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Isolation and identification of phytosterols from *Anogeissus pendula* (Edgew) and their antimicrobial potency

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Abstract
Phytosterol are significant bioactive molecules present in plant cells. These are similar compounds like cholesterol in structure. The plants have many secondary metabolites in abundance. The pharmacological properties of plants have been attributed due to the presence of phytochemicals in the plant materials. Due to the presence of bio-active chemicals which are present in plants, the pharmacological properties are found in them. *Anogeissus pendula* (Edgew) grows in dry, hot regions of India occurring in the dry tropical forest and deciduous forest of Rajasthan. The present observation showed presence of three sterols namely β-sitosterol, Stigmasterol and Campesterol in the leaves and bark of this plant (respectively in mg/gdw is 1.02, 1.09 and 0.87 in leaves and in bark 0.49, 0.67 and 0.58). Phytosterols isolated from *Anogeissus pendula* showed antimicrobial potential against some pathogenic microbes. The GC-MS profile showed various compounds found in the plant. Leaves of *Anogeissus pendula* (Edgew) showed maximum antibacterial potential against *Streptomyces grisveus* (14 mm) while minimum were recorded against *E. coli* (7 mm) while bark of *Anogeissus pendula* (Edgew) showed resistance against all microbes tested.

Keywords: Phytosterol, *Anogeissus pendula* (Edgew), β-sitosterol, stigmasterol and Campesterol, antimicrobial, GC-MS

Introduction
Phytosterols belongs to the family of lipid present in plant cells, it can be classified as β-sitosterol, stigmasterol and campesterol. However, there are over 200 different kinds of sterols structures that have been discovered in different plant species. Phytosterols are triterpene in nature, play significant role in plant membranes. Phytosterols are structurally similar like cholesterol that act to stabilize phospholipid bilayers in plants [1]. *Anogeissus pendula* Edgew (Family: Comretaceae) commonly known as "Dhok, Dhokra" in Hindi language and in English "Button tree" [2]. *Anogeissus pendula* (Edgew) is a specific species of the Aravalli region of the arid and semi-arid region, rich in fodder, fuel, timber and medicinal properties. It covers more than half of the sum forest area in Rajasthan [3]. It is deciduous gregarious shrub or small tree in nature, it grows in dry and mixed forest. It has maximum height of 9-15 m and 1m in girth [4, 5]. The occurrence of phytochemicals like alkaloidal and phenolic compounds have been reported in stem leaves and fruits [6, 7]. Various Ethanomedicinal properties of *Anogeissus pendula* (Edgew) reported are: leaves and twigs paste is used in swellings [8], bark used in Anemia and dysentery [9, 10].

Materials and Methods
Collection and Identification of Plant Materials
Collection of *Anogeissus pendula* (Edgew) plant for study was made from Jhalana and Nahargarh biological park area of Jaipur in December to march. The plant materials were taxonomically identified and authenticated by Department of Botany, University of Rajasthan (RUBL 211662), Jaipur. The plant materials were cleaned, shade dried and pulverized to powder in a mechanical grinder. The powdered materials were stored in air tight containers till use.

Extraction
Dried and powdered plant materials were defatted in petroleum ether (60-80^0 C) for 24 hours on a water bath. Defatted material were air dried and hydrolyzed in 30% HCl (v/v) for 4 hours. Each hydrolyzed sample was washed with distilled water till pH 7 was achieved and was dried later. The dried preparation was again extracted with benzene for 24 hours. The extract was

filtered and dried *in vacuo*. The crude extract was dissolved in benzene before chromatographic examination [11].

Thin layer chromatography (TLC)

Glass plates coated with silica gels G were used. All of the extracts were co-chromatographed separately with authentic sterols as marker. These plates were developed in an airtight chromatographic chamber, saturated with solvent mixture (Hexane: Acetone, 8:2) [12]. Other solvents such as benzene and ethyl acetate (85:15) [13]. benzene: ethyl acetate (3:1), was also used but hexane: acetone (8:2) gave better separation. These plates were air dried and visualized under UV light and fluorescent spots corresponding to that of standards marker were marked. These developed plates were sprayed with 50% sulphuric acid [14]. and anisaldehyde reagent, separately and heated at 110 ^{0}C for 10 min.

