Nigerian Journal of Scientific Research (NJSR)
ISSN-0794-0378

ISOLATION OF *β*-SITOSTEROL AND ANTIBACTERIAL ANALYSIS OF HEXANE STEM BARK EXTRACT OF *FICUS ABUTILIFOLIA* (MIQ.) MIQ. (MORACEAE)

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ABSTRACT

Ficus abutilifolia (Miq), Miq. belongs to the family Moraceae and is commonly called "Large-leaved rock fig" in English. The plant is widespread in tropical and subtropical regions but a few extends into temperate regions and used traditionally to treat various ailments such as typhoid fever, chronic dysentery, sexually transmitted infections, malaria and infertility. The aim of the study was to extract stem bark of the plant using *n*-hexane and carry out its preliminary phytochemical screening, isolate compound(s) using chromatographic techniques and screen it for antibacterial activity using standard procedures. The preliminary phytochemical screening of the *n*-hexane stem bark extract revealed the presence of steroids/triterpenes. Repeated silica gel column chromatography of the *n*-hexane stem bark extract led to isolation of a compound coded B6 which was characterized as *β*-Sitosterol using spectroscopic techniques and comparison to literature data. The *in-vitro* antibacterial activity of the crude *n*-hexane stem bark extract was assayed at concentrations of 12.5, 25, 50 and 100 mg/mL using agar well diffusion and broth dilution methods against two Gram positive bacteria (*Staphylococcus aureus* and *Baccillus subtilis*) and three Gram negative bacteria (*Escherichia coli*, *Salmonella typhi* and *Pseudomonas aeruginosa*). The antibiotic disc used was Ciprofloxacin (5 µg/mL). The crude *n*-hexane stem bark extract exhibited activity against one Gram positive (*Staphylococcus aureus*) and one Gram negative (*Pseudomonas aeruginosa*) bacteria because *Staphylococcus aureus* exhibited susceptibility of 10.67±0.67 mm at 12.5 mg/mL and 25 mg/mL; 10.33±0.33 mm at 50 mg/mL and 11.33±0.67 mm at 100 mg/mL as compared to 26 mm at 5 µg/ml of ciprofloxacin. While *Pseudomonas aeruginosa* with susceptibility of 15.33±0.33 mm at 12.5 mg/mL; 14.67±0.33 mm at 25 mg/mL; 20.33±0.33 mm at 50 mg/mL and 29.67±0.33 mm at 100 mg/mL as compared to 31 mm at 5 µg/mL of the standard drug. Thus, it can be drawn that the ethnomedicinal uses of *Ficus abutilifolia* in the treatment of diseases related to bacterial infections has scientific basis and the antibacterial activity observed could be linked to perhaps presence of some bioactive compounds present in the plant extract.

Keywords: Antimicrobial studies, *Ficus abutilifolia*, NMR spectra, TLC plates, *β*-Sitosterol
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INTRODUCTION

Natural products are chemical compounds or substances isolated from living organisms. The chemistry of the natural products includes their biosynthesis, extraction, identification, quantification, structural elucidation, physical and chemical properties and reactions. They are produced by the pathway of primary or secondary metabolism [1].

Medicinal plants have been used by man for the treatment of varieties of diseases, including microbial infections of several organs of the body. The use of extracts from various parts of medicinal plants has therefore significantly supported primary health care among and within human communities [2]. Infectious diseases are the number one cause of deaths worldwide, accounting for approximately 50 % of all deaths in tropical countries. Infectious diseases in humans are caused mainly by parasites such as bacteria, fungi, viruses, and protozoa which are generally referred to as microbes [3]. There has been an increasing incidence of multiple resistances in human pathogenic microorganisms in recent years. This has forced scientists to search for new antimicrobial substances from various sources like the medicinal plants [4].

