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Antimicrobial studies of stem bark extract and their phytoconstituent from *Semecarpus anacardium* L.

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Abstract

Background & Objective: To investigate the antimicrobial properties of stem bark extract of *S. anacardium* and their phytoconstituent using agar well diffusion and *in silico* docking methods. **Methodology:** *In vitro* antibacterial activity of petroleum ether, chloroform, methanol stem bark extract of *S. anacardium* was screened against both gram negative and gram positive bacteria. **Results:** The methanol extract of stem bark and amentoflavone, a bioactive compound showed significant antibacterial activity against *Klebsiella pneumonia* and antifungal activity against *Candida albicans. In silico* docking of amentoflavone with bacterial glucosamine-6-phosphate synthase and three fungal targets, mevalonate-5-diphosphate decarboxylase (1FI4), Sec3 - Rho1 complexes (3A58), Kre2p/Mnt1pa1, 2-mannosyltransferase (1S4N) showed significant inhibition with minimum binding energy, when compare to standard drug ciprofloxacin and amphotericin B. **Conclusion:** This study clearly showed that the amentoflavone used as broad spectrum of antimicrobial drug.

Keywords: Antimicrobial activity, *Klebsiella pneumonia,* Ciprofloxacin, Amphotericin B, Docking studies

1. Introduction

The *Semecarpus anacardium* L., belongs to a family anacardiaceae. The word *Semecarpus* is derived from simeion in Greek means marking/tracing and *carpus* in Greek means nut. Hence, it is popularly known as marking nut. *S. anacarium* called Bhallataka (Bhalltak), Antahsattva, Arusharah, Aruskara (Arukara), Arzohita, Balla´ta (Bhallata, Ballata), Bhallatakah, Viravrksa, Visasya (Sanskrit); Indian marking nut, Marsh nut, Oriental cashew nut (English); Bhela (Bhel), Bhelwa, Bhilawa (Bhilv), Bhilwa (Hindi); Erimugi or Erimuki (Tamil); Nallajeedi (Telugu); Bhilamu (Gujarati); Kaadu geru (Kannada) and distributed in sub-Himalayan region, tract east of the beas, ascending to 1050 m in Assam (Khasia hills), Madhya Pradesh, Gujarat, Konkan, Kanara forests of Tamilnadu state (Gothoskar *et al.,* 1971),Western Peninsula, North Australia (Kirtikar and Basu, 1975). The plant is very sparsely distributed in the Western ghats of Karnataka (Manjunath *et al.,* 2004).

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The plant high priority and applicability in indigenous system of medicine with caution, because it has potential to produce allergic symptoms through contact dermatitis (Chopra *et al.,* 1982; Khare *et al.,* 1982). In Ayurvedic medicine the nut (Bhallatak*)* has been used in the form of *Madhur, Kashay ras, Ushna vitya, Madhur vipak, Madhur laghu, Snigdha, Tikshna* and *Ushna gunas* (Gogte, 2000). The fruits of plants are largely used in Ayurvedic system of medicine for various ailments such as helmintic infection (Chattopadhayaya and Khare, 1969), leprosy, rheumatic pain, piles, asthma, cough, sexually transmitted diseases viz syphilis and gonorrhea, skin diseases such as leucoderma (Nadkarni, 1954, 1976), rejuvenating properties, increasing longevity, bringing a glow to the face, sweetness in tone and improvement in vision (Sreenivasacharyulu, 1931), heart, blood pressure, respiration, neurological disorders (Kurup *et al.,* 1979, Raghunath and Mitra, 1982, Sharma *et al.,* 1995), wound healing, diabetes, urinary diseases (Gaur, 1991) and promoting hair growth (Semalty *et al.,* 2008).

