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Probing the mechanism of the glaucoside from *Eugenia jambolana* in reversal of drug resistance of a few uropathogens -An in vitro and in silico study

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Abstract: Plants are a good source of bioactive compounds with negligible side effects. Syzgium cumini is a plant of interest from historic times due to different culinary and medicinal use. Hence this study was carried out to evaluate the antibacterial activity of the seeds of the chosen plant against drug resistant bacterial pathogens. The bacterial isolates that cause Urinary tract infection were isolated and identified. The drug resistant bacteria were identified and antibacterial activity of the chosen plant and isolated compound was determined by disc diffusion assay and plasmid curing effect was analysed by reversal of antibiotic resistance. Also docking was performed to predict the mode of action of the plant extract and compound. The qualitative phytochemical analysis of ethanol extract has revealed the presence of alkaloids, carbohydrates, tannins, saponins, phenolic compounds, carotenoids, flavonoids, diterpenes and phytosterols. The methanol extract and the glaucoside compound showed significant antibacterial activity by disc diffusion assay. The zones of inhibition against E.coli, Pseudomonas aeruginosa, Proteus mirabilis and P. vulgaris and Klebsiella pneumonia ranged from 13mm to 20mm respectively. These results were comparable with standard antibiotics as streptomycin and gentamycin. The bacteria resistant towards a few antibiotics tested before treatment demonstrated sensitivity after treatment, which may be due to plasmid curing. Hence, the plant is considered as a good candidate for bacterial infections. Further work is in progress.

Keywords: *Syzgium cumini*, bacteria; Zone of inhibition; Plasmid; Resistant

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Infectious diseases are one of the leading causes of mortality worldwide, especially in developing countries (Ziegler, 2005; Yala et al. 2001).Such infections are treated with several antibiotics for human therapy, but emergence of drug resistance has become a menace for physicians. Several bacteria have developed resistance mechanisms including the efflux of antibiotics (Yala et al. 2001). Several mechanisms have been proposed, such as target site modification, expression of the efflux pumps, and metabolic inactivation, which are the factors that contribute to the drug resistance in MDR bacteria (Hooper, 2001). This has limited the choice of drugs. Hence, alternate source of drugs are in demand. Therefore scientists have focused their attention on alternate natural drugs. According to World health organization (WHO) more than 80% of the world population relies on traditional medicine for their primary health care needs (Vashist and Jindal, 2012). A wide range of medicinal plants are used against various microbes (Vashist and Jindal, 2012). Plants are rich in a wide variety of secondary metabolites such as tannins terpenoids, alkaloids, flavonoids, etc, which have been found in vitro to have antimicrobial properties (Dahanukar et al. 2000). Hence, several plants have been screened for their potential uses as alternative remedies for the treatment of many infectious diseases (Tepe et al. 2004). Data from literature reveal great potential of plant extracts/ fractions for therapeutic treatment and the importance of plant extracts/fractions, when associated with antibiotics to control multidrug resistant pathogenic bacteria, a major threat to human health. Resistance to antimicrobials is a natural biological phenomenon that can be amplified or accelerated by a variety of factors, including human practices. Resistance to antimicrobials is a natural biological phenomenon that