Preparative thin layer chromatography

PTLC was performed using silica gel G coated plates (0.4-0.5mμ) along with the reference markers. These plates were developed in hexane: acetone (8:2), air dried and examined under UV light. Each spot coinciding with that of standard marker was marked, scraped from 50 plates, and eluted with chloroform. The eluted reactions were subjected to crystallization, separately and their melting point, mixed melting point were determined. The isolated compounds were also subjected to UV and IR spectral studies. Melting point and IR spectra of each of the isolated compounds was taken and a comparison of the TLC colour reaction was made, which was found to be in accordance with that of studied authentic compounds.

Gas Chromatography and Mass Spectroscopy (GC-MS)

Preparation of sample

Extraction of various metabolites rich fraction, such as flavonoids, steroids and alkaloids were done using standard protocols mentioned in extraction procedure section. Isolated fractions were filtered with Whatman No.1 filter paper and the residue was removed. It was again filtered through acrodisc syringe filter of having size 0.45mm in order to remove the traces of moisture. Flavonoid fractions were taken up in small volume of ethanol (2-5mL), steroid extracts were taken up in small volume of benzene and alkaloid extracts were taken up in small volume of methanol for GC-MS analysis.

Preparation of Extract

The isolated samples were dissolved in their respective solvent for further studies.

Gas Chromatography and Mass Spectroscopy (GC-MS) analysis

The Gas chromatography-Mass spectrometry (GC-MS) analysis of the extracts was performed using a GC-MS instrument. The GC-MS analysis was conducted at USIC (University Science Instrumentation Centre), University of Rajasthan, Jaipur. The Mass spectrometer used for GC–MS analysis was Thermo GC 1300 and "TSQ 8000 "Triple quadrupole GC-MS SYSTEM with auto sampler Al 1310 under the following conditions: capillary column, TG-5MS (30m × 0.25mm × film thickness 0.25μm); temperature program, 70 °C for 1min, then at 8 °C/min to 270 °C, hold for 1min, injector temperature, 280 ºC; carrier gas, helium, at flow rate of 1.0ml/min. GC-MS analysis was conducted using TSQ8000 with transfer line temperature 280 °C and ion source temperature 230 °C in EI mode. The MS scan parameters included electron impact ionization voltage of 70eV and a mass range of 50–500m/z. The identification of the components was based on the comparison of their mass spectra and retention time with those stored in NIST library or with mass spectra from published literature.

Antimicrobial Activity

Antimicrobial activity of phytosterols from *Anogeissus pendula* (Edgew) was studied against four bacterial and fungal strains for the primary screening.

Microorganisms Used

Clinical laboratory bacterial isolates of *Staphylococcus aureus* (MTCC-3381), *Bacillus subtilis* (MTCC-10619), *Escherichia coli* (MTCC-443) and *Streptomyces grisveus* (MTCC-4734) fungal isolates namely *Aspergillus niger* (ATCC-9029), *Fusarium oxysporum* (ATCC-62506), *Trichoderma reesei* (ATCC-13631) *and Penicillium funiculosum* (ATCC-11797) were collected from the stock cultures of Microbiology Laboratory, SMS Medical College Jaipur, India.

Determination of Antibacterial Assay

In vitro antibacterial activity of the phytosterols were studied against bacterial strains by the agar well diffusion method [15]. Mueller Hinton agar no. 2 (Hi Media, India) was used as the bacteriological medium. The extracts were diluted in benzene at the concentrations of 5 mg/mL. The Mueller Hinton agar was melted and cooled to 48 – 50 ºC and a standardized inoculum (1.5×108 Colony Forming Unit (CFU) /mL, 0.5 McFarland) was then added aseptically to the molten agar and poured into sterile petri dishes to give a solid plate. Wells were prepared in the seeded agar plates. The test compound (100 μl) was introduced in the well (6 mm). The plates were incubated overnight at 37 ºC. The antimicrobial spectrum of the extract was determined for the bacterial species in terms of zone of inhibition around each well. The diameters of zone of inhibition produced by the agent were compared with those produced by the commercially available control antibiotic, ciprotoxin. For each bacterial strain controls were maintained using pure solvents were used instead of the extract. The experiment was performed three times to minimize the error and the means values are presented.