Isolated and characterized many phytochemical such as nallaflavanone, semecarpetin, anacardoflavanone (Murthy, 1988; Rastogi and Mehrotra, 1995), catechol, anacardol (Naidu, 1925) and glucoside, anacardoside (Gil *et al.,* 1995). Amentoflavone from *Selaginella*

tamariscina has been used a therapeutic agent for human invasive fungi and antibacterial activity (Jung et al., 2006; Hwang et al., 2013). First time reporting the isolation of amentoflavone from stem bark of *S. anacardium* and *in silico* docking studies in support to antimicrobial activity. The scanty report available on the phytochemical screening and pharmacological properties stem bark extract. Traditional practitioners residing in vicinity of the central Western Ghats of Karnataka are using the stem bark of this plant to cure infectious wounds, diabetes and liver disorders. Hence, the present investigation was under taken to support the antimicrobial properties of stem bark and their phytoconstituent amentoflavone from *S. anacardium.*

2. Materials and methods

Plant collection, extraction, preparation of crude extract and phytochemical analysis has been described in previous work (Lingaraju et al., 2011; 2012). Different solvent system (Methanol:Petroleum ether, Methanol:Ethyl acetate, Methanol:Butanol) and ratio of methanol:ethyl acteate (9:1; 8:2; 7:3) were used for isolation of phytoconstituent. Characterization of the isolated compound was performed by subjecting it to qualitative analysis followed by IR, ^{1}H NMR, ^{13}C NMR, and mass spectral studies.

2.1 Antimicrobial activity

Antimicrobial activity was tested by employing agar-well diffusion method (Mukherjee *et al*., 1995; Nair *et al*., 2005) and was used to assess the antimicrobial activity of the test samples. Six strains of most common pathogenic gram positive bacteria namely, *Staphylococcus aureus*-NCIM-2079, *Pseudomonas aeruginosa*-NCIM-2036 and gram negative bacteria namely, *Proteus vulgaris* - NCIM-2027, *Salmonella typhi* - NCIM- 2501*, Klebsiella pneumoniae* - NCIM-2957 and *Salmonella paratyphi*- MTCC - 735 were obtained from National Chemical Laboratory, Pune and Microbial type culture collection and gene bank, Chandigarh, India and four clinically isolated pathogenic fungi such as *Aspergillus niger*, *Aspergillus fumigatus, Aspergillus flavus* and *Candida albicans* obtained from Shivamogga Institute of Medical Sciences, Shivamogga were used as a test organisms. All the bacterial microorganisms were maintained at -30°C in brain heart infusion (BHI, pH – 6.5) containing 17% (v/v) glycerol. Before testing, the suspensions were transferred to LB broth (pH – 7.2) and cultured overnight at 37°C. Fungal cells were obtained by centrifugation at 1500 × g, 4°C for 15 min and diluted in PBS, pH 7.2. One hundred µl of fungal spores were spread on BHI agar plates and wells were made using a sterilized cork borer and 50 µl of test compounds were loaded into each well. The plates were refrigerated for 2 hrs in order to stop fungal growth and facilitate diffusion of the substances.

The petroleum ether (50 mg/ml), chloroform (50 mg/ml) and methanol extract (50 mg/ml) and amentoflavone (5mg/ml and 10 mg/ml), reference antibacterial agent ciprofloxacin (1 mg/ml; Cipla, Mumbai) and antifungal agent amphotericin B (1 mg/ml; Medico Remedies Pvt. Ltd, Mumbai) were loaded in the corresponding wells (100 µg per 100 µl of sterilized distilled water). As a control, the wells were loaded with the same volume of sterile distilled water. Plates were then incubated at 37°C for 48 h. At the end of the incubation period, inhibition zones formed on the medium were determined and data was statistically evaluated by Tukey's pair wise comparison test.

2.2 Minimum inhibitory concentration (MIC) of extract and isolated constituent

Minimal inhibitory concentration (MIC) values were determined by broth dilution method. Serial dilutions (final volume of 1 ml) petroleum ether, chloroform and methanol extract and the phytoconstituent of *S. anacardium* (0.5 to 0.05 mg/ml) were performed with 0.9% saline. Following this, 9 ml of nutrient broth was added. Broths were inoculated with 100 µl of each bacterial suspension (5×10^4CFU) and incubated for 24 h at 37°C. Ciprofloxacin was used as the positive control and 0.9% saline as negative control. After 24 h, bacterial growth was assayed by measuring absorbance at 625 nm. MIC was defined as the lowest concentration in mg of stem bark of *S. anacardium* and amentoflavone to restrict the growth to ‹0.05 absorbance at 595 nm (National Committee for Clinical Laboratory Standards, 2005).

