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Original Article

ATOMIC FORCE MICROSCOPIC STUDY FOR THE ANTIBACTERIAL STUDY OF *GARCINIA XANTHOCHYMUS* HOOK. F. LEAF EXTRACT

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ABSTRACT

Objective: The present study was undertaken to study the antibacterial effect of *Garcinia xanthochymus* Hook. f. (Clusiaceae), leaf extract against *Staphylococcus aureus, Bacillus cereus, Escherichia coli* and *Pseudomonas aeruginosa* using atomic force microscope.

Methods: Antibacterial study was done by disc diffusion and minimum inhibitory concentration. Atomic force microscopy study was carried to find out morphological changes in bacterial cells.

Results: Among the tested extracts (petroleum ether, chloroform and methanol), methanol extract inhibited the growth of *Staphylococcus aureus, Bacillus cereus* and *Escherichia coli*. The minimum inhibitory concentration varied from 25 to 50 µg/ml. The atomic force microscope was used to study the morphological alterations induced by the leaf extracts in *S. aureus, B. cereus* and *E. coli*. Reduction in the cell size, the formation of clusters, indicating the maximum damage in *S. aureus* when treated with leaf methanol extract, whereas cell rupture and surface roughness were observed in *B. cereus* and *E. coli*. *P. aeruginosa* was susceptible to leaf extracts of *G. xanthochymus*.

Conclusion: The methanol extract of *Garcinia xanthochymus* leaf extract was found to be the most effective antibacterial property against *S. aureus, B. cereus* and *E. coli*.

Keywords: *Garcinia xanthochymus*, Antibacterial activity, Atomic force microscopy, Phytochemical study.

INTRODUCTION

There is a continuous and an urgent need to discover new antimicrobial compounds with diverse chemical structures and novel mechanism of action because there has been an alarming increase in the incidence of new and re-emerging infectious diseases. Another big problem is the development of resistance to the antibiotics in current clinical use [1]. The use of traditional medicine has expanded globally during the last decade and it has being continued to use, due to its primary health care of the poor in developing countries and also in countries where conventional medicine is predominantly used in the national health care system. About 80% of the world's population's health depending on herbal medicine, especially rural areas peoples of developing countries [2]. Atomic force microscopy (AFM) is a form of scanning probe microscopy developed recently for imaging the fine surface structures of various types of specimens at high-resolution [3]. It provides three-dimensional views and cross sections of microbial cells at any point, with simultaneous measurement of their sizes [4]. Compared to epifluorescence microscopy (EFM) and electron microscopy (EM) pre-treatment, such as fixation, staining or metal coating is not required. Atomic force microscopy were used study the flagella [5] and morphological changes [6] of bacteria.

Garcinia xanthochymus Hook. f. (Clusiaceae), commonly known as yellow gamboge, is a tree endemic to India. The fruits of this plant are used in traditional medicine for treating diarrhea, dysentery, dispelling worms, clearing away fire and removing food toxin [7]. Plants belonging to the Clusiaceae family are rich sources of xanthones, biflavonoids and benzophenones [8]. These compounds present in *G. xanthochymus* have shown a variety of bioactivities, including cytotoxicity [9], antimicrobial [4, 10], antioxidant [8, 12], antiviral [13] and antimalarial activity [14]. The present work carried to study the effect of *Garcinia xanthochymus* leaf extracts on *S. aureus, B. cereus, E. coli* and *P. aeruginosa* bacteria using an atomic force microscope.

MATERIALS AND METHODS

Plant material and sample preparation

Garcinia xanthochymus Hook. f. (Clusiaceae) leaves collected from forests near Siddapur of Uttar Kannada district, Karnataka, India. The plant identification was done by Dr. M. Jayaraj and a voucher specimen (KU/BOT-213) deposited in the Herbarium, Department of Botany, Karnatak University, Dharwad, India. The leaves were washed with water, dried under shade for two weeks, grounded into a fine powder by a mechanical grinder and stored in airtight bottles. The dried seed powder (50 g) extracted using 200 ml of petroleum ether till the colourless solvent runs; the same powder, dried and subsequently used for chloroform and methanol extraction using a soxhlet extractor. The solvents removed completely under vacuum; the extracts thus obtained were stored at 4 °C till used for further analysis.