Ficus abutilifolia belongs to the Moraceae family and is commonly called "Large-leaved rock fig" in English. It is a small-medium sized, deciduous-semi- deciduous tree up to 50 m high. The plant grows in semi-humid lowland-savannah, thicket-savannah, sea-level up to 800m as its habitat. *Ficus abutilifolia* can be found in Cameroon, Central African Republic, Dahomey, Nigeria, Somalia and Sudan and the species is reported from almost all tropical Africa [5]. The bark is whitish yellow or white and smooth, powdery

or somewhat flaking and it is this conspicuous bark that is immediately apparent to the eye. In Nigeria, it is called *Waa* in Hausa; *Ogunro* in Yoruba; *Okan Ovi* in Edo State and it is called *Pulaar* by Fulani tribe in Senegal [6]. *Ficus abutilifolia* (Family: Moracea) has been used traditionally to treat various ailments such as typhoid fever, chronic dysentery, sexually transmitted infections, malaria, and infertility. A decoction of the leaf of the plant was also reported to be used in promoting fertility in humans and the milky latex is used to remove skin warts [2]. In south eastern and Northern parts of Nigeria the stem bark decoction is taken by men as a strengthening tonic [7]. The aim of the study was to carry out preliminary phytochemical screening on the *n*-hexane stem bark extract of *Ficus abutilifolia*, isolate compound(s) and to also screen it for antibacterial activity.

MATERIALS AND METHODS

Collection, identification and preparation of plant material

Whole plant growing in the wilderness was collected from Samaru, Zaria, Kaduna State, Nigeria and authenticated at the Herbarium, Botany Department, Ahmadu Bello University Zaria-Nigeria where a voucher specimen number (No. 0188) was given. The stem bark was cut, air-dried and made into powder using pestle and mortar. It was subsequently referred to as powdered plant material.

Extraction of plant material

Extraction was done extensively using successive extraction with first *n*-hexane, then dichloromethane and finally methanol (in order of increasing polarity). The powdered plant material was macerated with each solvent for 4 days and the various extracts were evaporated to dryness at room temperature and coded as *n*-HEX, DCM and ME for *n*-hexane, dichloromethane and methanol extracts respectively.

Phytochemical screening

The *n*-hexane stem bark extract was subjected to preliminary phytochemical screening using standard methods [8, 9].

Column chromatographic separation of *n*-hexane extract

The *n*-hexane stem bark extract was subjected to column chromatographic separation using column of dimension 50 X 4.5 cm packed manually with 500 g silica gel with mesh size of 240-400 µm mounted unto a stand and allowed to settle and packed for 24 hours. The sample was applied using dry load method, where 10 g of the sample was dissolved in small amount of *n*-hexane and mixed with a small quantity of silica gel, dried and loaded on top of the packed column. The column was eluted in a gradient-wise manner with *n*-hexane (100%), then alternating with *n*-hexane: ethyl acetate (95:5%, 90:10%, 80:20%, 70:30%, 60:40% and 50:50%) respectively until ethyl acetate (100%) was attained. A total of 49 collections were made (50 mL each). The collections were pooled on the basis of TLC profiles. A total of 11 pooled fractions were obtained and coded FA (1-8), FA (9-10), FA (11), FA (12-13), FA (14-17), FA (18-20), FA (21-22), FA (23-27), FA (28-31), FA (32-37) and FA (38-49). The coded pooled fraction FA (14-17) gave 4 distinct spots which was subjected to further purification using isocratic elution with *n*-hexane: ethyl acetate (90:10). Subsequently, a total of 63 collections of 2 mL each were collected from which collections 49-63 were pooled and concentrated because they gave similar single spots on TLC plates. This afforded a white solid material coded B6 which was soluble in DCM and chloroform.

Antibacterial studies

Five (5) clinical isolates were collected from Department of Medical Microbiology, Ahmadu Bello University Teaching Hospital (ABUTH), Shika – Zaria and were maintained on nutrient agar slants in the refrigerator at (4^{o}C) after authentication prior to use [10]. The antibacterial studies were carried out using agar well diffusion method as described by Bereksi *et al.* and Srinivasan *et al.* [10, 11]. The clinical isolates used were three Gram negative bacteria (i.e. *Escherichia coli, Salmonella Typhi* and *Pseudomonas aeruginosa*) and two Gram positive bacteria (i.e. *Staphylococcus aureus* and *Bacillus subtilis.*

Preparation of stock solution

A 1g of the *n*-hexane stem bark extract was weighed and dissolved in a sample bottle containing 2 mL of dimethyl sulfoxide (DMSO) and later diluted with 8 ml water to obtain a concentration of 100 mg/mL. This was the initial concentration of the extract used to determine the antibacterial activity from the plant. A four-fold serial dilution of the extract was made to obtain the concentrations of 100, 50, 25, and 12.5 mg/ml. Ciprofloxacin (µg/mL) was used as the positive control drug for the bacteria [12].