The *in vitro* fungicidal activity (MFC) was determined described by Espinel-Ingroff *et al.* (2002). After 72 h of incubation, 20 µl was subcultured from each well that showed no visible growth (growth inhibition of over 98%), from the last positive well (growth similar to that for the growth control well), and from the growth control (extract-free medium) onto potato dextrose agar plates. The plates were incubated at 27°C until growth was seen in the growth control subculture. The minimum fungicidal concentration was regarded as the lowest extract concentration that did not yield any fungal growth on the solid medium used.

2.3 Molecular docking studies

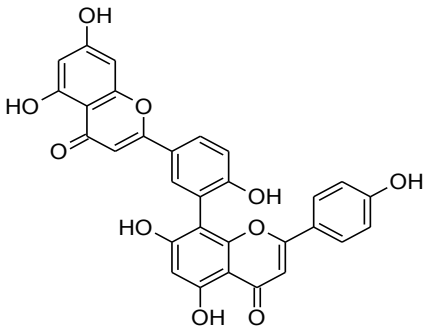
The target proteins, glucosamine-6-phosphate synthase (2VF5; Mouilleron et al., 2008), mevalonate 5-diphosphate decarboxylase (1FI4; Bonanno et al., 2001), 1,2-mannosyltransferase (1S4N; Lobsanov et al, 2004), and exocyst complex component SEC3 (3A58; Yamashita et al., 2010) was downloaded from the RCSB protein Data Bank. The chemical structure of phytoconstituent was designed and analyzed by using ChemDraw Ultra 6.0. Energy minimization and 3D coordinates were prepared using PRODRG server (Schuettelkopf and Van Aalten, 2004). The chemical structure of standard drug Ciprofloxacin and Amphotericin B was obtained from DrugBank database (Wishart et al., 2017). The PDB coordinates of the target proteins and ligand molecules were optimized and converted to PDBQT file by AutoDock 4.2. These PDBQT files were docked in AutoDock Vina (Trott & Olson, 2010)

The active sites are the coordinates of the ligand in the target protein, and these active pockets of target protein were obtained and analysed using the CastP server, which could be considered as the probable best

accurate region (Joe-Dundas et al., 2006). The grid map was centered at the residues of the target proteins 2VF5, 1FI4, 1S4N, and 3A58 were predicted from Ligplot and generated by using Autogrid.

3. Results and discussion

The fraction eluted through the solvent system (8:2) methoanol:ethyl acteate is a yellow amorphous compound and the yield was 825 mg/ 10g of the crude extract. The compound showed the positive results for Shinoda's test, Ferric chloride test, Zinc hydrochloric acid, alkaline test, lead acetate test and it was found to be a flavonoid. The ^{1}H NMR spectra the HO-5 of flavones were disappeared in the spectrum due to high acidic nature and exchange of protons with Deuterium in methanol used as solvent to record ^{1}H NMR (**Fig. S1A, S1B & S1C**). However the corresponding two doublets at 7.61 and 7.22 (J = 8.6 Hz, 2H), and signals at 7.09 (br,s, 2H), 7.52 (s, 1H), 6.88 (d, 8.0Hz, 2H), two hydrogen meta coupled [δH 6.73 (d, 2H, 2Hz)] and three singlets of protons at δ 6.43, 6.52, 6.96, (**Fig. S2**) and also by the mass M/Z was found to be 539.09 (M+1) (**Fig. S3A, S3B, S3C, S3D & S3E** respectively) were consistent with the biflavonoids. Based on the spectral analysis, the compound C-2 is identified as Amentoflavone. Based on spectral details, the isolated compound is characterized as amentoflavone with Molecular formula ($C_{30}H_{18}O_{10}$). The chemical structure of the compound drawn with the help of Chemdraw software is as follows (**Fig. 1**).