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can be amplified or accelerated by a variety of factors, including human practices. The use of an antimicrobial for any infection, real or feared, in any dose and over any time period, forces microbes to either adapt or die, in a phenomenon known as "selective pressure". The microbes, which adapt and survive, carry genes for resistance, which can be passed on when bacteria replicates. The resistance mechanisms in microorganisms are based on four strategies: i) Inactivation of the drug, ii) Prevention of the drug to reach its target, iii) Reduction of target's susceptibility, or iv) Acquisition of new, insensitive target (Brigitte, 2002). One of the promising methods in coping with bacterial resistance is, along the use of alternative classes of antimicrobial agents. The problem of microbial resistance is growing and the outlook for the use of antimicrobial drugs in the future is still uncertain (Nascimento et al. 2000). Plants have provided a source of inspiration for novel drug compounds, as plant derived medicines have made large contributions to human health and wellbeing (Iwu et al. 1999). Phytomedicine derived from plants have shown great promise in the treatment of intractable infectious diseases including opportunistic AIDS infections (Iwu et al. 1999). The problem of microbial resistance is growing and the outlook for the use of antimicrobial drugs in the future is still uncertain. Therefore, action must be taken to control the use of antibiotic, develop research to better understand the genetic mechanism of resistance, and to continue studies to develop new drugs, either synthetically or natural (Nascimento et al. 2000). The prevalence of antimicrobial-resistant human pathogens is rapid increasing, but the discovery and development of new antimicrobial drugs that are active against multidrug-resistant organisms have slowed dramatically (Gold and Moellering, 1996). Thus, the plants may have evolved compounds which evade multidrug resistance mechanisms and that plant antimicrobials might be developed into broad spectrum antibiotics in combination with inhibitors of multidrug resistance. Plants have now been utilized as herbal drugs and screening for more plant based drugs is on the rise. Few plants have been reported to possess reversal of antibiotic resistance. These have been frequently referred to as resistance modifying agents or biopotentiators. Reversal of multidrug resistance may be another attempt to mitigate the spread of resistance.

The present work is oriented in screening the drug resistance reversing efficacy of lead compound obtained from crude methanolic extract of seeds of *Sygium cumini.* This report states that the glaucoside may act as biopotentiators or bioenhancers with resistant drug to overcome antimicrobial drug resistance in microbes.

MATERIALS AND METHODS

Plant material

Seeds of *Sygium cumini* were collected during May 2004 from a tree growing at Holy Cross College, Tiruchirapalli, Tamil Nadu and authenticated by Dr. Rosalie, a taxonomist from the Department of Botany, Holy Cross College, Tiruchirappalli. A voucher (HCH:171) specimen was deposited at the herbarium of the above department. The seeds were air-dried and powdered. The powder (1kg) was extracted with *n*-hexane, acetone, and methanol sequentially in a Soxhlet apparatus and evaporated to dryness under reduced pressure in a rotary evaporator. The yields of the *n*-hexane, acetone, and methanol extracts were 10.9, 12.1, and 14.3 g, respectively. The dry residues of the crude extracts were stored at 4°C.

Isolation and identification of the compound

The shade-dried plants were powdered and1kg powder was extracted using hexane, acetone and methanol in a soxhlet apparatus sequentially and the extracts were evaporated to dryness under reduced pressure in a rotary evaporator. The yield of the hexane extract was14.3g,acetone extract was 10.9g and the methanol extract was 12.1g. The dry residues of the crude extracts obtained were stored at 4^0C for further use. The acetone extract was further chromatographed on a silica gel column (Merck70-230mesh, 400g, 3.5 i.d. 60 cm) and successively eluted with stepwise gradient of ethylacetate and hexane, then with acetone and ethylacetate, with acetone and chloroform and finally with chloroform and hexane. Seventeen fractions were collected and each fraction was spotted on a precoated Silica gel 60F254, 0.25mm thick TLC plate (Merck) and fractions with similar Rf values in TLC pattern were pooled to get her into 5 fractions. Fraction 3 showed single spot in TLC and hence fraction 3 was subjected to spectral study. Based on the results obtained from nuclear magnetic resonance (NMR), mass spectrometry (MS), ultraviolet (UV) and infra-red (IR) spectrometry, the structure of the active compound was elucidated.

Antibacterial Activity

Clinical Samples

The clinical study included urine samples obtained from patients suffering from symptomatic and asymptomatic urinary tract infection admitted in the CSI Mission General Hospital, Tiruchirappalli, India. The urine specimens were transported to the bacteriology laboratory within 2 hours of collection or refrigerated for 4 hours before processing.

Culture

Specimens were cultured on UTI agar /cystine lactose electrolyte-deficient medium, (Hi-Media, India) by the semi quantitative method and the specimens yielding colony counts = 10^4/ml were interpreted as diagnostic of UTI (Colle et al. 1996). Bacterial counts less than this was considered insignificant. Specimens where a single pathogen was isolated, were termed monomicrobial; where two were isolated were termed as polymicrobial infection. Growth of 3 or more types of organisms or diphtheroids was considered as contamination.