Determination of Antifungal Assay

Anti-fungal activity of the experimental plant was investigated against fungal strain by agar well diffusion method [16]. The fungi strains were subcultured onto potato dextrose agar (PDA) (Merck, Germany) and respectively incubated at 37 °C for 24 hours and 25 °C for 2 - 5 days. The plates were dried at room temperature for 15 min. Wells of 10 mm in diameter and about 6 mm apart were punctured in the culture media using sterile glass tube. 0.1 ml of several dilutions of fresh extracts was administered to fullness for each well. Plates were incubated at 37 °C. After incubation of 24 hours bioactivities were determined by measuring the diameter of inhibition zone (in mm). The diameters of zone of inhibition produced by the agent were compared with those produced by the commercially available antifungal, ketoconazole. All experiments were made in triplicate and means were calculated.

Result and Discussion

Phytosterols

Three sterols were spotted which were common in plant parts

on thin layer chromatography. The R_f values of the spots matched with authentic standards and were identified as β-sitosterol, stigmasterol, and campesterol. Among the various solvent systems tested best results were obtained in the solvent system Hexane: Acetone (8:2) with R_f values viz., β-sitosterol, 0.89; stigmasterol, 0.83; and campesterol, 0.29. The characteristic colours were also developed when TLC plates were sprayed with anisaldehyde reagent (β-sitosterol - pink; stigmasterol - Purple; campesterol - Gray;) and with 50% sulphuric acid (β-sitosterol-Purple brown; stigmasterol- Gray; campesterol- Gray;) corresponding to their authentic samples. The isolated sterols were also identified and characterized with their mp, which also corresponded with those of their respective standards separately (β -sitosterol 136-137ºC; stigmasterol; 167-169ºC; and campesterol-157-158ºC). The characteristic peaks of IR spectra of isolates (β-sitosterol, stigmasterol, and campesterol) were also found to be superimposable with the IR spectra of reference compounds.