Amentoflavone

M/Z 538 (mass calculated)

M/Z 539 (M^{+}1) (Mass found)

Figure 1: Structure of Amentoflavone

3.1 Antimicrobial activity

The antibacterial activity of stem bark extract of *S. anacardium* showed varying magnitudes of inhibition patterns with standard drug ciprofloxacin a well-known broad-spectrum antibacterial agent. The mean inhibitory zone of extracts and the standard drug ciprofloxacin against eight bacterial species is summarized in **Table 1.** In the agar well diffusion method, stem bark methanol extract of *S. anacardium* showed a highly significant level of bacterial inhibition against *K. pneumoniae* (29.83±0.60), *P. aeruginosa* (29.00±0.73), *S. typhi* (27.17±0.60), *S. aureus* (24.50±0.85), *S. paratyphi* (23.50±0.76) and *P. vulgaris* (23.17±0.79). The petroleum ether extract showed moderate activity against *P. aeruginosa* (16.67±0.67), *K. pneumoniae* (16.67±0.49), *S. typhi* (12.00±0.58), and very less against *S. aureus* (9.50±0.43). The chloroform extract showed less activity in *P. vulgaris* (7.33±0.49) and moderate activity in *K. pneumonia* (13.67±0.80). The isolated constituent amentoflavone from methanol extract showed significant activity against *K. pneumoniae* (17.50±0.76) and moderate activity in *P. vulgaris* (15.00±0.58), *S. aureus* (13.67±0.71), *S. typhi* (13.17±0.70) and *P. aeruginosa* (12.33±0.56). The MIC value determined by broth dilution method indicated that methanol extract at the concentration of 100 µg/ml showed significant antibacterial activity against *P. aeruginosa, K. pneumoniae, S. typhi,* and *S. aureus.* The petroleum and chloroform stem bark extracts at 50 - 500 **µ**g/ml against the six tested bacterial strains were presented in the **Table S1.**

Among the four fungal isolates cultured for antifungal assay, the zone of inhibition of methanol extracts of *S. anacardium* were found to be maximum on *Candida albicans* (8.23±0.08), *Aspergillus fumigatus* (7.42±0.09), *Aspergillus niger* (6.28±0.09) and *Aspergillus flavus* (5.47±0.14) respectively. The isolated constituent amentoflavone showed maximum inhibition zone against *Candida albicans* (5.12±0.14) and very less activity against *Aspergillus flavus* (2.05±0.08), *Aspergillus niger* (2.38±0.09), *Aspergillus fumigatus* (2.22±0.09). The details of antifungal activity are

Table 1: Antibacterial activity of stem bark extracts and the constituent amentoflavone of *S. anacardium*

Sl. No.	Drugs tested	*P.vulgaris-NCIM-2027*	*S. aureus-* NCIM-2079	*S .typhi-* NCIM-2501	*S. paraty-phi-* MTCC-735	*K. pneumo-niae-NCIM-2957*	*P. aerugi-nosa - NCIM-2027*
01.	Ciprofloxacin (1 mg/ml)	20.83±0.31	21.33±0.49	19.67±0.42	15.50±0.43	22.17±0.60	22.00±0.63
02.	Pet. ether (50 mg/ ml)	11.17±0.48	9.50±0.43	12.00±0.58	11.67±0.67	16.67±0.49	16.67±0.67
03.	Chloroform (50 mg/ ml)	7.33±0.49	10.50±0.43	9.170.60±	11.67±0.67	13.67±0.80	10.33±0.76
04.	Methanol (50 mg/ ml)	23.17±0.79	24.50±0.85	27.17±0.60	23.50±0.76	29.83±0.60	29.00±0.73
05.	Amentoflavone (5 mg/ ml)	15.00±0.58	13.67±0.71	13.17±0.70	10.83±0.60	17.50±0.76	12.33±0.56

depicted in **Table 2**. The MIC value determined by broth dilution method indicated that methanol extract at the concentration of 50 µg/ml showed significant antifungal activity against *Candida albicans*. While the petroleum and chloroform extracts of stem bark showed the value ranges from 50 - 500 µg/ml against the four tested fungal strains and the data is depicted in **Table S2.**

