combined fraction BASBCL10-D2 in chloroform: ethyl acetate (1:4)

Analysis of the combined fraction BASBCL10-D2 was carried out and the result from the gas chromatogram shows (Figure 3) a single peak at retention time 14.57 min. This confirmed that BASBCL10-D2 is a pure compound and it was renamed as Compound 1 and 22 mg of this compound was obtained.

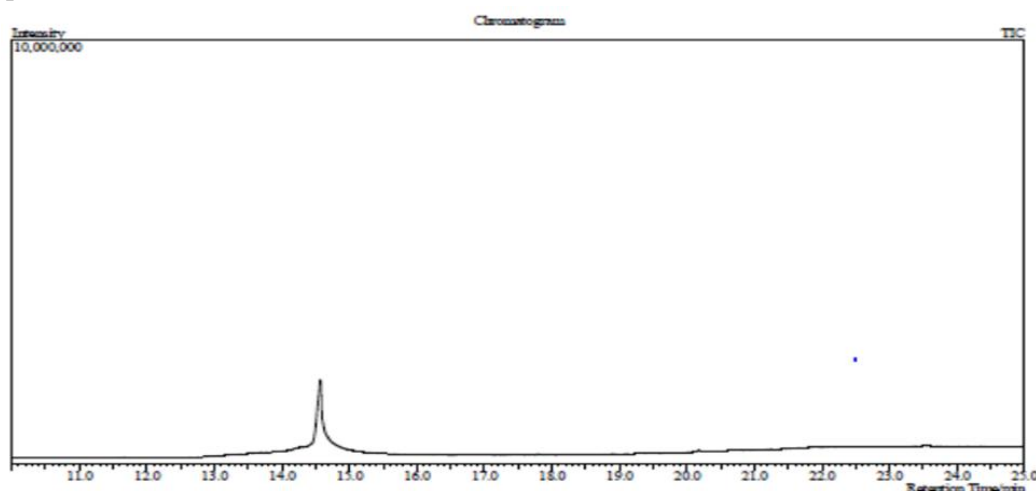

Fig. 3: Gas chromatogram of Compound 1

Structural Elucidation

Compound 1 was isolated from the chloroform crude stem-bark extract of *Barringtonia asiatica*. Its physical appearance is yellowish solid with a melting point of 134 ^oC. The mass spectrum of Compound 1 (Figure 4) show a similarity index of 97% with the mass spectrum of the compound suggested by the NIST library in Figure 5. The mass spectrum of Compound 1 has one of its molecular ion peak at *m/z* 148 and this corresponded to the same molecular ion peak and molecular weight of the suggested compound by the NIST library with a chemical formula of $C_9H_8O_2$.

