ISSN 0974 – 5211

Journal of Natural Products
(Only ON-LINE, ANNUAL, Science Journal published from INDIA)
Volume 10 (2017)
www.JournalofNaturalProducts.com

Research Paper

Isolation and characterization of Stigmasterol from *Xylocarpusgranatum*and Anti-microbial activity Study

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(Received 04 Dec..2016; Revised 22 March-10April 2017; Accepted 24 April 2017; On-lined 06 August 2017)

ABSTRACT

Isolation and characterization of Stigmasterol from the fruit of *Xylocarpusgranatum*. The structure of the compound was established on the basis of spectroscopic data, especially UV, IR, ^{1}HNMR, ^{13}C-NMRand 2D-NMR. To the best of our knowledge, this is the first time Stigmasterol was isolated as a natural product from this plant and Anti-microbial activity study also observed.

Key Words: *XylocarpusGranatum;* Stigmasterol; Antimicrobial activity

INTRODUCTION

Xylocarpusgranatum a marine mangrove plant, is a source of structurally unique limonoids and recycled as a folk medicine in Southeast Asia for the treatment of diarrhoea, cholera, and fever-causing diseases such as malaria (Jianxin Cui, 2007,2009; Wu YB, 2015; Jun Wu, 2006). Limonoids which have been found to date only in plants of the order Rutile's, are tetra-nor-tri-terpenoids with aβ-furyl ring substituent located at C17 that is derived from a 4,4,8-trimethyl-17-furanyl steroid skeleton. They are classified by the ring structure in the intact tri terpene core unit, and these are usually oxidized and designated as A, B, C and D. The mangrove *Xylocarpusgranatum*, is known to produce anti-feedentlimonoids, especially phragmalins and mexicanolides. Previous investigations on the seeds of two Meliaceae plants, the mangroves, *X.granatum* and *X.moluccensis*, uncovered one obacunol, two phragmalins, three andirobins, and 14 mexicanolides, including the Xyloccensins A-K(Jun Wu, 2008).*X.granatum*is a rich source of structurally unique limonoids. Their structures can be classified into phragmalin, mexicanolideanfandirobin-types based on the ring systems. All naturally occurring citrus limonoids contain a furan ring attached to the D-ring, at C-17, as well as oxygen –containing functional groups at C-3, C-4, C-7, C-16 and C-17. Limonoids have been found in all *Xylocarpus* plants investigated so far. The triterpenes are supposed to be biogenetically derived from a 4,4,8-trimethyl-17-furanyl steroid skeleton. A previous chemical investigation of *X.granatum*, growing in a different

mangrove area, resulted in the isolation and characterization of more than 40 limonoid type derivatives (Jianxin Cui, 2008).

The isolation and identification of novel mexicanolides has been recently reported(Jun Wu, 2007, 2006, 2005). 9-hydroxy-3-methoxy-6H-pyridol[1,2-a] pyrazine-6-one, a new pyridol[1,2-a] pyrazine alkaloid named Xylogranatinin, was isolated from the Chinese mangrove *Xylocarpusgranatum* (Yuan Zhou, 2007). Research on limonoids from the Meliaceae family is of interest due to their wide range of biological activities, such as insect antifeedant and growth regulator, antibacterial, antifungal, antimalarial, anticancer and antiviral activities.The mangroves *Xylocarpusgranatum* is known for producing antifeedantlimonoids, especially phragmalins and mexicanlodes(Yuan Zhou, 2007, Khisal A, 1991).

In the current paper, we present the isolation and characterization of Stigmasterol isolated from the fruit of *Xylocarpus*.granatum. The structure of the compound was established on the basis of spectroscopic data,especially UV, IR, ^{1}HNMR, ^{13}C-NMRand 2D-NMR.To the best of our knowledge, this is the first time Stigmasterol was isolated as a natural product from this plant and Anti-microbial activity study also observed.