Table 2: Antifungal activity of stem bark extracts and the constituent amentoflavone of *Semecarpus anacadium*

Sl. No.	Drugs tested	*Aspergillus niger*	*Aspergillus fumigatus*	*Aspergillus flavus*	*Candida albicans*
I.	Standard drug Amphotericin B (1 mg/ml)	8.98±0.08	7.80±0.09	5.27±0.11	6.60±0.14
II.	Pet. ether (50 mg/ ml)	2.75±0.13	2.18±0.10	2.83±0.09	4.18±0.06
III.	Chloroform (50 mg/ ml)	3.42±0.12	3.83±0.14	4.77±0.15	3.25±0.08
IV.	Methanol (50 mg/ ml)	6.28±0.09	7.42±0.09	5.47±0.14	8.23±0.08
V.	Amentoflavone (50 mg/ ml)	2.38±0.09	2.22±0.09	2.05±0.08	5.12±0.14

3.2 Molecular docking analysis

The highest binding energy correlates with the highest binding efficiency in protein and drug interactions. The grid box surround the region of Active site or all the amino acid residue in the macromolecule. Among 10 conformation, best affinity or binding energy between protein and ligand was considered (Muhammad and Fatima, 2015). The enzyme glucosamine-6-phosphate (GlcN-6-P synthase) is play an important role to build peptidoglycan of bacterial cell wall (Chmara et al., 1984). Hence, present authenticate antibacterial drug target this GlcN-6-P synthase for inhibition of this bacterial life sustaining enzyme or temporary inactivation was lethal to microorganisms (Pradeepa et al., 2014). The isolated Phytoconstituent Amentoflavone molecule were docked against GlcN-6-P synthase with a comparison to standard drug Ciprofloxacin. The Amentoflavone interaction with GlcN-6-P synthase showed highest binding energy of -10.0 by involving with 5 hydrogen bond with Ser 303, Gln 348, Ser 401, Ala 602, Val 399 amino acid residues (Fig. 2). Singh et al., 2015 performed the virtual screening through molecular docking studies against potential antifungal targets, Mevalonate-5-diphosphate decarboxylase (1FI4) is a single-domain α/β protein that catalyzes the successive ATP-dependent reactions which convert mevalonate to isopentenyl diphosphate. Isopentenyl disphosphate isomerase (IDI) is an α/β metalloenzyme that catalyzes interconversion of isopentenyl diphosphate and dimethylallyl diphosphate, which condense for sterol/isoprenoid biosynthesis (Bonanno et al., 2001). Sec3-Rho1 complexes (3A58) involved in phosphoinositide-dependent localization to membrane. Cell polarization is a critical process for differentiation and proliferation in eukaryotes. The required proteins and lipids are carried in secretory vesicles and transported to the target membrane by