Bacterial strains and growth conditions

In this study, two gram positive bacterial strains, *Staphylococcus aureus* (NCIM 5021), *Bacillus cereus* (NCIM 2106) and two gram negative bacterial strains, *Pseudomonas aeruginosa* (NCIM 5029) and *Escherichia coli* (NCIM 2931) strains were used to test the antibacterial activity of *G. xanthochymus* leaf extracts. Bacterial strains were obtained from National Chemical Laboratory, Pune.

Antibacterial activity

Antibacterial activity was tested using a modified disc diffusion method [15]. 100 µl of bacterial culture were spread on the nutrient agar plates. Sterile filter paper discs (6 mm in diameter) impregnated with 10 µl the plant extract (50 mg/ml) were placed on the cultured plates and incubated at 37 °C. The Dimethyl sulfoxide (DMSO) without extracts served as negative control standard gentamicin (30 µg/Disc) was used as positive control. After 24 h of incubation, the diameter in mm of clear zones around the discs was recorded.

Determination of minimum inhibitory concentration

The broth microdilution method of minimum inhibitory concentration was determined [15]. Bacteria were grown in nutrient broth until the number of bacteria reaches 0.2 OD at 600 nm. 10 µl of plant extract dissolved in DMSO, gentamicin and control DMSO was pipetted into corresponding wells containing 190 µl of nutrient broth and twofold serially diluted. The concentrations used for plant extracts were in the range of 1.56 to 200 µg/ml while the

concentrations used for gentamicin were in the range of 0.78 to 50 µg/ml. Five microliter of inoculum was pipetted into each corresponding well of a 96-well plate. After 24 h, the optical density at 600 nm of each well was recorded using a multi mode plate reader (Tecon, Switzerland).

Atomic force microscopy

The bacteria which were sensitive to the leaf extracts were used for the AFM study. One hundred microliter of the suspension containing log-phase bacterial cells (OD at 600 nm=0.2) cultured in nutrient broth medium were centrifuged at 4000 rpm for 5 min in 4 °C. The cells were gently washed with 500 µl of sterile 20 mM phosphate buffer of pH 6.8 and centrifuged to collect bacterial pellets. Then the bacteria were incubated for 24 h without (control) and in the presence of plant extract at 28 °C in 20 mM phosphate buffer of pH 6.8 in a final volume of 550 µl. After centrifugation, the bacterial pellet was gently washed thrice with 500 µl of sterile double distilled water. After the final centrifugation, the bacteria were suspended in 10 µl of sterile double distilled water. The samples were applied to a surface of the clean microscopic slide and allowed to dry overnight at 28 °C before imaging by atomic force microscopy [16]. All measurements in contact modes were carried out by using a Nanosurf Easy Scan 2 AFM with a maximum scan range of 10 µm × 10 µm for *x, y* and 3 µm for *z*-axis. A rectangular Si cantilever/tip (Tap 190 ALG) with a spring constant of 48 N/m and resonance frequency of 190 kHz was used. A resolution of the obtained scans was 256 × 256 pixels at scan rate 0.7 Hz. Two kinds of images: a topography image and a deflection (a contact mode) or amplitude (a tapping mode) image was obtained simultaneously. From the topographic images of both treated and untreated cells, mean length, width and mean height of each cell were measured using the imaging software (Nanosurf Easy Scan 2, Switzerland). In each sample (control as well as treated), an average of 5 cells were imaged to find out the effect of plant extracts on cell surface morphology.

RESULTS AND DISCUSSION

Antimicrobial study

Methanol extracts of leaf showed good antibacterial activity against *S. aureus* (15.33±0.33 mm*)*, *B. cereus* (17.33±0.88 mm) and *E. coli* (12.00±0.57 mm), whereas the petroleum ether, chloroform extract of leaf and DMSO did not show any activity against all the tested bacteria (table 1). The MIC values obtained in this study from all the extracts tested ranged from 1.56 to 200 µg/ml for broth dilution method. The MIC value of 25 µg/ml was observed against *S. aureus* and *B. cereus*. highest and of 50 µg/ml were observed against *E. coli* (table 1).

