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Antimicrobial Activity of Crude Fractions of *Gossypium barbadense* Leaves and Isolation of Stigmasterol

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ABSTRACT

Plants have been used in traditional medicine in different parts of the world to treat several diseases. Many studies have confirmed their medicinal values, which have led to the isolation and characterization of various potential drug candidates. This study focuses on the antimicrobial activity of crude fractions of *Gossypium barbadense* leaves and the isolation of active compound. The pulverized plant leave was soaked in ethanol (98% v/v), and a solvent-based fractionation was carried out using *n*-hexane, chloroform, ethyl acetate, and ethanol. The obtained crude fractions were screened for antimicrobial activity against four (4) clinical isolates: *Escherichia coli*, *Pseudomonas aeruginosa, Staphylococcus aureus*, and *Candida albicans*. NMR and FTIR analyses of the most active fraction were carried out. The ethanol fraction showed the highest extraction yield and premier antimicrobial activity against the tested organisms. At 500 mg/mL, the fraction showed inhibition of 28.50±1.14, 26.40±1.46, 21.00±0.05 and 18.03±0.41 mm against *E. coli*, *P. aeruginosa, S. aureus*, and *C. albicans* respectively. The lower Minimum Inhibitory Concentration (MIC) values of 12.5 mg/mL were obtained against *E. coli* and *P. aeruginosa*. NMR and FTIR analyses revealed that the isolated compound is stigmasterol, which is known to possess antimicrobial properties. However, compounds that were not isolated could have also contributed significantly to the antimicrobial activities of the fraction. Results of this study justifies the use of leaves extract of *Gossypium barbadense* as an antimicrobial agent.

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INTRODUCTION

Pathogens that are resistant to antimicrobial agents continue to pose a serious challenge to the proper transformation of healthcare systems (WHO, 2014). The gravity of the inefficiency of the existing antimicrobial drugs is unanimous among the scientific community, and hence the treatment of microbial infections in patients with immunodeficiency remains worrisome (Theuretzbacher & Mouton, 2011; Laxminarayan et al*.,* 2013). The cause of the occurrence of resistance to antimicrobial agents is mainly associated with the misuse of the drugs, while the scarcity of alternative antimicrobial agents in the drug discovery pipeline has enabled the growing emergence of many types of infections that are difficult to treat (Munita & Arias, 2016). The World Health Organization recognizes medicinal plants as one of the sources of new drug varieties (WHO, 2021). This is because plants are naturally endowed with phytochemicals capable of curing a number of diseases.

Gossypium barbadense L. short as *G. barbadense* is a cotton wool producing plant, 1–3 m high perennial shrub, an indigenous of South America and now extensively bred in the tropics with significant existence in Nigeria and Senegal. It usually grows as a tree, produces greenish-yellow flowers with black seeds, and yields cotton with an unusually long silky fiber (Ansell & Mwaikambo, 2009). However, its extracts have significant pharmacological potentials as antibacterial, antifungal, antimalarial, antihypertensive, antiulcer, and anti-inflammatory (Eugene et al., 2012). The leaves and roots are usually decocted and used against diarrhea and dysentery (Salako & Awodele, 2012). Several studies have established the potency of this plant, and further studies have been carried out to identify the compounds responsible for the observed activities (Muhammad et al., 2014; Agbor, 2015). Ikobi et al. (2012) studied the antibacterial and wound-healing properties of methanolic extract from dried fresh *G. barbadense* leaves. The extract was found to be effective against bacterial pathogens, and the result further confirmed the biological potential of the leaves in speedy wound healing properties in healthy albino rats (Ikobi et al., 2012). In this work, the antimicrobial activity of the crude fractions of *G. barbadense* leaves was carried out, and further study led to the isolation as well as the characterization of stigmasterol.

MATERIALS AND METHODS

General Instrumentation

Nuclear Magnetic Resonance spectroscopic technique was performed on Bruker NMR DBX-400 MHz, Germany using deuterated chloroform ($CDCl_3$) as solvent. The Fourier Transform Infrared spectrum data were recorded on Agilent Technology's 630 FTIR, India to study the available functional groups. The sample was fixed in potassium bromide (KBr) disc and the sample was scanned in the range of 4000-650 cm^{-1} wavenumber.