Preparation of culture media and standardization of inocula

The Mueller Hinton agar was prepared according to manufacturer's instructions. The prepared sterile medium was then poured into sterilized petri dishes and was allowed to cool and solidify. McFarland 0.5 Barium sulphate Turbidity Standard was used which was prepared by dissolving 0.5 mL of 0.004 M BaCl in 99.5 mL of 0.3 N sulphuric acid using normal saline containing the isolates to give a turbid solution. The turbidity was equivalent to $1.5x10^{8}$ colony forming units per ml (cfu/mL).

Susceptibility studies

The susceptibility studies of the extract were carried out using agar well diffusion method. The inocula were poured and spread evenly over the surface of the

sterilized solid medium (MHA) by the use of a sterile swab. Four wells each were bored into the solidified inoculated MHA plates using cork borer number 4 (6 mm in diameter) which were labelled according to the prepared concentration of the extracts 100, 50, 25, and 12.5 mg/mL respectively. The wells were filled with equal volume of 0.2 mL extract each. Ciprofloxacin disc was placed on separate agar plate. The plates after introducing the plant extracts at different concentrations were left on the table for one hour for the extract to diffuse into the agar after which the plates were incubated overnight at 37ºC for 24 hours [13]. At the end of incubation period, diameter of inhibition zone was measured using ruler and recorded in mm. The extracts and standard antibiotic were tested in triplicates and mean zone of inhibitions were calculated and expressed as mean±SEM.

Minimum Inhibitory Concentration (MIC)

MIC was determined using broth dilution technique [14]. A 0.1 g of the crude *n*-hexane stem bark extract was weighed and dissolved into 10 ml of the sterilized nutrient broth to obtain concentration of 10 mg/mL of the extract in the broth. Four test tubes were arranged in a row, dilution of the extract in broth was done to obtain concentrations of 100, 50, 25 and 12.5 mg\mL. Using a sterile syringe, 0.1 ml of the microorganism suspension in normal saline was transferred into each dilution in the test tubes and the test tubes were incubated at 37ºC for 24 hours, after which turbidity was observed in each test tube. Tubes containing broth and crude extracts without inocula served as positive control while tubes containing broth and inocula served as negative control. The test tube with lowest concentration of the extract showing less turbidity was taken as the MIC [10].

Minimum Bactericidal Concentration (MBC)

The minimum bactericidal concentration (MBC) was determined by assaying the test tube content resulting from MIC determination that showed no evidence of growth. A loop full of the content of each tube was inoculated by streaking on solidified MHA plate and then incubated at 37ºC for 24 hours, after which the plates were observed for microbial growth. The lowest concentration of the sub-cultured solution with no growth was considered as minimum bactericidal concentration [10].

RESULTS AND DISCUSSION

The extraction from 1,000 g of *Ficus abutilifolia* stem bark using hexane yielded 20 g (i.e. 2.0 % w/w) of dark green gummy mass of the crude extract. The preliminary phytochemical examinations of the extract revealed the presence of steroids and triterpenes (Table 1) which is in accordance with the reported phytochemicals of the plant [15, 16].

Table 1: Preliminary phytochemical screening of *n*-hexane stem bark extract of *Ficus abutilifolia*

SN	Phytoconstituents	Inference
1.	Alkaloids	-
2.	Anthraquinones	-
3.	Cardiac glycosides	-
4.	Flavonoids	-
5.	Glycosides	-
6.	Steroids/Triterpenes	+
7.	Tannins	-