The highest antibacterial activity of *G. xanthochymus* was observed for *B. cereus* with a mean diameter of inhibition of 17.33±0.88 mm followed by *S. aureus* (15.33±0.33 mm) and *E. coli* (12.00±0.57 mm). The MIC values obtained in this study from all the extracts tested ranged from 1.56 to 200 µg/ml for broth dilution method. The highest MIC value of 50 µg/ml was observed against *E. coli* where as a lowest MIC value of 25 µg/ml was observed against *S. aureus* and *B. cereus*. The petroleum ether and chloroform extract did not inhibit the growth of any tested bacteria. None of the extracts showed MIC against *P. aeruginosa* (table 1). According to the MIC studies of the *G. xanthochymus* leaf extracts, the *S. aureus* and *B. cereus* were highly sensitive to the leaf extracts of *G. xanthochymus* followed by *E. coli* and *P. aeruginosa*. This may be due to the cell wall nature of the Gram-negative bacteria which is different from that of Gram-positive bacteria, *S. aureus* and *B. cereus* in having a lipopolysaccharide layer. The methanol extract of the leaf was more effective against *S. aureus* and *B. cereus* among all the tested bacteria, but *P. aeruginosa* was resistant to all the tested extracts (table 1). Extensive phytochemical studies have shown that a variety of oxygenated and prenylated xanthones are rich in *Garcinia* spp [17]. Since xanthones have phenolic functional groups, they show a wide range of biological activities including antimicrobial activities [18].

There are some reports on the antibacterial effects of *G. xanthochymus* plant extracts, Manohar *et al.* [10] reported the inhibitory effects of *G. xanthochymus* seed oil over *Staphylococcus aureus, Bacillus subtilis, Micrococcus spp*, and *Staphylococcus epidermidis* and Tandon *et al.* [4] reported the *Streptococcus faecalis and Klebsiella pneumonia* inhibited by the xanthochymol of *G. xanthochymus* fruits.

Atomic force microscopy

Only the methanol extract of the leaf was considered for the AFM study of *S. aureus, B. cereus* and *E. coli*. The images of the untreated cells and the cells treated with DMSO showed the normal coccus shape for *S. aureus,* rod shape for *B. cereus* and *E. coli* (fig. 1 a-d, 2 a-d and 3 a-d). The mean length, width and height and root-mean-square roughness (R_{rms}) of these strains are represented in table 2. Distinct morphological changes were observed in bacteria treated with methanol extract of *G. xanthochymus* leaf and gentamicin (fig. 1 e-h, 2 e-h and 3 e-h).

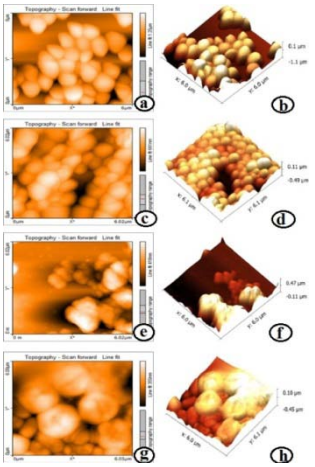

Fig. 1: 6×6 µm² AFM scans of *S. aureus* in tapping mode before and after 12 h treatment with leaf extracts of *G. xanthochymus* (2D and 3D scans): a-b) The untreated bacteria, c-d) Bacterial cells after treatment with DMSO, e-f) Bacterial cells after treatment with the methanol extract of leaf, g-h) Bacterial cells after treatment with gentamicin

Table 1: Antibacterial activity of leaf extracts of *G. xanthochymus* (n*=3)

	Zone of inhibition in mm				Minimum inhibitory concentration in µg/ml			
	S. aureus	*B. cereus*	*E. coli*	*P. aureginosa*	*S. aureus*	*B. cereus*	*E. coli*	*P. aureginosa*
Petroleum ether	-	-	-	-	-	-	25	-
Chloroform	-	-	-	-	-	-	25	-
Methanol	15.33±0.33	17.33±0.88	12.00±0.57	-	25	25	50	-
Dimethylsulfoxide	-	-	-	-	-	-	-	-
Gentamicin	22.00±1.15	27.66±0.33	27.00±0.57	22.33±0.88	0.78	0.78	1.56	1.56

*Number of replicates used for experiments.

The AFM studies which were carried out to investigate the antibacterial activity of the leaf extracts showed significant morphological changes in the cell surface of the bacterial strains treated with leaf extracts when compared to control. The methanol

extracts of *G. xanthochymus* leaf showed the highest damage of *B. cereus* cells compared to *S. aureus* and *E. coli* (fig. 1 e-f, 2 e-f and 3 e-f). The cell surface of all the bacteria which were used as a control was smooth with a typical shape, whereas the *S. aureus* treated with leaf extracts showed the cluster formation with smaller size cells. *B. cereus* treated with leaf methanol extract also showed changes in size when compared to the control (fig. 2 e-f).