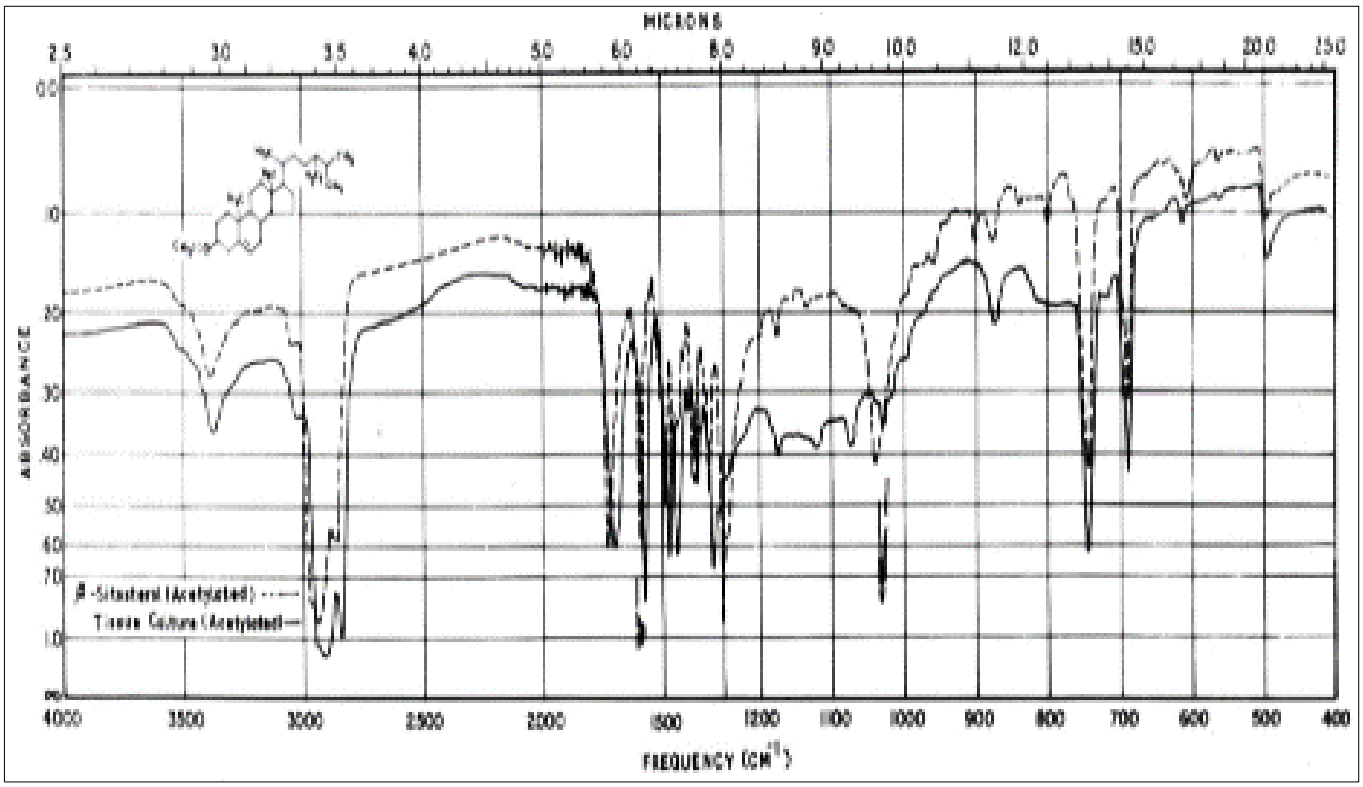

Fig 1: Infra-red Spectra of Isolated and Standard β-sitosterol

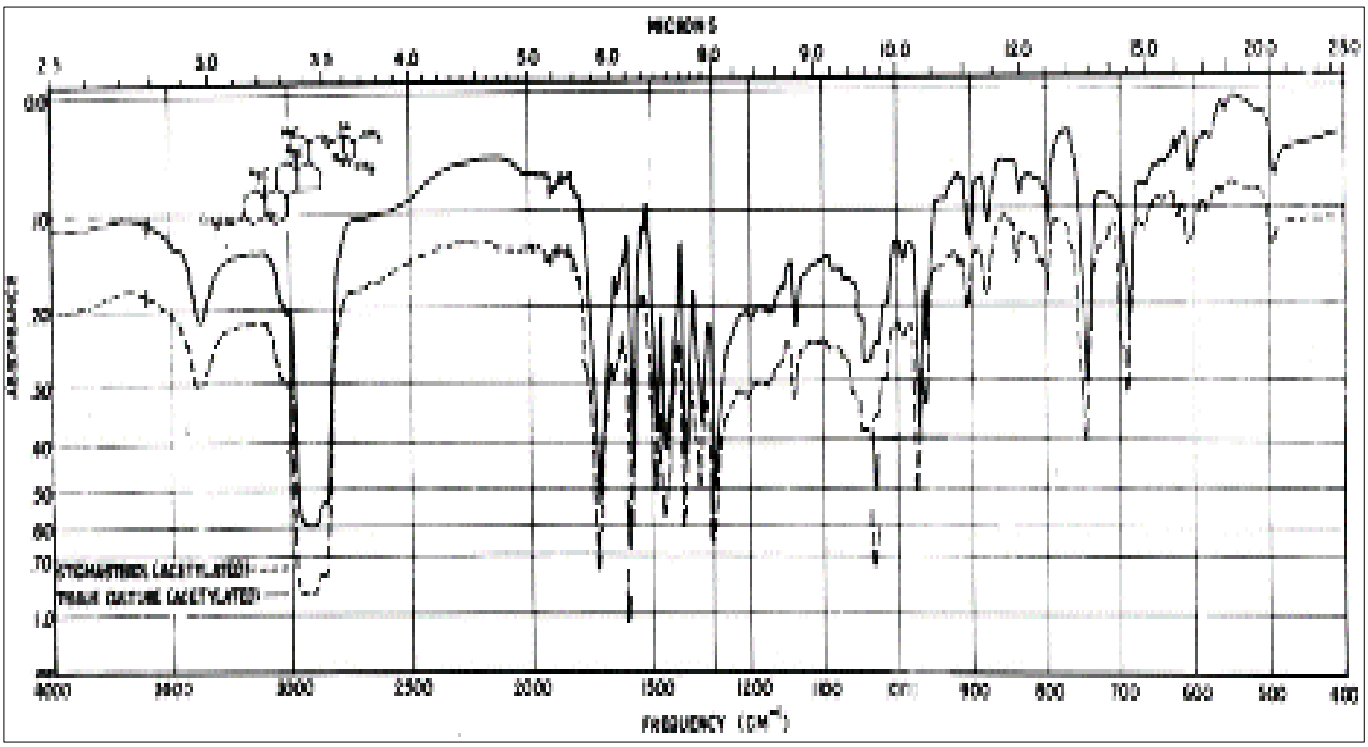

Fig 2: Infra-red Spectra of Isolated and Standard Stigmasterol

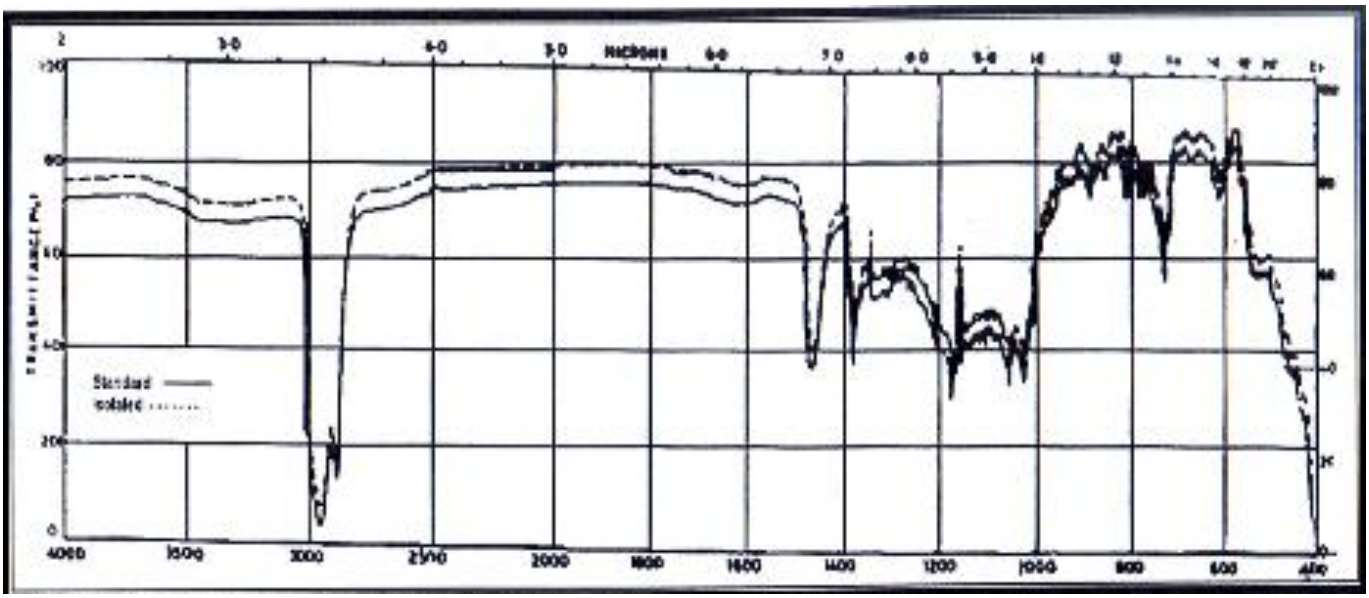

Fig 3: Infrared spectra of standard and isolated Campesterol

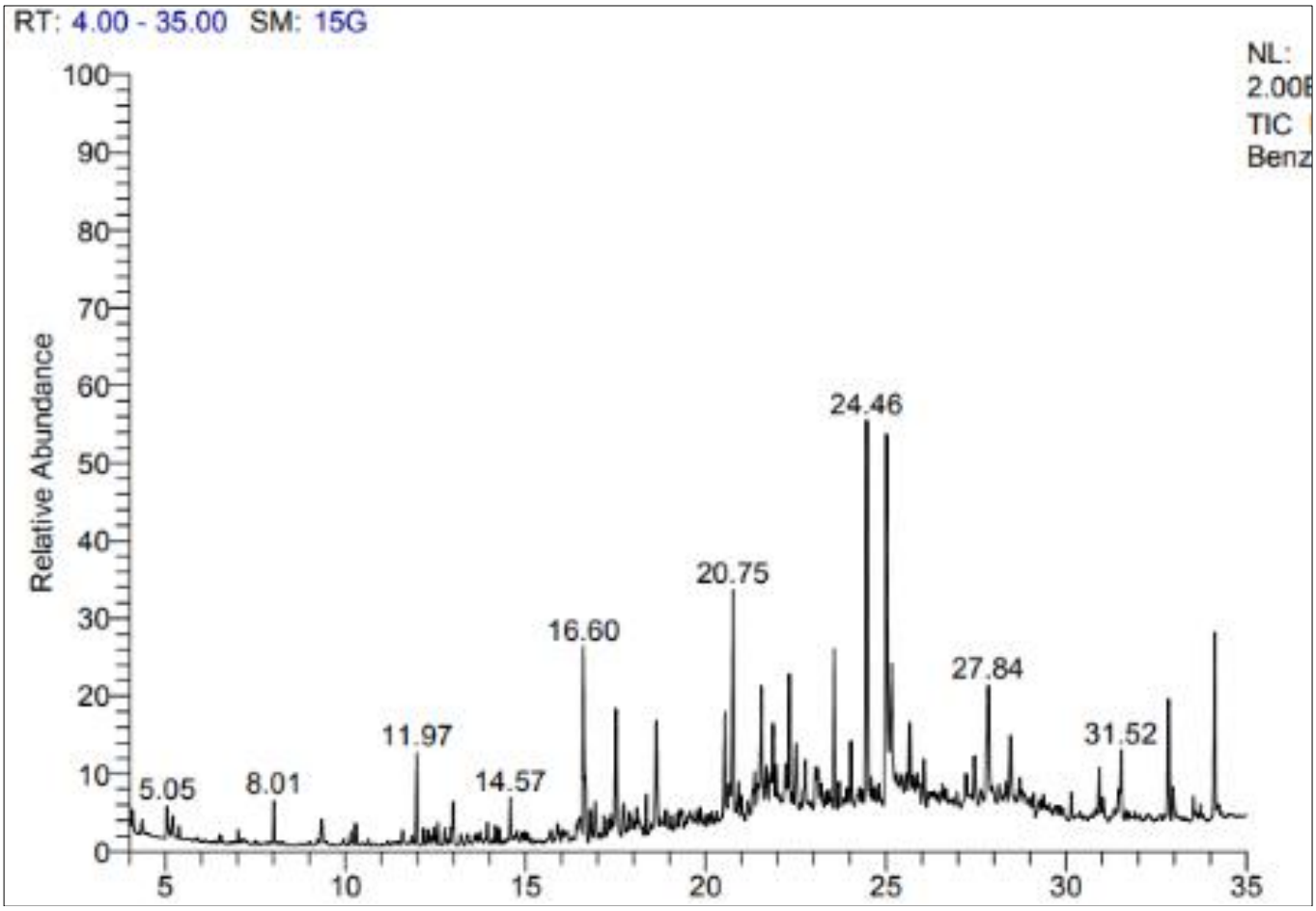

Fig 4: GC-MS profile of phytosterols