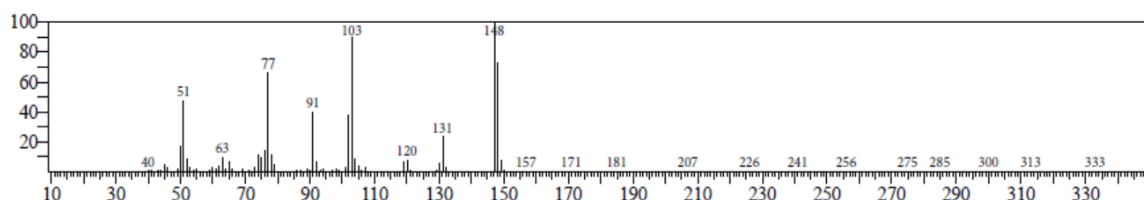

Fig. 4: Mass spectrum of Compound 1

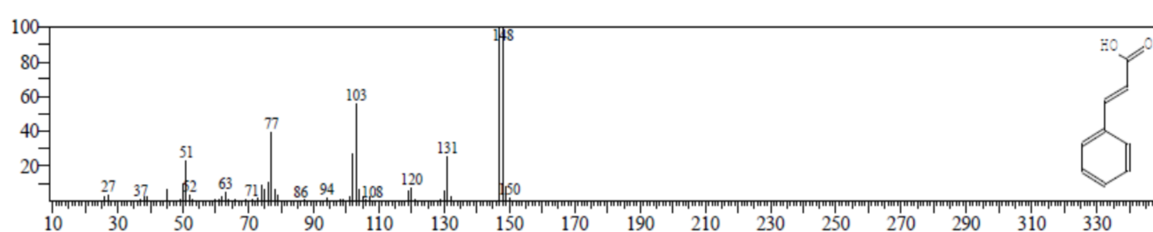

Fig. 5: Mass spectrum of suggested structure of Compound 13 by NIST library

Chemical structure of Compound 1 has functional group of (OH) which appeared at 3064.64 cm^{-1} as illustrated in IR spectrum (Figure 6). An absorption bands of C-H was observed at 2970.28 cm^{-1} and 2825.08 cm^{-1} which indicated the presence of methine group in the chemical structure. A signal was observed at 1670.98 cm^{-1} which indicated the presence of C=O band. A signal of 1080.28 cm^{-1} was observed which matched with the carboxylic bond in the suggested structure. A single bond C-C stretching was observed at 913.99 cm^{-1} as shown in the IR spectrum of Compound 1.

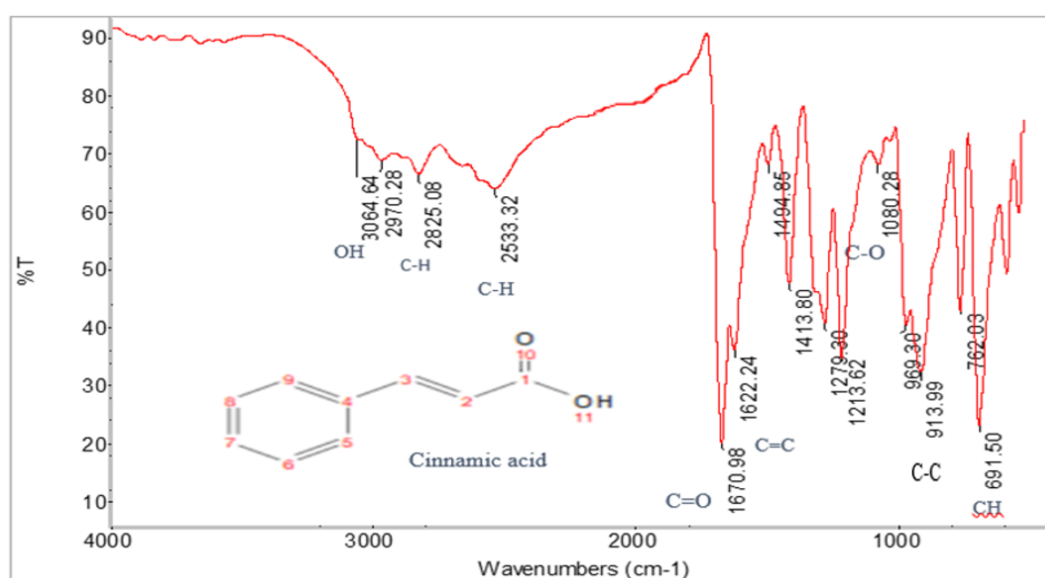

Fig. 6: IR spectrum of Compound 1

NMR analysis was further performed for the elucidation of the chemical structure of Compound 1 and the result are shown in Figure 7 (^{1}H-NMR) and Figure 15 (^{13}C-NMR) based on the table of ^{1}H-NMR characteristics absorption and ^{1}H-NMR peak splitting pattern as reported in Organic Chemistry by Silverstein *et al*. (2005). The proton signals were all integrated and were assigned to every proton NMR of Compound 2 as proposed in the chemical structure.