Identification of isolates was done using standard microbiological techniques (Colle et al. 1996).

Bacterial isolates

The clinical isolates obtained for the study were *Esherichia coli, Klebsiella pneumoniae, Pseudomonas aeruginosa, Staphylococcus aureus,*. The isolates were maintained on Nutrient agar slants and were subcultured periodically.

Antimicrobial agents

The antimicrobial agents used in the present study were ampicillin and cefixime, a product of Cipla pharmaceutical Pvt. Ltd, India. The antibiotics were stored in sealed containers in the dark at 4°C with a desiccant.

Antibacterial activity

Disc diffusion Test

The antibacterial activity of the glaucoside was determined by the disc diffusion method (Bauer et al. 1966).The zones of inhibition were calculated by measuring the diameters of the zone around the disc.

Determination of Minimal Inhibitory Concentration (MIC)

The compound was subjected for determining the minimal inhibitory concentration (MIC) by broth dilution method. The minimum inhibitory concentration of the extract was estimated for each of the test organisms in triplicates. To 100µl to 200µl of varying concentrations of the extracts, 100µl of nutrient broth was added and then a loopful of the test organism previously diluted, the microwell plate was incubated for 24hrs and each dilution was plated and observed for the bacterial growth inhibition. The MIC values were interpreted as the highest dilution of the sample, which showed no growth. Results of minimum inhibitory concentration (MIC)

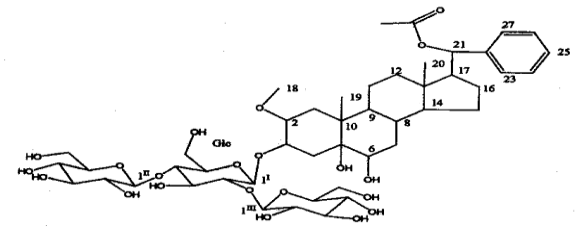
Docking

To investigate the mechanism of reversal of the resistance pattern of the bacteria to antibiotics, the compound was docked with Fim H, the flagellar protein used in attachment of bacteria to host cells by Gem dock. The Fim H structure was obtained from PDB and the compound was drawn using Chemdraw.

RESULTS AND DISCUSSION

Spectral analysis

The spectral data obtained through UV,FT-IR,ESI-MS, 1HNMR and 13C NMR provided the following structure (Fig.1). The compound was identified as glaucoside J .The above spectral data led to the structure of the compound as 5, 6-dihydroxy-3- [(4-hydroxy-6- (hydroxymethyl)-3,5-di [3,4,5-trihydroxy-6-(hydroxymethyl) tetrahydro-2*H*-2-pyranyl]oxytetrahydro-2*H*-2-pyranyl) oxy]-2-methoxy-10, 13-dimethylperhydro cyclopenta[a] phenanthren-17-yl(phenyl) methylacetate from *Sygium cumini* seeds.



The name of the compound :

"5,6-dihydroxy-3-[(4-hydroxy-6-(hydroxymethyl)-3,5-di[3,4,5-trihydroxy-6-(hydroxymethyl)tetrahydro-2*H*-2-pyranyl]oxytetrahydro-2*H*-2-pyranyl)oxy]-2-methoxy-10,13-dimethylperhydrocyclopenta[*a*]phenanthren-17-yl(phenyl)methyl acetate"

Figure1 Structure of the isolated glaucoside from *Sygium cumini* seeds

Antibacterial activity

The antibacterial activity of the compound was determined by Disc diffusion assay and the diameters of the zone of inhibition (ZOI) were recorded. Activity of the compound was determined by measuring the zones of inhibition. Because zone of inhibition were often asymmetrical, experiments were repeated three times and the average was recorded.Table.1 displays the results of antibacterial testing for the glaucoside from *Sygium cumini* seeds. The results illustrate that *Sygium cumini* showed a wide spectrum of antibacterial activity.