Collection, Identification, and Preparation of Plant Material

Fresh leaves of *G. barbadense* were collected from Batagarawa town, Batagarawa Local Government Area, Katsina State, Nigeria. The plant was taxonomically identified and authenticated by a Botanist from the Biology Department, Umaru Musa Yar'adua University Katsina, Nigeria. A voucher specimen (Accession number UMYUH-2331) containing the identification characteristics was kept at the herbarium. The leaves were cleaned with tap water, plucked into small pieces, and dried under shade for ten days at room temperature. An electric blender was used to grind the leaves into fine powder and store them in a clean, airtight container prior to extraction.

Preparation of Crude Ethanol Extract

The crude extract was prepared according to the method used by Tiwari et al. (2011) and Pandey & Tripathi (2014) with some modifications. The pulverized leaves (300 g) were macerated in 2 L of 98% ethanol (solute to solvent ratio of 1:7 w/v) with occasional shaking for 72 h at room temperature. The suspension was filtered, and the filtrate was concentrated *in vacuo* to furnish the crude extract, which was weighed, and the percentage yield was also calculated.

Fractionation of Crude Ethanol Extract

The crude ethanol extract obtained was subjected to solvent-solvent partitioning using *n*-hexane, chloroform, and ethyl acetate following the procedure reported by Liu (2011) and Ingle et al. (2017) with some modifications. After treating the crude ethanol extract with the different solvent systems, the final residue was concentrated and labeled as GEF. The remaining fractions were labeled as GHF, GCF, and GEAF for *G. barbadense n*-hexane, chloroform, and ethyl acetate fractions, respectively. Their colors, textures, and yields were also recorded.

Antimicrobial Susceptibility Test

Antimicrobial activities of *G. barbadense* fractions (GHF, GCF, GEAF and GEF) were determined by disc diffusion method as contained in the European Committee on Antimicrobial Susceptibility Testing and Clinical and Laboratory Standards Institute manual (EUCAST, 2013; CLSI, 2018 & 2021). Four (4) different concentrations of the fractions i.e., 500 mg/mL, 250 mg/mL, 125 mg/mL and 62.5 mg/mL were prepared using serial doubling dilution method. Thus, prepared sterile culture media were poured into sterile plates (approximately 25 mL in each plate so that uniformity remained maintained) and then kept at room temperature to solidify. A colony of 24 h culture of each isolate adjusted to 0.5 McFarland standard was spread evenly on the surfaces of the culture media plates using sterile swab sticks dipped into the isolate's suspension. After the cotton swab was streaked throughout the agar plates, the plates were kept aside for 5 mins and fractions impregnated discs (6 mm) were aseptically placed on the agar surface with the aid of sterile forceps and ensured complete contact between the disc and the agar surface. Discs (6 mm) impregnated with nystatin and ciprofloxacin (10 µg/mL) and 200 µg/mL of 20% DMSO were used as positive and negative controls on separate media plates inoculated with the test organisms. The plates were incubated for 24 to 48 h at 37°C respectively. The susceptibility test was determined by measuring the diameter of zones of growth inhibition (mm) surrounding the discs and the readings were taken in 2 different fixed directions (EUCAST, 2013; CLSI, 2018 & 2021).

Determination of Minimum Inhibitory Concentration (MIC)

The MIC was determined using serial dilution method (EUCAST, 2013; CLSI, 2018 & 2021). Thus, varying amounts of the fractions in the following concentration order; 100 mg/mL, 50 mg/mL, 25 mg/mL, and 12.5 mg/mL were prepared. The least concentration of the fractions that does not permit any visible growth of the inoculated test organisms in broth culture was taken as the minimum inhibitory concentration in each case. Control experiments of the antibiotics (nystatin & ciprofloxacin) were also performed.