MATERIALS AND METHODS

Melting points were determined on a VEB-Analytic Dreader HMK hot plate and are uncorrected. IR spectra were reported on a Perkin-Elmar 841 IR Spectrometer in $CHCl_3$ solution. UV spectra were recorded on a Milton Roy Spectronic 1201 spectrometer in $CHCl_3$. ^{1}H NMR spectra were measured on a Bruker Advance DRX 600 and JEOL JNM EX-90 spectrometers. ^{13}C NMR spectra were measured on a Bruker Advance DRX 600 spectrophotometer at 400 MHz and JEOL JNM EX-90 spectrophotometer at 22.5 MHz using $CDCl_3$ as a solvent and tetra-methyl Selane as an internal reference. Mass spectra were obtained on a JEOL JMS-300 spectrometer.

***Plant material*:** The underlying foundations of *Xylocarpus* were tranquil from Corangi Mangrove backwoods close Bhiravapalem of Godavari Estuary (160 58' N scope and 820 15' E longitudes) in March 2002 and was set up by Prof.B.KondalaRao, Dept.of Marine Living Sources, Andhra University, and Visakhapatnam. Voucher examples (Code: AU 1/60) have been saved at the Marine Museums of School of Chemistry, Andhra University and National Institute of Oceanography, Goa.

Extraction, Fractionation and Isolation Procedures:The powdered fruit material (6kg) of *Xylocarpusgranatum*was extracted with n-hexane for 24 hours the supernatant was filtered. The extract was then left to dry under room temperature. The quantity extracted was 35g. The crude extract was subjected to Column Chromatography using silica gel (100-200 mesh) as a stationary phase. Gradient elution was applied to the column using mixture solvent of hexane,EtoAc (0%-100%). The total volume used was 300ml to 120 fractions were collected and left to dry at room temperature. Fractions were mixed according to TLCs analysis. The use of concentrated sulphuric acid and vanillin as a spraying reagent revealed the presence of sterol from fraction 6 (650mg). The white power crystallized from fraction 6 was named compound 1.

RESULTS AND DISCUSSIONS

Different spectroscopic methods were applied to elucidate the structure of isolated compound (1), Including: IR, ^{1}HNMR and ^{13}C NMR.

The melting point of Compound (1) was 165^0C; the UV λ_{max}value of Compound (1) was 257nm. Mas Spectrum of Compound (1) showed parent molecular ion [M^+] peak at m/z 412 which corresponds to the molecular formula**$C_{29}H_{48}O$.**

In IR Spectrum of Compound (1) a very intensely broad band at 3584.46 cm^{-1} and moderately intense band at 1371 cm^{-1} and 780 cm^{-1} were observed for the OH bond vibrations of hydroxyl group. The out of plane CH-vibrations of the unsaturated part was observed at 890cm^{-1}. The corresponding C=C vibrations was shown around 1648 cm^{-1} as weakly intense band. The stretching and bending vibrations of methyl part were noticed by the intense band 3113 $cm^{-1}$and medium intensity band at 1502cm^{-1}. The vibrations of methylenic part was shown by the band at 2867 cm^{-1} and the medium band at 1450 cm^{-1}.The moderately intense band at 730 $cm^{-1}$was attributed to the rocking movement of methylenicpart. The corresponding C-C vibration was shown as weak intense band at 1055 cm^{-1}.

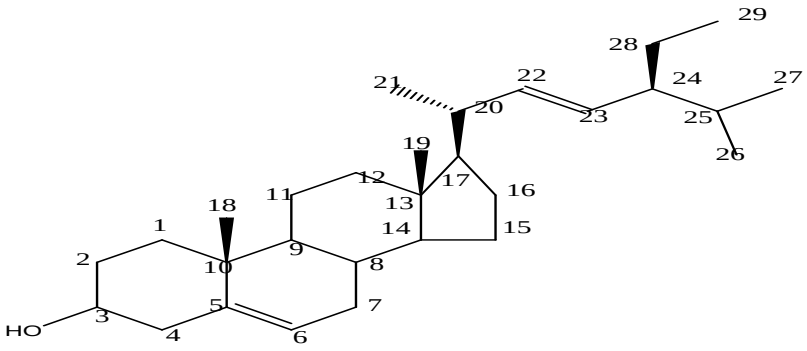
In ^{1}HNMR spectrum of Compound (1) H-3 proton appeared as a multiplet atδ 3.6 (J=4.5 and 1.1 MHz) and H-6 olefin proton showed a multiplied at δ 5.4. Two olefinic protons appeared downfield at δ 5.1 (m) with chemical shift of H-22, respectively of Stigmasterol. Methyl protons also appeared at δ 1.2 (d, 3H), δ1.2 (d,3H) and δ 0.74 (d,3H).