Table 1 Effect of the glaucoside from Sygium cumini seeds against few human pathogens by Disc Diffusion method

CULTURE	Zones of Inhibition (mm)	
	10 µL	20 µL
Proteus vulgaris	12	10
E. coli	14	14
Klebsiella pneumoniae	10	12
Pseudomonas aeruginosa	10	14

Minimal Inhibitory Concentration of the extracts by Microbroth Dilution method

The MIC values of the glaucoside from *Sygium cumini* seed extract against drug resistant bacterial isolates are given in table 2 as shown.

Table 2 Minimal Inhibitory Concentration of the glaucoside from Sygium cumini against human pathogens

Bacterial Isolates	Minimal Inhibitory Concentration (µl)
Proteus vulgaris	50
E.coli	50
Klebsiella pneumoniae	100
Pseudomonas aeruginosa	100

Antibiogram Pattern

The antibiogram pattern shows that the five isolates chosen for the study were resistant to the drugs, ampicillin and cefixime. On treatment with the glaucoside, there was a reversal in the resistance pattern as shown in table 3.

Table 3 Reversal of antibiotic resistance by glaucoside on bacterial pathogens

Bacterial Isolates	Before Treatment		After treatment	
	AA	CFM	AA	CFM
Proteus vulgaris	R	R	S ZOI = 12 mm	R
E. coli	R	R	S ZOI = 13 mm	R
Klebsiella pneumoniae	R	R	S ZOI = 12 mm	S ZOI = 12 mm
Pseudomonas aeruginosa	R	R	S ZOI = 12 mm	S ZOI =12 mm

Docking result

The docking study was taken up to confirm the results of antibacterial activity of the glaucoside. To determine the mode of action of the glaucoside, the compound was docked with FimH receptor. FimH is a protein involved in attachment of bacteria to the host cells through flagella. The docking results indicate that the compound has the capacity to bind with FimH and prevent the attachment of bacteria to host cells, thereby controlling bacterial infections as shown in figure 2 and 3.

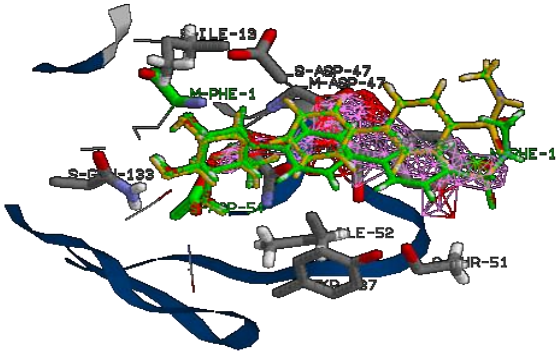


Figure 2 Docking the compound glaucoside with FIM H (3ZL1)

Natural products of higher plants may possess a new source of antimicrobial agents with possibly novel mechanisms of action (Ahmad and Aqil, 2007; Barbour et al. 2004). They are effective in the treatment of infectious diseases while simultaneously mitigating many of the side effects that are often associated with conventional antimicrobials (Iwu et al, 1999). Reversal of multidrug resistance may be another attempt to mitigate the spread of resistance. One of the promising methods in coping with bacterial resistance is, along the use of alternative classes of antimicrobial agents, also the application of synergistic activity between antibiotics and non-antibiotics. Many plants have direct antimicrobial activity but also resistance modifying/modulating activities (Gibbons, 2004). Resistance modifying agents may inhibit multidrug resistance mechanism. This ability of plant extracts to enhance antibiotics has not been well defined. Plasmid-mediated multidrug resistance is one of the most upcoming problems in the treatment of infectious diseases, as bacteria have reached the resistance to most of the antibiotics that are available for treatment. Identification of a novel curing agent derived from plant is significant, since majority of natural products are non-toxic to human and environment. Previous reports of plant derived curing agents are limited. Curing of R-plasmids in *E. coli* by Plumbagin from *Plumbago zeylanica* has been reported by Lakshmi et al. (1987), while Shriram et al. (2010) has earlier reported potential plasmid curing agents from plants including *Dioscorea bulbifera* and *Helicteres isora*.