Determination of Minimum Bactericidal/Fungicidal Concentration (MBC/MFC)

The minimum bactericidal and fungicidal concentrations (MBC and MFC) were determined by sub-culturing from the tubes with concentrations equals to the MIC and above on fresh media plates (MHA and SDA). The lowest concentrations of the fractions associated with no visible bacterial/fungal growths were recorded (EUCAST, 2013; CLSI, 2018 & 2021).

Statistical Analysis

All experiments were performed in triplicate to safeguard the effectiveness of the method used. The zone of inhibition data is expressed as mean±standard error of the mean (SEM). The statistical difference of the mean zone of inhibition of the fractions for each individual isolate was carried out by employing One-Way Analysis of Variance (ANOVA).

Column Chromatography of Fraction GEF

The most active fraction, GEF was subjected to chromatography using a glass column of internal diameter 80 mm and length 100 cm. A column grade silica gel G (70 g) of 60-120 mesh size was wet-packed using *n*-hexane:EtOAc (60:40) as the solvent system and loaded into the column. After the silica had set, 3.5 g amount of the crude extract (GEF) was first dissolved in 10 ml of the solvent system and the slurry was loaded onto the packed column and continuously eluted with different ratios of *n*-hexane/EtOAc mixture to obtain fifty fractions (50). Based on the TLC pattern of the fractions as monitored using UV lamp as well as spray solutions ($KMnO_4$) and iodine, a total of nine fractions were pooled together for further purification. Re-chromatography of the pooled fractions using a micro-column (50 mm × 30 cm) and a solvent system, *n*-hexane:EtOAc (80:20) afforded a pure compound. The isolated compound was fully subjected to NMR and FTIR spectroscopic techniques for characterization.

Table 1. Yield of crude ethanol extract and fractions of *G. barbadense* leaves

S/N	Fraction	Weight (g)	Yield (%)	Color	Texture
1	GHF	8.57	14.51	Greenish-brown	Sticky oil
2	GCF	10.15	17.19	Green	Sticky oil
3	GEAF	12.26	20.76	Green	Sticky oil
4	GEF	16.45	27.86	Deep green	Sticky oil
5	Crude Ethanol Extract	59.05	19.68	Deep green	Sticky oil

GHF= *G. barbadense n*-hexane fraction, GCF= *G. barbadense* chloroform fraction, GEAF= *G. barbadense* ethyl acetate fraction and GEF= *G. barbadense* ethanol fraction

Table 2. Susceptibility pattern of fractions of *G. barbadense* against clinical isolates

Fractions	Concentration (mg/mL)	Organisms			
		E. coli	*P. aeruginosa*	*S. aureus*	*C. albicans*
		Mean Zone of Inhibition (mm) ± SD			
GHF	500	18.03±0.41	18.00±0.80	12.05±0.30	10.03±0.41
	250	15.00±0.05	13.23±0.74	10.42±0.68	08.63±0.00
	125	12.00±1.22	10.84±0.46	08.25±0.13	06.50±0.23
	62.5	10.25±0.06	08.50±0.00	6.50±0.00	6.33±0.75
GCF	500	18.81±0.00	17.51±1.84	14.63±0.00	12.73±0.42
	250	12.00±1.25	14.26±1.22	11.00±0.50	11.00±1.22
	125	10.73±0.05	12.23±0.74	10.03±0.41	08.00±0.05
	62.5	08.04±1.25	10.55±0.44	08.62±0.57	06.50±0.00
GEAF	500	23.50±1.25	20.20±2.26	18.26±1.22	14.83±0.37
	250	20.20±2.26	18.20±1.84	15.63±0.00	11.55±0.10
	125	18.63±0.00	12.27±0.49	10.50±0.25	08.00±0.50
	62.5	10.51±1.84	10.00±0.25	08.25±0.00	06.50±0.00
GEF	500	28.50±1.14	26.40±1.46	21.00±0.05	18.03±0.41
	250	22.60±2.26	20.23±1.17	18.00±3.68	12.00±0.50
	125	18.20±1.84	14.70±0.03	12.04±0.63	10.04±0.37
	62.5	13.80±0.46	10.30±1.11	10.5±0.07	08.23±0.74
+ve control		36.0±0.00[1]	32.0±0.00[1]	30.00±0.00[1]	32.0±0.00[2]
-ve control		06.00	06.00	06.00	06.00

GHF= *G. barbadense n*-hexane fraction, GCF= *G. barbadense* chloroform fraction, GEAF= *G. barbadense* ethyl acetate fraction, GEF= *G. barbadense* ethanol fraction, -ve control = DMSO and +ve control= [1]Ciprofloxacin &[2]Nystatin.