The ^{13}C NMR spectrum of Compound (1) given signal at 140.7 ppm and 11.8 ppm for double bond respectively, 77.3ppm for C3 β–hydroxyl group, 19.0ppm and 12.2ppm for angular methyl Carbon atoms for C19 and C18 respectively.138.2 ppm for C-22 and 129.3 ppm for C-23. The C5, C6, C22 and C23 appeared to be alkane Carbons.

2D COSY (400 MHz, $CDCl_3$): δ Cross Peaks at 5.4 (S, 1H, H-6), 5.2 (m, 1H, H-23), 5.1 (m, 1H, H-22), 3.6 (m, 1H, H-3), 1.2 (d, 3H, H-18), 1.2 (d, 3H, H-21), 1.0 (t, 3H, H-29), 0.9 (d, 3H, H-27), 0.8 (d, 3H, H-26), 0.7 (d, 3H, H-19).

These assignments are in good agreement for the structure of Stigmasterol.

The ^{1}H and ^{13}C NMR values for all the protons and carbons were assigned on the basic of COSY were given in Table-2.



COMPOUND (1)

Figure 1: Chemical Structure of Stigmasterol

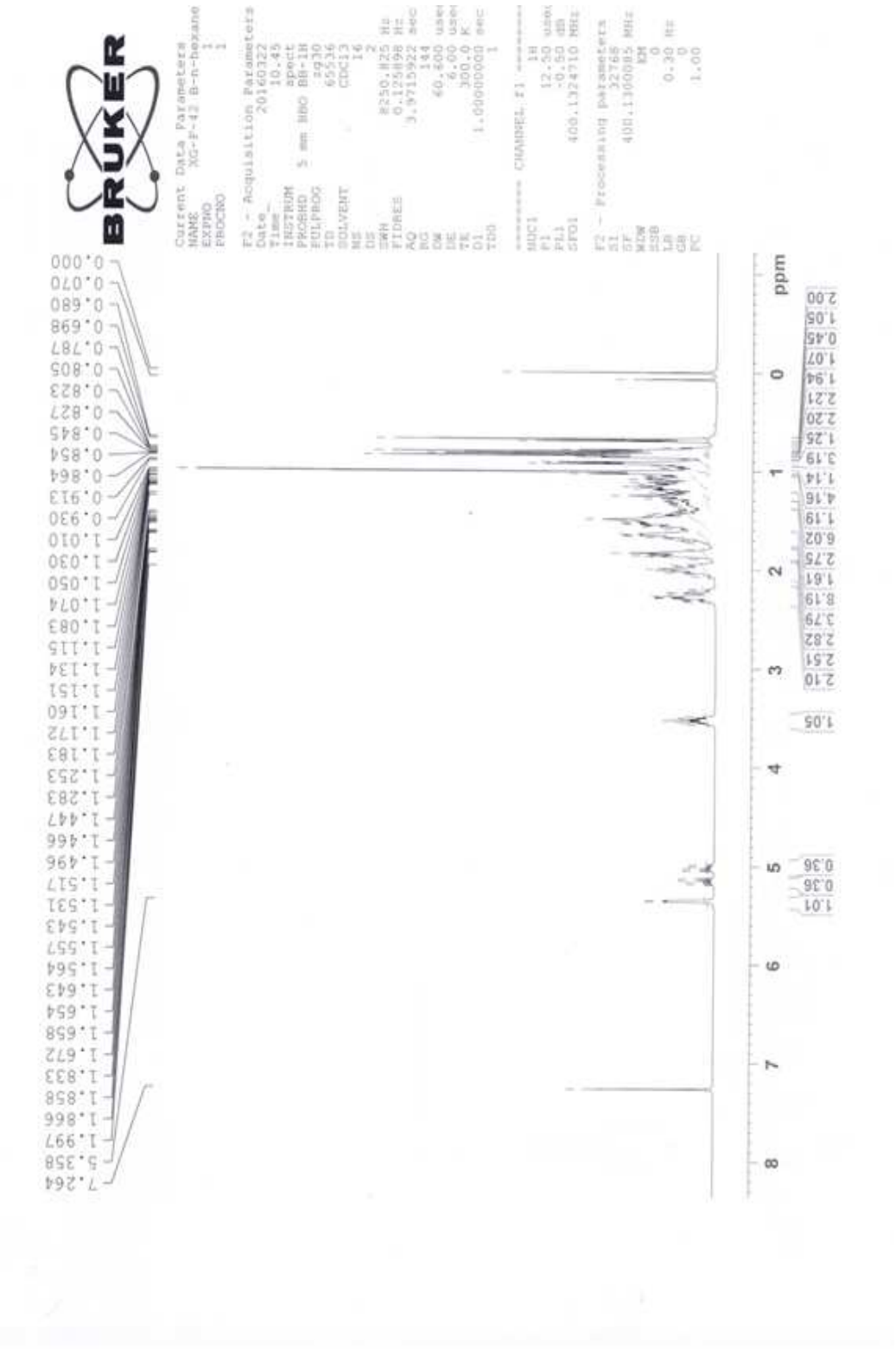


Figure-2: ^{1}H NMR Spectrum of Compound (1)