Table 1: Chromatographic Behavior and Physico-chemical Characteristics of Isolated Phyto-sterols

Isolated Compounds	Rf Value			Color After Spray		M.P. (ºC)	IR Spectral Peaks (rept.) ν (KBr) cm^{-1}
	S_1	S_2	S_3	R_1	R_2		
β-sitosterol	0.89	0.90	0.71	PU-BN	PK	136-137	3350 (O-H), 2830, 1665 (C=C), 1470, 1300, 1052 (C-O) and 880
Stigmasterol	0.83	0.64	0.65	GY	PU	167-69	3400 (O-H). 2950, 1750, 1640 (C=O), 1035 (C-O), 991, 957, 935, 810 and 715
Campesterol	0.29	0.23	0.21	GY	BL	157-158	3400 (O-H), 2950, 2850, 1640 (C=O), 1470, 1380, 1035, 880 and 820

Abbreviations: S_1 - Hexane : acetone (8 : 2), S_2 - Benzene : acetone (2 : 1), S_3 – Benzene : ethyl acetate (3 : 2),R_1 - 50% H_2SO_4, R_2 - Anisaldehyde reagent, BN - Brown, PK- Pink, PU – Purple, BL – Blue, GY – Gray.

Table 2: GC- MS analysis of compounds in phytosterols extract of leaves of *Anogeissus pendula* (Edgew).