Key: SN = Serial number; - = absent and + = present

Characterization of Compound B6

The IR spectrum of compound **B6** showed absorption bands at 3429 cm^{-1} (broad) which corresponds to O-H bond vibration, 2926 cm^{-1} (highly intense) and 2855 cm^{-1} vibrations is due to CH stretching resulting from asymmetric and symmetric vibrations, absorption band at 1654 cm^{-1} was due to C=C bond vibration. The ^{1}H NMR spectrum of this isolated compound showed one olefinic proton at δ_H 5.35 which appeared to be characteristic of the sterols, and it was assigned to H-6 proton in the steroid. Another proton which correspond to *β*-Sitosterol, the hydroxyl group also appeared as a multiplet at δ_H 3.52 which was assigned to H-3 Proton. Six other proton signals were evident which includes four secondary methyl groups at δ_H 0.92, 0.86, 0.86 and 0.83 (all doublets) and two methyl singlets at δ_H 0.72 and 1.02 assigned to angular methyl proton at H-18 and H-19. The ^{13}C NMR spectra exhibited 29 carbon signals characteristic of phytosterols [18, 19]. The ^{13}C NMR showed recognizable signals at δ_C 140.99 and 121.95 which were assigned to C-5 and C-6 respectively. The chemical shifts at δ_C 12.09 and 19.62 were assigned to the angular methyl groups at C-18 and C-19 Respectively. The signals observed at δ_C 31.90, 50.36 and 56.90 were assigned to carbon at C-8, C-9 and C-14 respectively. The upfield chemical shifts were assigned to cyclohexyl and cylopentyl of A, B, C and D rings. The DEPT spectrum revealed signals for six methyl carbons at C-18, C-19, C-21, C-26, C-27 and C-29; eleven (11) methylene carbon atoms viz: C-1,

C-2, C-7, C-11, C-12, C-15, C-16, C-22, C-24, C-25 and C-28; nine (9) methine carbons at C-3, C-4, C-8, C-9, C-14, C-17, C-20, C-24 and C-25; then three (3) quaternary carbons with signals at C-5, C-10 and C-13. From the ^{1}H NMR, ^{13}C NMR and DEPT analysis with comparison to literature data, the structure of the compound was confirmed to be *β*-Sitosterol [17, 18] in agreement with the studies carried out by Soumia *et al.* [16] as shown in Table 2.

β-Sitosterol (Figure 1) is a natural micronutrient found in the cells and membranes of oil producing plants, fruits, vegetables grains and seeds. It is commercially available in preparative amounts only as mixtures with other phytosterols like stigmasterol and campesterol. *β*-Sitosterol has been proven to be safe, non-toxic and effective nutritional supplement. *β*-Sitosterol has also been proven to have amazing potential health benefit in many diverse applications including antibacterial and antifungal activities [19, 20].

The increasing failure of chemotherapeutics and antibiotic resistance exhibited by pathogenic agents has led to the screening of several medicinal plants for their potential antimicrobial activity [21]. In this study, antibacterial activity of crude *n*-hexane bark extract of *Ficus abutilifolia* was evaluated using diameter of zones of inhibition, MIC and MBC methods against test organisms Tables 3 and 4. From the results, the *n*-hexane extract had activity against *Staphylococcus aureus* and *Pseudomonas aeruginosa.* The highest zones of inhibition observed in the extract against *Pseudomonas aeruginosa* was at 100 mg/mL with 29 mm and the least was at 12.5 mg/mL having 10 mm zone of inhibition observed against *Staphylococcus aureus.* The standard antibiotic had zones of inhibition between 25-38 mm against the isolates (Table 3). It is observed in this work that the control drug used was ciprofloxacin (5µg/ml) which presented a wide diameter of zones of inhibition compared to the individual extracts against tested organisms which may be due to the fact that the control drug is a purified form of antimicrobial agent whereas the stem bark extracts have compounds in their unpurified forms [10].

Staphylococcus aureus can cause skin diseases including superficial and deep follicular lesion [13] in humans. The highest activity shown by the plant extract against *Pseudomonas aeruginosa* in this study indicates that the plant can be a source of compound that can really suppress the growth of *Pseudomonas aeruginosa* and can be used effectively against diseases caused by this microorganism, which justifies its use by traditional medical practitioners in the treatments of UTI and other infectious diseases [23]. The antimicrobial activities observed could be attributed to the presence of some of the phytochemicals detected in the plant which have been associated with antibacterial activity. It was observed also in this study that the antibacterial activity of the extracts was enhanced by an increase in the concentration of the extracts as earlier reported [24] i.e. the higher the concentration of the plant extracts the greater the zones of inhibition. In this study, the *n*-hexane bark extract did not inhibit the growth of *Escherichia coli*, *Bacillus subtilis* and *Salmonella typhi* (Table 3) which could be due to absence of polar secondary metabolites like alkaloids, cardiac glycosides flavonoids, tannins, e.t.c. (Table 1) that have been reported to possess antimicrobial activities. The MIC for the *n*-hexane extract against *S. aureus* was 25 mg/ml and *P. aeruginosa* at 50 mg/mL indicating the extract was bactericidal (i.e. able to kill) or able to inhibit growth of *S. aureus* and *P. aeruginosa* at 25 mg/mL and 50 mg/mL respectively (Table 4).