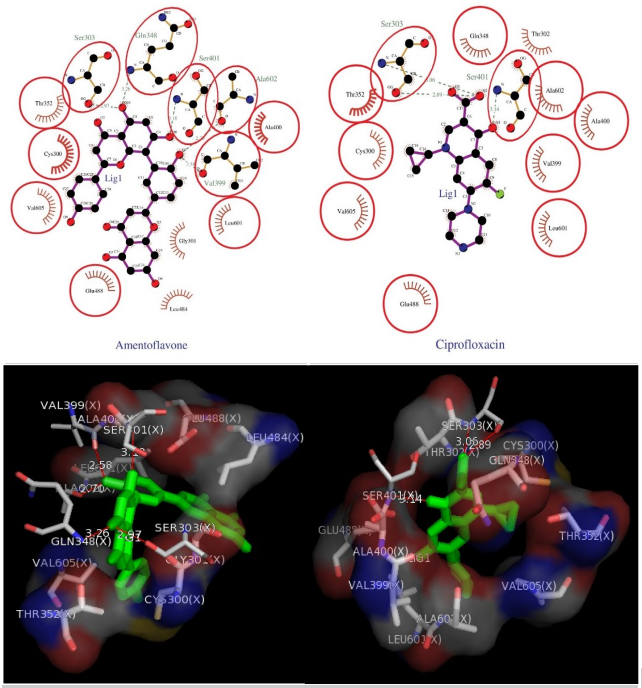


Figure 2: 2D Ligplot and docking result showing the structure of docking pose of Amentoflavone and Ciprofloxacin with GlcN-6-P synthase.

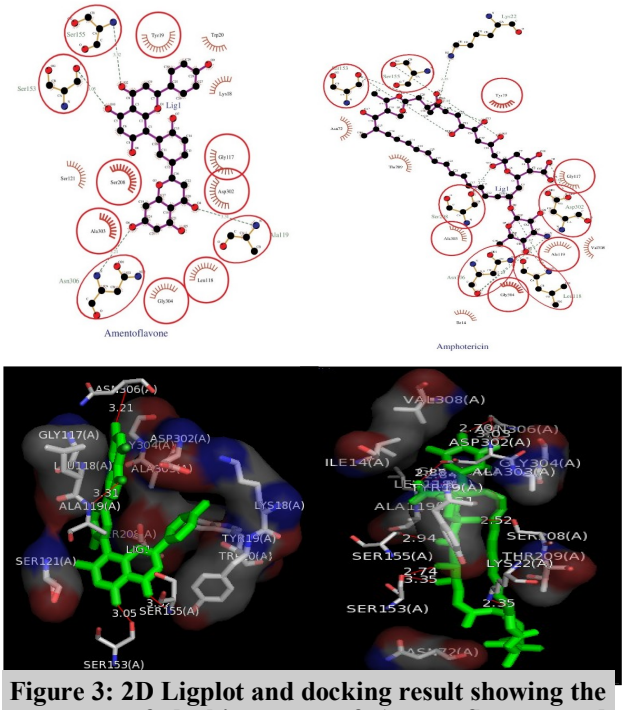
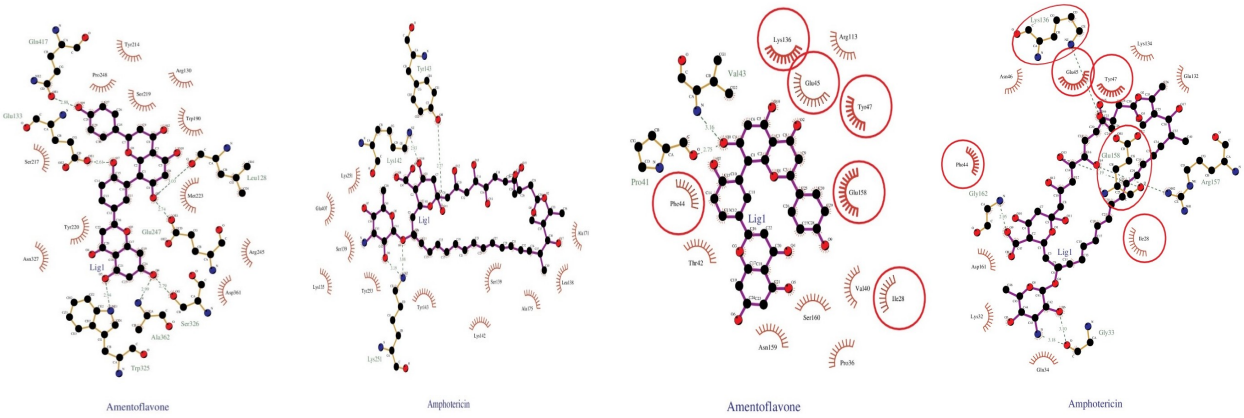


Figure 3: 2D Ligplot and docking result showing the structure of docking pose of Amentoflavone and Amphotericin B with mevalonate 5-diphosphate decarboxylase.