The ^{1}H-NMR spectrum of Compound 1 exhibited seven proton resonates. A signal was observed at δ 6.59 (1H, d) indicating the presence of methine group of the proton adjacent to hydroxyl group of the structure and another methine signal of δ 7.83 (1H, d) was observed, they were both assigned to H-2 and H-3. A doublet signal was observed at δ 7.57 (2H, t), δ 7.42 (2H, t) and 7.40 (2H, d) as the methine proton of the carbon ring and were assigned to H-5/H-9, H-6/H-8, and H-7, respectively.

From the result of the ^{13}C-NMR spectrum (Figure 8) of Compound 1 every carbon NMR signal that was observed was assign to the proposed chemical structure of Compound 1 which is based on the table of ^{13}C-NMR characteristic absorption reported in Organic Chemistry by Silverstien, (2005).

A total of seven carbon resonates were observed in the ^{13} C-NMR of Compound 1. At the down field region signals were observed at δ 168.94, δ 116.19, δ 144.61, δ 135.88, δ 129.02, δ 129.46, and δ 128.06, and were assigned to C -1, C-2, C-3, C-4, C-5, C-6 and C-7, respectively.

The chemical shift of every proton and carbon for Compound 1 is shown in Table 5 and Table 8, respectively and comparison was made with NMR data of the similar compound, reported by Gao et al. (2012).

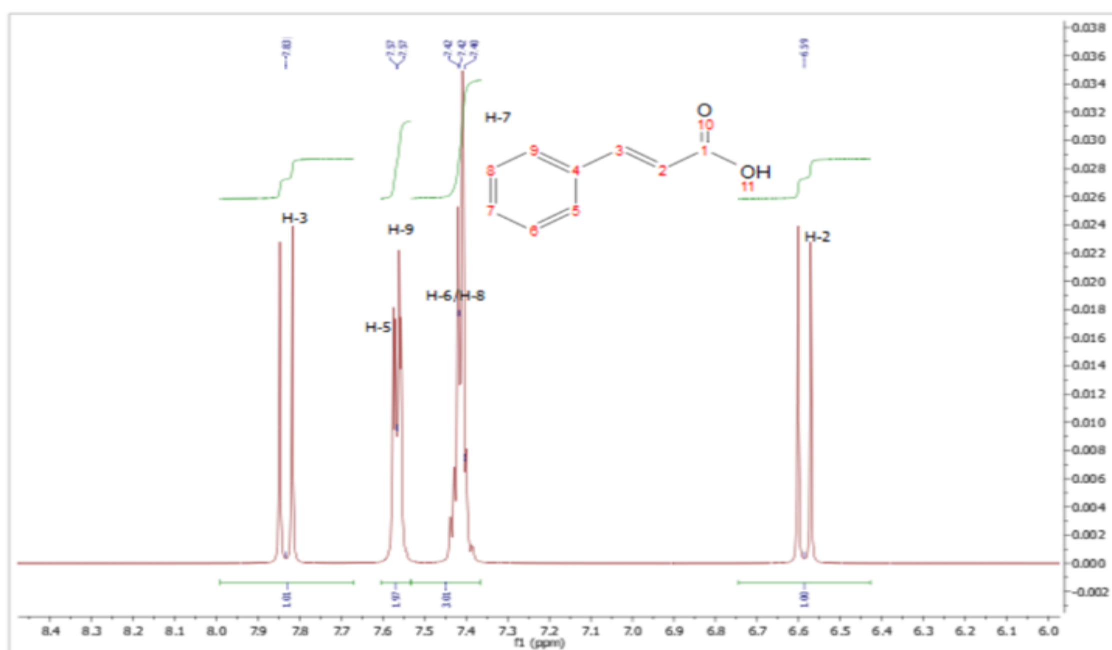

Fig. 7: ^{1}H-NMR spectrum of Compound 1 from δ 6.0 to δ 8.4 (500 MHZ, $CDCl_3$)