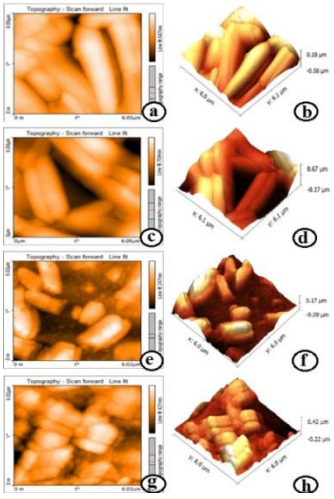

Fig. 2: 6×6 µm² AFM scans of *B. cereus* in tapping mode before and after 12 h treatment with leaf extracts of *G. xanthochymus* (2D and 3D scans):a-b) The untreated bacteria, c-d) Bacterial cells after treatment with DMSO, e-f) Bacterial cells after treatment with the methanol extract of leaf, g-h) Bacterial cells after treatment with gentamicin

The shrinkage and formation of grooves were observed in the cells of *B. cereus*. The cells treated with plant extracts and gentamicin (fig. 1 g-h, 2 g-h and 3 g-h) showed smaller clusters with collapsed cells, rough surface and large amount of debris closing to the cells, in addition, cells also showed characteristic damage such as the reduction in size. The *E. coli* treated with plant extracts did not show any significant changes in size, shape, cellular rupture and roughness of cells (fig. 3 e-f). The methanol extract of *Garcinia xanthochymus* leaf was more effective against Gram positive bacteria than Gram negative bacteria this can be attributed even to the difference in the structure and cell wall composition of the both types of strains of bacteria [19].

The cells treated with gentamicin have shown more cellular rupture, roughness and grooves on the surface of cells compared to the leaf extracts in all three bacterial species.

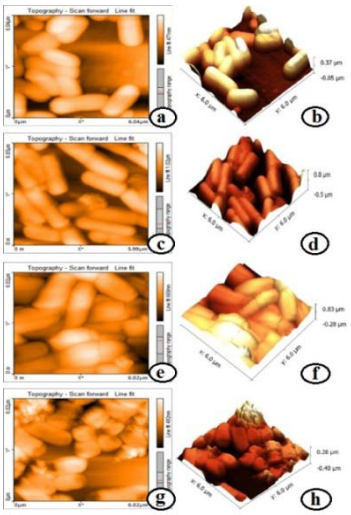

Fig. 3: 6×6 µm² AFM scans of *E. coli* in tapping mode before and after 12 h treatment with leaf extracts of *G. xanthochymus* (2D and 3D scans):a-b) The untreated bacteria, c-d) Bacterial cells after treatment with DMSO, e-f) Bacterial cells after treatment with the methanol extract of leaf, g-h) Bacterial cells after treatment with gentamicin

Table 2: Size and roughness of the bacterial cells treated with the leaf extracts of *G. xanthochymus* measured by AFM in tapping mode

	Test samples	Size of the bacterial cells in µm			Rrms
		Length	Width	Height	
S. aureus	Control	1.10±0.06	0.90±0.07	0.55±0.06	0.012±0.31
	DMSO	1.08±0.13	0.96±0.98	0.44±1.02	0.012±0.94
	Leaf Methanol	0.99±0.14	0.63±0.04	0.19±0.01	0.053±4.17
	Gentamicin	0.35±1.35	0.44±0.68	0.08±0.36	0.131±1.35
B. cereus	Control	2.87±0.86	1.10±0.05	0.33±0.06	0.038±0.65
	DMSO	2.91±0.62	1.14±0.74	0.36±0.83	0.041±0.58
	Leaf Methanol	1.72±0.36	0.91±0.21	0.25±0.04	0.088±6.48
	Gentamicin	1.73±0.64	0.58±1.00	0.09±1.51	0.112±0.98
E. coli	Control	1.91±0.09	0.80±0.09	0.18±0.04	0.011±0.02
	DMSO	1.88±0.53	0.82±0.94	0.19±0.57	0.011±0.64
	Leaf Methanol	1.15±0.14	0.78±0.07	0.17±0.02	0.038±3.18
	Gentamicin	1.04±0.58	0.59±0.72	0.10±0.39	0.135±1.02

CONCLUSION

The results of this study indicated that methanol leaf extracts of *Garcinia xanthochymus* showed potent antibacterial activity against gram positive, *S. aureus* and *B. cereus* and gram negative, *E. coli* bacteria. Further work needs to carried out to isolate active compound responsible for antibacterial activity.

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CONFLICT OF INTERESTS

Declared None

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