Table 2: The ^{1}H NMR and ^{13}C NMR data of compound B6 compared to literature data

	Compound B6		**Sumia *et al.*, 2012**	
Position	**δ_C ^{13}C**	**δ_H ^{1}H, *J* (Hz)**	**δ_C ^{13}C**	**δ_H ^{1}H, *J* (Hz)**
1	37.48	1.99(^{1}H, *m*, 1a), 1.14(^{1}H, *m*, 1b)	37.2	1.90(^{1}H, *m*, 1a); 1.13(^{1}H, *m*, 1b)
2	31.80	1.85(^{1}H, *m*, H-2a);1.58(^{1}H, *m*, H-2b),	31.6	1.88(^{1}H, *m*, H-2a);1.56(^{1}H, *m*, H-2b)
3	72.04	3.52(^{1}H, *m*, *J*=7.76, 8.92Hz, H-3)	71.8	3.58(^{1}H, *m*, *J*=7.76,8.92Hz, H-3)
4	42.54	2.31(^{1}H, *m*, H4a); 2.30(^{1}H, *m*, H4b)	42.3	2.34(^{1}H, *dd*, H4a); 2.30(^{1}H, *td*, H4b)
5	140.99	-	140.0	-
6	121.95	5.35(^{1}H, *t*, *J*=3.72, 6.24Hz, H-6)	121.7	4.0(^{1}H, *dd*, *J*=5.2, 2.34Hz, H-6)
7	31.91	1.51(^{1}H, *m*, H-7)	31.90	1.50(^{1}H, *m*, H-7)
8	31.90	2.01(^{1}H, *d*, *J*=7.1Hz, H-8)	31.80	2.03(^{1}H, *td*, *J*=12.4, 2.4Hz, H-8)
9	50.36	0.95(^{1}H, *m*, H-9)	50.1	0.98(^{1}H, *m*, H-9)
10	36.74	-	36.5	-
11	21.31	1.54(^{1}H, *m*, H-11a);1.51(^{1}H, *d*, H-11b)	21.1	1.55(^{1}H, *m*, H-11a);1.50(^{1}H, *d*, H-11b)
12	40.01	2.03(^{1}H, *d*, 4.62Hz, H-12a);1.27(^{1}H, *d*, H-12b)	39.7	2.03(^{1}H, *d*, 4.62Hz, H-12a);1.27 (^{1}H, *d*, H-12b)
13	42.54	-	42.30	-
14	56.90	1.01(^{1}H, *m*, H-14)	56.7	1.0(^{1}H, *m*, H-14)
15	24.53	1.60(^{1}H, *m*, H-15a);1.12(^{1}H, *m*, H-15b)	24.30	1.63(^{1}H, *m*, H-15a);1.11(^{1}H, *tm*, H-15b)
16	28.47	1.89(^{1}H, *m*, H-16a),1.29(^{1}H, *d*, H-16b)	28.20	1.89(^{1}H, *m*, H-16a);1.30(^{1}H, *d*, *m*, H-16b)
17	56.28	1.16(^{1}H, *m*, H-17, *J*=9.6Hz)	56.0	1.16(^{1}H, *t*, H-17, *J*=10.0Hz)
18	12.09	0.72(^{1}H, *s*, H-18)	11.8	0.74(^{1}H, *s*, H-18)
19	19.62	1.02(^{1}H, *s*, H-19)	19.8	1.06(^{1}H, *s*, H-19)
20	36.37	1.43(^{1}H, *m*, H-20)	36.10	1.40(^{1}H, *m*, H-20)
21	19.01	0.92(^{1}H, *d*, H-21)	18.80	0.93(^{1}H, *d*, *J*=6.5, H-21)
22	34.18	1.40(^{1}H, *m*, H-22a);1.05(^{1}H, *m*, H-22b)	33.90	1.36(^{1}H, *m*, H22a);1.07(^{1}H, *m*, H-22b)
23	26.31	1.26(^{1}H, *d*, H-23)	26.0	1.2(^{1}H, *m*, H-23)
24	46.07	0.93(^{1}H, *m*, H-24)	45.8	0.97(^{1}H, *m*, H-24)
25	29.66	1.83(^{1}H, *m*, H-25)	29.1	1.71(^{1}H, *m*, H-25)
26	20.00	0.86(^{1}H, *d*, H-26)	19.8	0.88(^{1}H, *d*, H-26)
27	19.01	0.83(^{1}H, *d*, H-27)	19.0	0.87(^{1}H, *d*, *J*=6.9Hz, H-27)
28	23.29	1.30(^{1}H, *m*, H-28)	23.0	1.31(^{1}H, *m*, H-28)
29	12.21	0.86(^{1}H, *t*, H-29)	12.0	0.89(^{1}H, *t*, H-29)