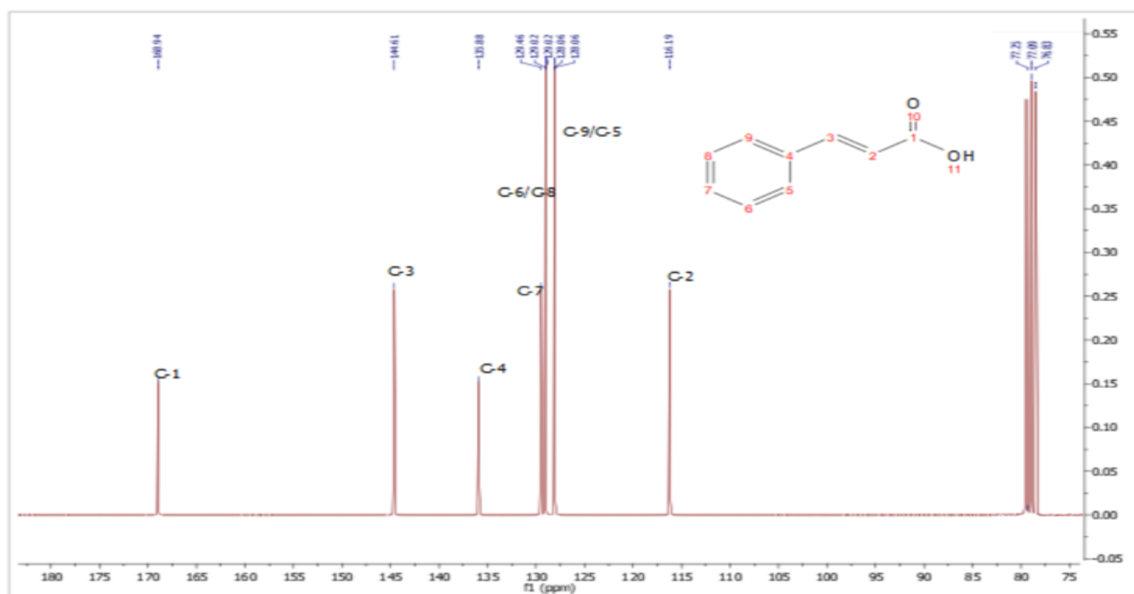

Fig. 8: ^{13}C-NMR spectrum of Compound 1 from δ 75 to δ 180 (125 MHz, $CDCl_3$)

Table 5: Proton NMR signal of Compound 1 and that reported by Gao et al., (2010) for cinnamic acid

Proton assigned to Compound 1	Proton chemical shift (ppm) of Compound 1	Proton assigned to cinnamic acid by Gao *et al.* (2012)	Proton chemical shift (ppm) of cinnamic acid (Gao *et al.*, 2012)
H-2	6.59 (1H, d)	H-2	6.51 (1H, d)
H-3	7.83 (2H, d, *J*=7.2)	H-3	7.51 (2H, d)
H-5/ H-9	7.57 (2H, t, *J*=7.2)	H-5/H-9	7.39 (1H, d)
H-6/H-8	7.42 (2H, t, *J*=7.2)	H-6/H-8	7.34 (2H, d)
H-7	7.40 (1H, d)	H-7	7.30 (1H, d)

Table 6: Carbon NMR signal of Compound 2 and that reported by Gao et al., (2012) for cinnamic acid

Carbon assigned to Compound 1	Carbon chemical shift (ppm) of Compound 1	Carbon assigned to of cinnamic acid by Gao *et al.* (2012)	Carbon chemical shift (ppm) of cinnamic acid (Gao *et al.*, 2012)
C-1	168.94	C-1	175.80
C-2	116.19	C-2	127.00
C-3	144.61	C-3	140.80
C-4	135.88	C-4	137.60
C-5	129.02	C-5	127.00
C-6	129.46	C-6	130.00
C-7	128.06	C-7	128.60

Based on the spectroscopic information and comparison with the published information, Compound 1 was identified as cinnamic acid (1).