RT (min.)	Compound Name	Area	Area %
5.05	Ethanol, 2-(Methylamino)-	23853141	0.71
8.01	Nonanal	29058141	0.87
11.97	Undecane, 4,7-dimethyl-	58718495	1.75
12.99	Undecane, 4,7-dimethyl-	39645699	1.18
14.57	Tetradecane	33928258	1.01
16.60	Dodecane, 2,6,11-trimethyl-	176668345	5.28
16.79	Hexadecane	26219489	0.78
16.92	Phenol, 2,4-bis(1,1-dimethylethyl)-	34782483	1.04
17.50	Hexadecane, 2,6,11,15-tetramethyl-	115811554	3.46
17.70	Oxalic acid, 6-ethyloct-3-yl heptyl ester	35466992	1.06
18.08	Sulfurous acid, butyl decyl ester	21880299	0.65
18.34	Dodecane, 2,6,11-trimethyl-	28302620	0.85
18.63	Hexadecane	113860082	3.40
20.52	Heptadecane	68915596	2.06
20.64	2,2-Dimethyl-propyl 2,2-dimethyl propanesulfinyl sulfone	20872547	0.62
20.75	Sulfurous acid, butyl nonyl ester	170678729	5.10
20.90	Eicosane	25946227	0.78
21.35	Sulfurous acid, butyl nonyl ester	45206691	1.35
21.53	Oxalic acid, isohexyl neopentyl ester	102061559	3.05
21.68	2-methyltetracosane	27293184	0.82
21.80	2-Hexyl-1-octanol	26329761	0.79
21.86	2-Methyl-3-(-3methyl-but-2-enyl)-2-(4-methyl-pent-3-enyl)- oxetane	54930072	1.64
21.95	Oxalic acid, 6-ethyloct-3-yl-isohexyl ester	25685324	0.77
22.22	2-Cyclohexen-1-one,4-hydroxy-3,5,6-trimethyl-4-(3oxo-1-butenyl)-	36876767	1.10
22.31	Hexadecane	88028855	2.63
22.50	Eicosane	57166746	1.71
22.76	Isopropyl myristate	48852822	1.46
23.05	Oxalic acid, 6-ethyloct-3-yl heptyl ester	31821966	0.95
23.11	Sulfurous acid, butyl nonyl ester	33658382	1.01
23.56	Phthalic acid, hex-3-yl isobutyl ester	112913964	3.37
23.69	2-Hexyl-1-octanol	20745496	0.62
24.02	Hexadecane	49744078	1.49
24.46	Nonadecane, 2-methyl-	286405825	8.56
24.60	Sulfurous acid, butyl nonyl ester	14439453	0.43
25.01	n-Hexadecanoic acid	326521230	9.75
25.16	Eicosane	144724225	4.32

In present investigation leaves of *Anogeissus pendula* (Edgew) showed potent antibacterial activity. Leaves showed maximum potential activity against *Streptomyces grisveus* (14 mm) while minimum were recorded against *E.coli* (7 mm) and rest of bacterial strains were found to be resistant in bark all four type of bacterial strain were found to be resistant.

Table 3: Antibacterial activity of phytosterols isolated from *Anogeissus pendula* (Edgew).

S. No	Name of Bacterial strain	Leaves (Zone in mm)	Bark (Zone in mm)
1	*Bacillus subtilis*	8	Nil
2	*Staphylococcus aureus*	Nil	Nil
3	*Escherichia coli*	7	Nil
4	*Streptomyces grisveus*	14	Nil

I n present observation leaves of *Anogeissus pendula* (Edgew) showed potent antifungal activity. Leaves showed maximum potential activity against *Aspergillus niger* (14 mm) while minimum was investigated against *Trichoderma reesei* (7 mm) but *Penicillium funiculosum* strain was found resistant. In bark extract all four type of fungal strain were found resistant.

Table 4: Antifungal activity of phytosterols isolated from *Anogeissus pendula* (Edgew).

S. No.	Name of fungal strain	Leaves (Zone in mm)	Bark (Zone in mm)
1.	*Trichoderma reesei*	7	Nil
2.	*Fusarium oxysporum*	8	Nil
3.	*Penicillium funiculosum*	Nil	Nil
4.	*Aspergillus niger*	14	Nil

Discussion

Present investigation showed β-sitosterol, stigmasterol and Campesterol were present in *Anogeissus pendula* in the leaf and bark extracts similar results were obtained by Maima *et al.* (2008) [17]. Results from the present studies are in correlation with the studies made by Mann *et al.* (2015), Elsidding *et al.* (2015), Timothy *et al.* (2015) and Alhassan *et al.* (2016) [18, 19, 20, 21] they have also observed the potential

effect of *Anogeissus leiocarpus* against pathogenic microorganisms such as *Staphylococcus aureus*, *Klebseilla* species, *Candida albicans*, *E. coli*, *Shigella dysenteriae*, *Aspergillus niger* and *Pseudomonas aeruginosa*.

Conclusion

The extracts from *Anogeissus pendula* in this study proved significant bioactive components against some of the test pathogenic organisms. The isolate from the plant had more activity against the most of the selected pathogens. Therefore, the plant is a potential member for novel drug development for the treatment of diseases caused by these pathogens.

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