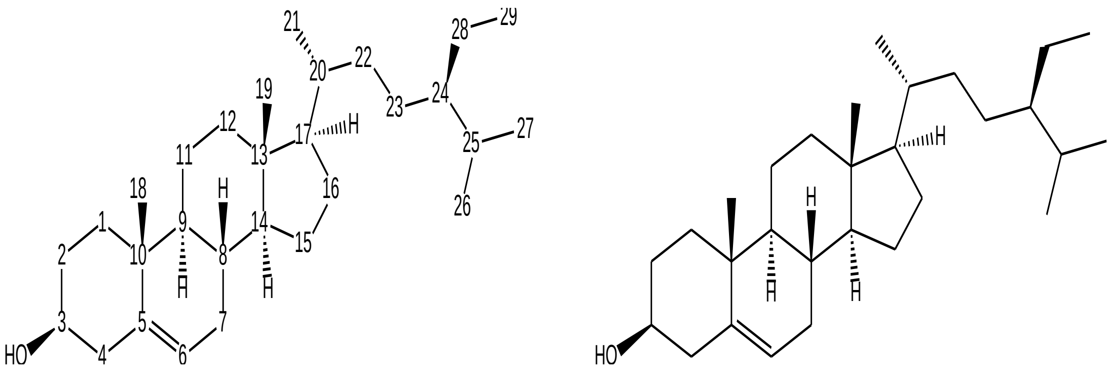

Figure 1: *β*-Sitosterol

Table 3: Antibacterial activity of *n*-hexane extract of *F. abutilifolia* against test organisms (zones of inhibition in mm)

Test organisms	*n*-hexane extract (mg/mL)/zones of inhibition (mm)				Standard drug Ciprofloxacin (5µg/mL)
	100	50	25	12.5	
S. aureus	11.33±0.67	10.67±0.67	10.67±0.67	10.33±0.33	26
B. subtilis	0.00	0.00	0.00	0.00	38
E. coli	0.00	0.00	0.00	0.00	25
S. typhae	0.00	0.00	0.00	0.00	32
P. aeruginosa	29.67±0.33	20.33±0.33	14.67±0.33	15.33±0.33	31

Key: *F. abutilifolia = Ficus abutilifolia;* Zones of inhibition in (mm); Values are presented as mean±SEM; *S. aureus = Staphylococcus aureus; B. subtilis = Bacillus subtilis; E. coli = Escherichia coli; S. typhae = Salmonella typhi; P. aeruginosa = Pseudomonas aeruginosa;* mg/mL = milligramme per liter and 5µg/mL = microgram per liter.

Table 4: Minimum inhibitory concentration (MIC) and the minimum bactericidal concentration (MBC) of *n*-hexane extract of *F. abutilifolia*

Test organism	*n*-hexane extract (mg\mL)	
	MIC	MBC
S. aureus	25	50
P. aeruginosa	50	-

Key: MIC = Minimum inhibitory concentration; MBC = minimum bactericidal concentration**;** *F. abutilifolia = Ficus abutilifolia*; mg/mL = milligramme per liter

CONCLUSION

The compound coded B6 was characterized as *β*–Sitosterol based on spectroscopic methods then compared to that in literature. The *n*-hexane extract of the plant stem bark exhibited moderate antibacterial activity against some of the tested pathogens. Thus, it can be concluded that the use of *Ficus abutilifolia* in the treatment of conditions caused by the test organisms has scientific basis and the anti-bacterial activity observed can be linked to the presence of some bioactive compounds present in the plant, this is the first report on the antimicrobial studies of *n*-hexane stem bark extract of *Ficus abutilifolia* to the best of our knowledge.

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