RESULTS AND DISCUSSION

Extraction and Fractionation

In this study, maceration method of extraction and solvent partitioning (liquid-liquid extraction) using solvents of increasing polarities were adopted. Among the solvents used, ethanol showed the highest fractionation yield (16.45 g) followed by ethyl acetate (12.26 g), chloroform (10.15 g) and *n*-hexane (8.57 g) using 59.05 g of the crude extract. This indicated that the extraction efficiency favored the highly polar solvent of ethanol with 27.86% yield (Table 1). Some works on medicinal plants also reported the fractionation of extracts with various organic solvents (Akor & Anjorin, 2009; Ofokansi et al., 2013). Solvents such as *n*-hexane, petroleum ether and chloroform are capable of extracting non-polar compounds mainly of terpenoids or highly methoxylated phenolics (Pandey & Tripathi, 2014; Azwanida, 2015). While ethyl acetate, acetone, ethanol, water or mixture of these solvents are responsible for extracting polar compounds (Das et al., 2010; Tiwari et al., 2011).

Extraction of bioactive compounds from plant material is complex and is promoted by the chemical nature, extraction process, particle size of the raw material, extractant properties, solvent-to-solid ratio, extraction temperature as well as the extraction duration (Li et al., 2014; Wen et al., 2020). The method of extraction play a significant role in assessing the biological activity of plant extract as it influence yield of the extracts, extractant capacity in extraction and quality parameter of the herbal preparations (Albuquerque & Hanazaki, 2006).

Antimicrobial Activity

In the present study, the antimicrobial potentials of the soluble fractions were tested against four potentially clinical isolates includes; *Escherichia coli*, *Pseudomonas aeruginosa*, *Staphylococcus aureus* and *Candida albicans* at different concentrations of 500, 250, 125 and 62.5 mg/mL. Although, some studies were reported on the antibacterial activity of the plant (Essien et al., 2011; Ikobi et al., 2012) but however, the antifungal activity was also determined in this study due to the differences in geographical areas of plant collection, extraction method, extractant, clinical isolates and the parts of the plant used to assure its antimicrobial activity.

All the fractions evaluated has varying degree of inhibitory effect against the tested bacteria (Gram-positive as well as Gram-negative) and fungi as proved in inhibition zones (Table 2). But, there were differences in spectrum of activity for the fractions indicating that they contained different bioactive compounds. GEF showed better activity against all the tested bacterial isolates with MIC range of 12.5–100 mg/mL (Table 3). A lower MIC value indicated high effectiveness lower toxicity of a sample. Among the tested clinical bacteria, *E. coli* was the most sensitive bacteria followed by *P. aeruginosa* and lastly *S. aureus*. An increase in growth inhibition was noted with increase in concentration of the fractions. The growth inhibition was in the order of *E. coli* >*P. aeruginosa* >*S. aureus* >*C. albicans* and GEF>GEAF>GCF>GHF for the fractions. Susceptibility of these bacterial strains to the fractions supports the traditional use of *G. barbadense* as curative agent for diarrhea, skin infections and ear pain (Daboor & Haroon, 2012; Iyamah & Idu, 2015).

Some species of *Gossypium* have been shown to possess antibacterial, anti-inflammatory, wound healing and anti-cancer properties (Velmurugan et al., 2012). On the other hand, all the fractions did not show appreciable activity on the growth of fungi as small or no inhibition zone was observed. The observed high MFC in all the fractions against *C. albicans* (>25 mg/mL) was not appreciable considering the prevalence of fungi in human infections (Table 3).