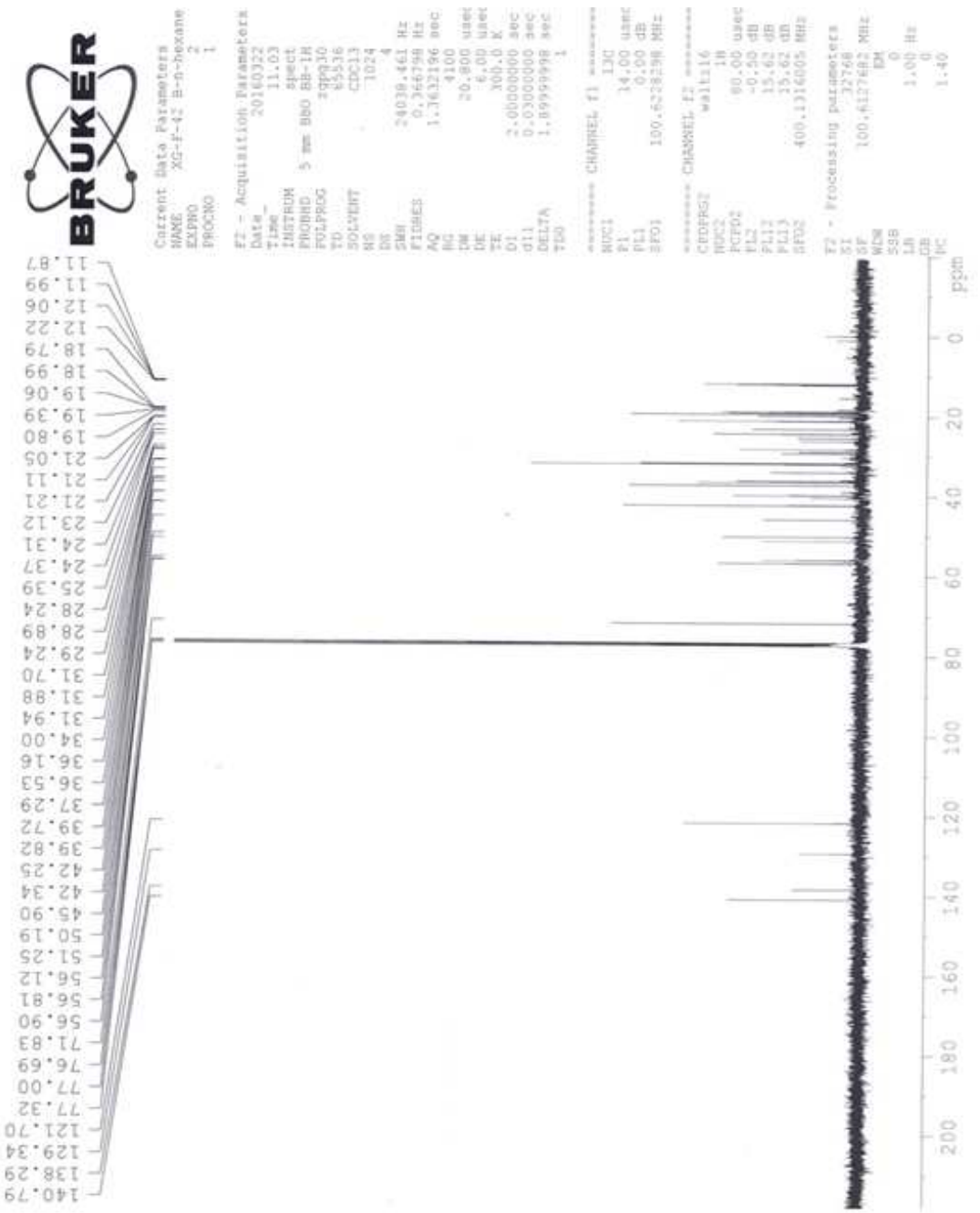


Figure-3: ^{13}C NMR Spectrum of Compound (1)