Cinnamic acid (**1**) was found to be quite potent lipid peroxidation inhibitors (Peperidou et al., 2017). Cinnamic acid (**1**) has been reported to have antibacterial, antiviral and antifungal properties (Sova, 2012). Cinnamic acid has shown the best antifungal effect against all *C. albicans* strains (Lima et al., 2018).

Table 7: Average death of brine shrimp (*Artemia salina*) at different concentration of isolated compounds

Solvent system	Average death of *Artemia salina* Concentration (µg/mL)						LC_{50} (µg/mL)
	1	10	25	50	100	500	
-ve Control	0	0	0	0	0	0	-
Cinnamic acid	5.71±0.42	7.10±0.69	7.36±0.46	9.07±0.69	10.0±0.00	10.0±0.00	**3.48**
Thymol	5.00±0.57	7.00±0.58	10.00±0.0	10.00±0.0	10.00±0.0	10.00±0.0	1.16

The result is Mean±SD. N = 30

There was an observed concentration dependant increment in mortality rate of the brine shrimp

Table 8: DPPH IC_{50} (µg/mL) Value of Isolated Compound

Standard and compound	R^2 and IC_{50} (µg/mL)	
	R^2	IC_{50} (µg/mL)
Standard Ascorbic acid	0.9657	17.27
Cinnamic acid (1)	0.9703	**12.22**

IC_{50} = Half maximal inhibitory concentration

Table 9: Antibacterial activity of isolated pure compounds of *Barringtonia asiatica* stem-bark

Concentration (µg/mL)	Bacteria	Tetracycline (30µg)	Cinnamic acid
25 µg/mL	*Staphylococcus aureus*	20.77 ±0.03	15.98±0.67
	Exiguobacterium aquaticum	19.79 ±0.06	**15.49±0.33**
	Escherichia coli	21.16 ±0.11	19.00±0.8
	Acinetobacter baumanni	20.76 ±0.18	17.98±0.67
50 µg/mL	*Staphylococcus aureus*	20.77 ±0.03	17.32±0.58
	Exiguobacterium aquaticum	19.79 ±0.06	20.00±0.8
	Escherichia coli	21.16 ±0.11	19.98±0.67
	Acinetobacter baumanni	20.76 ±0.18	21.98±0.66
100 µg/mL	*Staphylococcus aureus*	20.77 ±0.03	**23.67±0.3***
	Exiguobacterium aquaticum	19.79 ±0.06	21.98±0.66
	Escherichia coli	21.16 ±0.11	19.98±0.67
	Acinetobacter baumanni	20.76 ±0.18	21.49±0.33

Result is Mean ± SD. N = 3

*= significant activity was observed when compared to the control ($p<0.05$)

Concentration of standard is 30 µg/mL of tetracycline

Table 10: Antifungal activity of isolated pure compound (cinnamic caid)

Concentration	Bacteria	Tetracycline (30µg)	Cinnamic acid
25 µg/mL	*Aspergillus niger*	24.67±0.11	18.13±0.13
	Aspergillus flavin	23.4 ± 0.05	18.06±0.12
	Candida tropicalis	23.1 ± 0.08	19.06±0.08
	Fusarium oxysporium	23.2 ± 0.10	18.04±0.01
50µg/mL	*Aspergillus niger*	24.67±0.11	24.09±0.07
	Aspergillus flavin	23.4 ± 0.05	19.03±0.03
	Candida tropicalis	23.1 ± 0.08	23.01±0.09*
	Fusarium oxysporium	23.2 ± 0.10	24.04±0.06
100 µg/mL	*Aspergillus niger*	24.67±0.11	**24.70±0.07***
	Aspergillus flavin	23.4 ± 0.05	22.06±0.05
	Candida tropicalis	23.1 ± 0.08	21.73±0.13
	Fusarium oxysporium	23.2 ± 0.10	22.77±0.14

Result is Mean ± SD. N = 3

*= significant activity was observed when compared to the control ($p<0.05$)

Concentration of standard is 30 µg/mL of fluconazole

Brine shrimp bioassay has been established as safe, practical and economical method for the determination of bioactivities of isolated compounds. This established a significant correlation with *in-vitro* growth inhibition of human solid tumor cell lines as demonstrated by the National Cancer Institute (NCI, USA) where it shows the value of this bioassay as a pre-screening tool for antitumor drug research (Anderson et al., 1991).