Table 3. Minimum inhibitory and minimum bactericidal/fungicidal concentrations of fractions of *G. barbadense*

		Test organisms/concentration (mg/mL)							
		E. coli		***P. aeruginosa***		***S. aureus***		***C. albicans***	
S/N	**Fraction**	**MIC**	**MBC**	**MIC**	**MBC**	**MIC**	**MBC**	**MIC**	**MFC**
1	GHF	25	50	>100	R	50	50	>100	R
2	GCF	100	100	>100	R	>100	R	100	100
3	GEAF	50	50	50	100	>100	R	50	50
4	GEF	12.5	12.5	12.5	25	25	25	50	100
5	Control	12.5[1]	12.5[1]	12.5[1]	12.5[1]	12.5[1]	12.5[1]	25[2]	25[2]

GHF= *G. barbadense n*-hexane fraction, GCF= *G. barbadense* chloroform fraction, GEAF= *G. barbadense* ethyl acetate fraction, GEF= *G. barbadense* ethanol fraction, R = Resistant and Control= [1]Ciprofloxacin &[2]Nystatin

The results obtained clearly indicated GEF to be more efficient in growth inhibition against the tested clinical bacterial isolates in comparison with the control (Table 2). The result also agrees with previous reports on the antimicrobial activities of *G. barbadense* leaves (Ikobi et al., 2012; Muhammad et al., 2014).

The negative control (20% DMSO) did not show activity on the clinical isolates (Table 2). This agrees with the study conducted by Brito et al., (2017) where he demonstrated the use of DMSO as a chemical solvent in concentration not exceeding 80% as non-inhibitor against growth. Similarly, Macieira (2013) and Filipe et al. (2008) investigated the antimicrobial activities of marine cyanobacteria and C*ydonia oblonga* where DMSO was used as negative control and no interference with the growth of the tested organisms was detected at the concentrations used.

Spectroscopic Analysis

The isolated pure compound was identified as stigmasterol (Figure 1), a known steroid based on the ^{1}H and ^{13}C NMR as well as the FTIR spectroscopic techniques. As indicated in Table 5, the ^{1}H NMR spectrum showed signals at δ 3.52 for H-3 and a triplet at δ 5.36 for H-6.

Table 4.^{1}H, ^{13}C and DEPT-135 NMR spectroscopic data of stigmasterol

Position	^{1}H NMR (ppm)	^{13}CNMR (ppm)	DEPT-135	Type of C-atom
1	1.84 (m)	36.74	Down	CH_2
2	1.95 (m)	29.71	Down	CH_2
3	3.50 (m)	71.91	Up	CH
4	2.24 (d, *J*= 1.44)	42.39	Down	CH_2
5	--	140.81	Quaternary	C
6	5.34 (t)	121.83	Up	CH
7	1.99 (m)	31.69	Down	CH_2
8	2.00 (m)	29.47	Up	CH
9	0.94 (m)	50.17	Up	CH
10	--	36.24	Quaternary	C
11	1.02 (m)	24.87	Down	CH_2
12	1.16 (m)	39.71	Down	CH_2
13	--	42.32	Quaternary	C
14	1.00 (m)	56.83	Up	CH
15	1.06 (m)	24.87	Down	CH_2
16	1.25 (m)	28.07	Down	CH_2
17	1.12 (m)	56.11	Up	CH
18	0.90 (s)	11.94	Up	CH_3
19	1.01 (s)	19.82	Up	CH_3
20	1.16 (m)	39.83	Up	CH
21	1.03 (d, *J*= 7.2 Hz, 3H)	23.13	Up	CH_3
22	5.00 (dd, *J*=1.73 & 1.72 Hz)	139.41	Up	CH
23	5.14 (dd, *J*=1.75 &1.73 Hz)	129.17	Up	CH
24	1.54 (m)	50.50	Up	CH
25	1.45 (m)	33.84	Up	CH
26	1.02 (d, *J*= 13 Hz)	21.15	Up	CH_3
27	0.84 (d, *J*= 6.5 Hz)	23.02	Up	CH_3
28	1.16 (m)	26.49	Down	CH_2
29	0.81 (t)	12.07	Up	CH_3

S= singlet, m= multiplet, d=doublet, dd= doublet of doublet, t= triplet and *J* = coupling constant

*Pierre, L. L., & Moses, M. N. (2015).