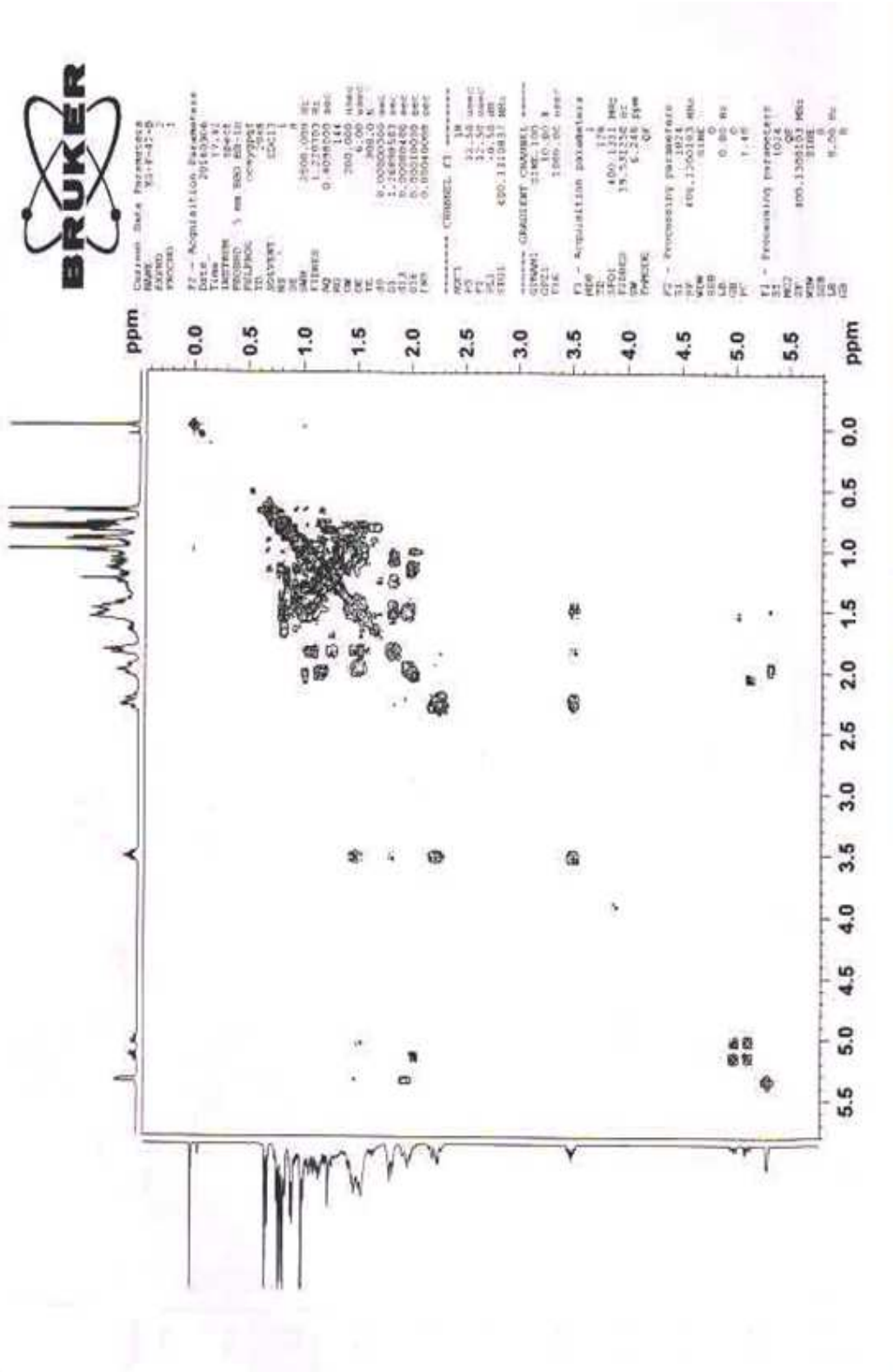


Figure-4: ^{13}C NMR Spectrum of Compound (1)

Table-1: ^{1}H and ^{13}C NMR Chemical Shift Values for Compound (1).

CARBON ATOM	^{1}H NMR	^{13}C NMR	NATURE OF CARBON
C-1		36.5	CH_2
C-2		29.2	CH_2
C-3	3.6(m,1H)	71.8	CH
C-4		42.2	CH_2
C-5		140.7	C=C
C-6	5.4(s,1H)	121.7	C=CH
C-7		31.8	CH_2
C-8		29.2	CH
C-9		50.1	CH
C-10		36.1	C
C-11		24.3	CH_2
C-12		39.7	CH_2
C-13		40.4	C
C-14		56.9	CH
C-15		25.3	CH_2
C-16		28.8	CH_2
C-17		56.0	CH
C-18	1.2(d,3H)	12.2	CH_3
C-19	0.74(d,3H)	19.3	CH_3
C-20		39.8	CH
C-21	1.2(d,3H)	23.1	CH_3
C-22	5.1(m,1H)	138.2	C=C
C-23	5.2(m,1H)	129.3	C=C
C-24		51.2	CH
C-25		34.0	CH
C-26	0.8(d,3H)	21.2	CH_3
C-27	0.9(d,3H)	22.8	CH_3
C-28		25.3	CH_2
C-29	1.0(t,3H)	12.0	CH_3

Anti-microbial Activity

Microbial Strains:*Bacillus puvuilis, Bacillus subtilis, Bacillus coagulans, Staphylococcus aureus, Bacillus licheniformis, Corynebacterium diphtheria, Klebsiella pneumonia, Pseudomonas aeruginosa, Shigellaflexneri, Sphingomonaspaucimobilis, Escherichia coli and Vibrio cholera.*

Agar Disc Diffusion Method:The agar disc diffusion method was employed for the determination of antimicrobial activities of the extracts according to some modification. Briefly, inoculum containing 120^{0}C was spread on nutrient agar medium with the respective bacterial strains of bacteria and medium potato dextrose agar for fungus strains. Testing sterile forceps, the sterile filter papers (6mm diameter) containing the crude extracts (1 or 1.5mg), standard antibiotics (30µg of chloramphenicol or 100µg of amphotericin B) or negative control (DMSO) were laid down on the coverage of inoculated agar plate. The plates were incubated at 37±2℃ for 24 h for the bacteria and at room temperature 28±2℃ for 12h for yeasts strains. Each sample was tested in duplicate and the zone of inhibition was measured as 50 micro litters diameter.