Horiuchi and coworkers (2007) stated that Carnosol, the active compound showed weak antimicrobial activity and greatly reduced the MICs of various aminoglycosides. The exact mechanism for the reduction of *β*-lactam (methicillin) resistance by the natural antimicrobials is unknown but is likely due to some structural change in the resistant bacteria. Epigallocatechin gallate from green tea was reported to inhibit the activity of penicillinase produced by *S.*

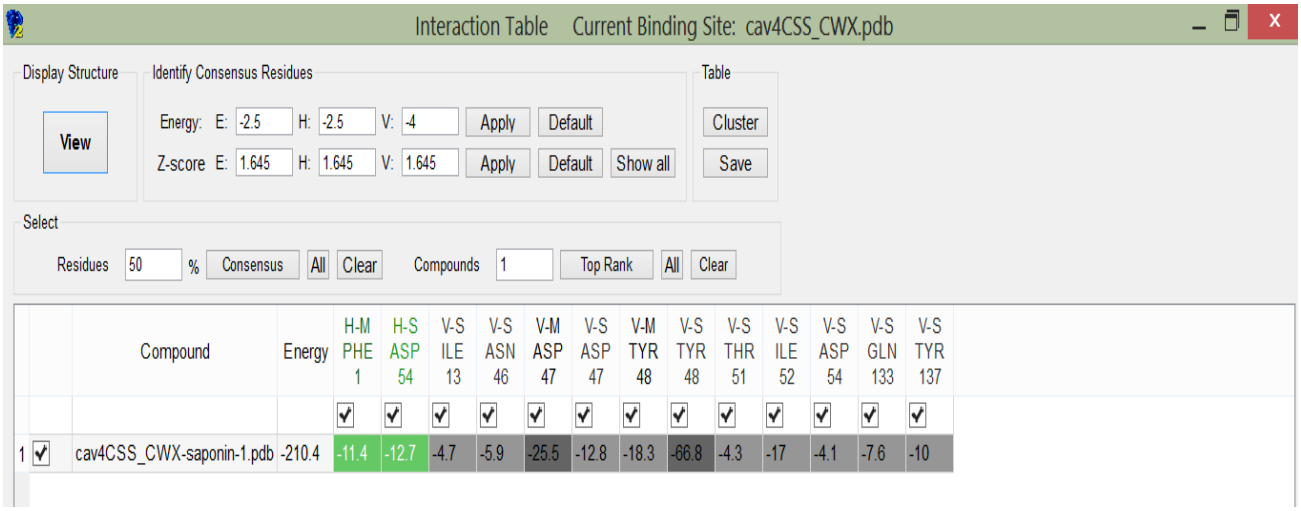


Figure 3 Depicting the energy of interaction of the FIM H with glaucoside

aureus by Zhao (2002) and was said to synergistically enhance the activity of carbapenems against methicillin-resistant *S. aureus*.

Resistance modifying agents may inhibit multidrug resistance mechanism. This ability of plant extracts to enhance antibiotics has not been well defined. Shiota et al. (2000) have reported that the synergistic combination of hydrolysable tannins Tellimagrandin I with tetracycline reduces the MICs of tetracycline against Methicillin-Resistant *Staphylococcus aureus* (MRSA). Gibbons (2000) findings state that Reserpine caused a fourfold reduction in the MIC of tetracycline against a variety of multidrug resistant MRSA and MSSA. This was also supported by Schmitz et al. (1998). According to Tegos (2002), the polypeptide derived Rhein and Plumbagin have striking activity against multidrug resistant *Staphylococcus aureus* and some gram negative bacteria. Nascimento et al. (2000) reported that synergistic effect of anacardic acid and totarol with Methicillin inhibited MRSA.

Hence, the present study indicates that the glaucoside from *Sygium cumini* is a promising drug candidate and can be used in the treatment of bacterial infections, since the compound exhibits strong affinity against FimH receptor. Hence, FimH receptor may become a prospective target for inhibition of bacterial attachment, which is a pre-requisite for establishment of infection and hence the compound might have inhibited bacterial adhesion and thus have reversed the resistance pattern. This may unlock a strong initiative in developing the natural novel ligand which is specified towards it.

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