Brine shrimp (*Artemia salina*) was used to test for cytotoxicity. The result of the isolated pure compounds Table 7 revealed significant toxicity of the chemical constituents with brine shrimp lethality activity of 3.48 µg/mL. Higher toxicity was observed when compared to the test control thymol of 1.16 µg/mL.

The most exploring feature of this study is that, this result obtained is not an affirmation of the toxicity of the isolated pure compound, but rather an indication of a proof the bioactive components of the plant products to have an antimicrobial activity (Abhilasha et al., 2013; Adelowotan et al., 2008; Prashith et al., 2010)

The isolated compounds cinnamic acid exhibited a significant antioxidant activity. The result of this compounds in table 8 shows cinnamic acid to exhibit higher antioxidant potential with IC_{50} of 12.22 when compared to the control Ascorbic acid of 17.27.4±0.16 µg/mL.

For centuries, plant extract has been known and the therapeutic efficacy of this plants in the treatment of several disorders and infectious diseases has been described by various practitioners of traditional medicine (Wakawa et al., 2017; Umaru et al., 2018b). Harmful microorganisms are being control with synthetic drugs, and continuous treatment results in emergence of multiple drug resistance bacteria which creates an alarming clinical situation. This explore researchers in search of natural product to cutile this menace associated to synthetic drug resistance pathogens (Siddiq et al., 2009).

Table 9 shows the inhibition values of isolated compounds on the selected pathogen. It was observed that the isolated compound cinnamic acid indicated a significant activity of all the concentrations with higher activity observed on *Staphylococcus aureus* with inhibition value at 23.67±0.3 mm higher when compared to the standard tetracycline at 20.77 ±0.03 mm. lower inhibition was observed at 25 µg/mL on *Exiguobacterium aquaticum* 15.49±0.33 mm when compared to the test control at 19.79 ±0.06 mm.

Table 10 show the mean values of zone of growth inhibition of antifungal activity of isolated pure compounds against *Aspergillus niger, Aspergillus flavus, Fusarium oxysporium and Candida tropicallis.* Fluconazole of 30 µg/mL was used as control for the isolated compound cinnamic acid, Significant antifungal activity of the pure compounds on all the selected pathogens with inhibition values ranged of 15.36±0.07 mm to 24.70±0.07 mm.

The isolated pure compound which happened to be the isolate of the plant parts *Barringtonia asiatica* gave a significant antifungal activity, provide better understanding of a novel mechanism of antifungal. It has also been reported that plant has continued to be a rich source of therapeutic drugs and the active principles of many drugs are found in plants or are produced as secondary metabolites (Boyan et al., 2005; Runyoro et al., 2006). Therefore, the findings in this study shows that stem-bark extract of *Barringtonia asiatica* has the potential to be used as a valuable source of natural antimicrobial agents.

CONCLUSIONS

The beneficial effect of cinnamic acid for antioxidant and pathogenic conditions may be justified by the results obtained in the cytotoxicity, antifungal and antioxidant assays. With respect to antimicrobial activity, cinnamic acid (1) was active against the fungi *Aspergillus niger, Aspergillus flavin, Candida tropicalis* and *Fusarium oxysporium.* Regarding the scavenging effect, the compounds were more effective than the test control. The results also support the

widespread use of this plant in traditional medicine. A simple analytical pattern was developed and fully validated for the quantification and isolation of pure compounds (Cinnamic acid).

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Conflicts of Interest: The authors declare no conflict of interest.

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