Screening for Anti-microbial Activity:The antimicrobial activity was carried out by the employing 24h young cultures with the given compounds by using Agar-well diffusion method. The medium was sterilized by autoclaving at 120^{0}C (15lb/in2).

About 20ml of the medium (Nutrient Agar Medium) with the respective bacterial strains of bacteria and medium (Potato Dextrose Agar) for Fungal strains were transferred aseptically into each sterilized petri Plate. The plates were left at room temperature for solidification. Each plate is made 5 wells with equal distance with of 6mm sterile borer. The test compounds were freshly reconstituted with suitable solvents (DMSO) and tested at various concentrations. The samples and the control along with standard (Ciprofloxacin) were places in 6-mm diameter well in Antimicrobial assays. Plates were incubated at 28±2□ for fungi for 24h and 37±2□ for bacteria for 12h. Standard with 5µg/ml used as a positive control for antibacterial activity. Activity diameter of the zone of inhibition was measured using Himedia antibiotic zone scale. Observations and results were represented in Table-2.

Table-2: Results of Anti.microbial Activity in *Xylocarpusgranatum*.

Gram Positive Bacteria's

S.No	Plant code	Organism/s	500mg/ml	250mg/ml	100mg/ml	Standard	Control
1	XG	*Bacillus puvuilis*	24mm	20mm	19mm	43mm	No Activity
2	XG	*Bacillus subtilis*	No Activity	No Activity	No Activity	40mm	No Activity
3	XG	*Bacillus coagulans*	No Activity	No Activity	No Activity	40mm	No Activity
4	XG	*Staphylococcus aureus*	No Activity	No Activity	No Activity	40mm	No Activity
5	XG	*Bacillus licheniformis*	15mm	11mm	No Activity	32mm	No Activity
6	XG	*Corynebacteriumdiphtheriae*	17mm	14mm	No Activity	34mm	No Activity

Gram Negative Bacteria's

S.No	Plant code	Organism/s	500mg/ml	250mg/ml	100mg/ml	Standard	Control
1	XG	*Bacillus puvuilis*	17mm	13mm	10mm	28mm	No Activity
2	XG	*Bacillus subtilis*	18mm	No Activity	No Activity	28mm	No Activity
3	XG	*Bacillus coagulans*	No Activity	No Activity	No Activity	32mm	No Activity
4	XG	*Staphylococcus aureus*	16mm	13mm	12mm	37mm	No Activity
5	XG	*Bacillus licheniformis*	19mm	No Activity	No Activity	28mm	No Activity
6	XG	*Corynebacteriumdiphtheriae*	16mm	13mm	12mm	40mm	No Activity

- Diameter of the well = 06mm; Volume of the Compound = 50 Micro liters

Figure-5: Anti-microbial activity of Compound (1)

The antimicrobial activity of the Compound (1) for strains: *Bacillus puvuilis, Bacillus subtilis, Bacillus coagulans, Staphylococcus aureus, Bacillus licheniformis, Corynebacterium diphtheria, Klebsiella pneumonia, Pseudomonas aeruginosa, Shigellaflexneri, Sphingomonaspaucimobilis, Escherichia coli and Vibrio cholera.* The Gram Positive Bacteria's are *Bacillus puvuilis, Bacillus licheniformis* and *Corynebacterium diphtheria* these strains with the values 24,20,19,15,11,17 and 14 respectively.*Bacillus subtilis, Bacillus coagulans and Staphylococcus aureus* these strains are no activity. The Gram Negative Bacteria's are *Klebsiella pneumonia, Pseudomonas aeruginosa*these straines with the values 17,13,10 and 18.*Sphingomonaspaucimobilis, Escherichia coli and Vibrio cholera these* strains with the values 16, 13,12,19,16,13and 12respectively.*Shigellaflexneri*has no activity.

CONCLUSIONS

The compound isolated from the root of *Xylocarpusgranatum* was given chemical structure **Compound (1)** based on the spectral analysis. Further work is in progress to obtain suitable derivatives and followed by Single Crystal X-RAY analysis.

Acknowledgements: We are thankful to Department of Chemistry K L University, Andhra Pradesh, India for making available the necessary facilities.

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