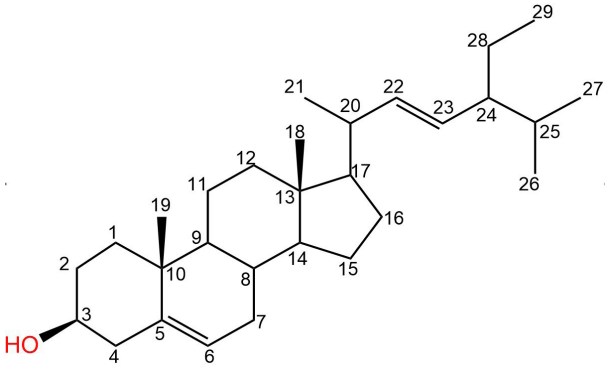

Figure 1: Structure of Stigmasterol

Table 5. FTIR spectroscopic data of stigmasterol

Wave number (cm^{-1})	Functional group	Assignment
3324	Alcohol	O-H stretch
2922	Aliphatic	C-H stretch
1610	Alkene	C=C stretch
1267	Alkoxy	C-O stretch

Two (2) singlets appeared upfield at δ 0.85 and 0.10 for the angular methyl protons, H-18 and H-19 respectively. The spectrum revealed the presence of signals in the downfield for olefinic protons at δ 5.00 and 5.15 for H-22 and H-23. Three (3) doublets assigned to the methyl groups at H-21, H-26 and H-27 were found at δ 1.03, 1.02 and 0.84 respectively. The trans stereochemistry of the double bond δ $^{22, 23}$ was consistent with the values of its highest coupling constant ($J_{22, 23}$ = 15.0 Hz).

The ^{13}C NMR and DEPT-135 indicated the presence of 29 carbons (Table 4). A total of six (6) methyl carbons at δ 11.94, 12.07, 19.82, 21.15, 23.13 and 23.02 were. Nine (9) methylene carbons at δ 24.87, 24.89, 26.49, 28.07, 29.71, 31.69, 36.74, 39.71 and 42.39 were found. Eleven (11) methine carbons at δ 29.47, 33.84, 39.83, 50.17, 50.50, 56.11, 56.83, 71.91, 121.83, 129.17 and 139.41. Three (3) quaternary carbons at δ 36.24, 42.32 and 140.81 were observed. These were supported by the DEPT-135 experiment (Table 4). Comparison of this NMR data with that of the literature shows that the compound isolated was a stigmasterol which is a known steroid (Pierre & Moses, 2015; Idowu et al., 2016; Talla et al., 2021).

The FTIR spectrum showed absorption peaks at 3324 cm^{-1} characteristic of O-H stretching, 2922 and 2855 cm^{-1} are due to C-H stretching of aliphatic. The absorption frequency at1610 $cm^{-1}$is due to C=C stretching, at 1461 and 1371 cm^{-1} are bending frequencies for cyclic $(CH_2)_n$ and $(CH_3)_n$, 1267 cm^{1} for C-O stretching and 1038 cm^{1} signifies cycloalkane (Table 5). These absorption frequencies resemble the absorption frequencies observed for stigmasterol (Kamboj & Saluja, 2011; Ahmed et al., 2013).

CONCLUSION

This study focused on the investigation of antimicrobial activity of various fractions of *G. barbadense,* and it was found that the fractions possessed reasonable antimicrobial activities against *Escherichia coli*, *Pseudomonas aeruginosa*, *Staphylococcus aureus* and *Candida albicans* especially the ethanol fraction. Column chromatographic isolation of the most active fraction GEF led to the isolation of stigmasterol — a known compound with antimicrobial activity. The structure of the compound was fully characterized by ^{1}H and ^{13}C NMR as well as FTIR spectroscopic techniques. The spectroscopic data obtained was found to be in agreement with the literature. Thus, the investigations carried out shed light on the traditional applications of *G. barbadense* leaves for the management of some disease conditions.

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CONFLICT OF INTEREST

None.

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