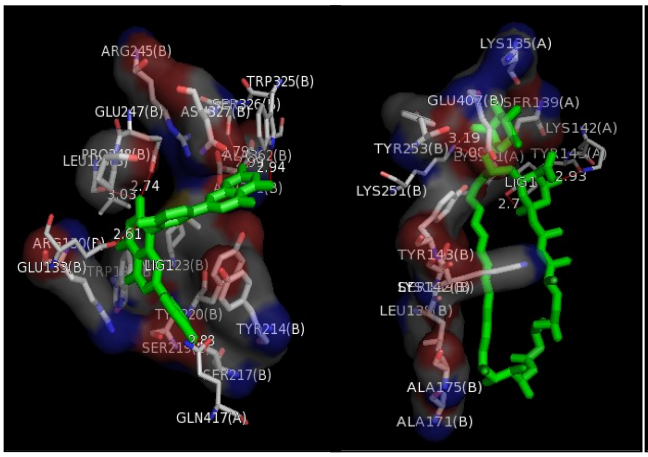


Figure 4: 2D Ligplot and docking result showing the structure of docking pose of Amentoflavone and Amphotericin B with 1,2-mannosyltransferase.

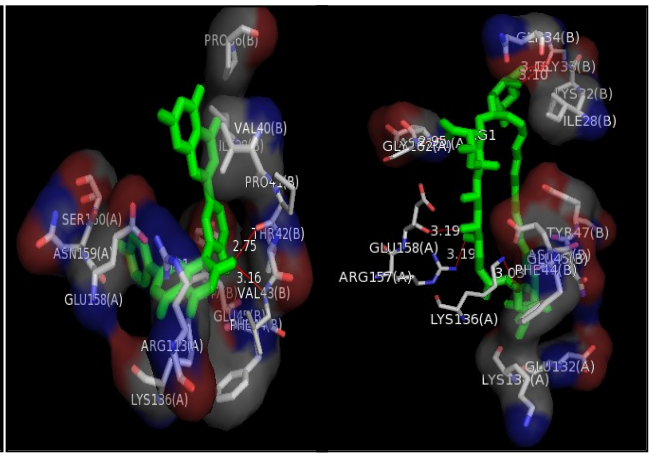


Figure 5: 2D Ligplot and docking result showing the structure of docking pose of Amentoflavone and Amphotericin B with exocyst complex component SEC3.

exocytosis that function during cell polarization. In yeast, exocyst subunit Sec3 binds to phosphatidylinositol 4,5-bisphosphate (PtdIns(4,5)P2 and the small GTPases Rho1 and Cdc42 via its N-terminal domain (Sec3-N), and these interactions target Sec3 to the plasma membrane (Yamashita et al., 2010). Kre2p/Mnt1p belongs to glycosyltransferase (GT) family 15 and has major a1,2-mannosyltransferase activity involved in the biosynthesis of yeast cell wall glycoproteins (Lobsanov et al., 2004). In the present study made an attempts to predict the orientation of ligand binding to target proteins that inhibition or regulation of the target protein functions by molecular docking. Amphotericin B is an antifungal drug used for *in vitro* activity of fungal strains. The highest binding energy of Amentoflavone was observed in all the selected targets, 1FI4, 1S4N and 3A58. The values of binding energy or affinity, number of hydrogen bond formation, hydrophobic interaction of ligand and protein was depicted in **Table 3 & 4 and Fig. 3, Fig. 4 & Fig. 5.**

.

Table 3: Docking of Amentoflavone with glucosamine-6-phosphate synthase (2VF5)

Ligands	Target Protein	Affinity (kcal/ mol)	H-Bonds	H-Bond Length (Å)	H-Bond With	Hydrophobic Interactions
Amentoflavone	2VF5	10.0	5	2.97 3.26 3.18 2.70 2.58	Ser 303 Gln 348 Ser 401 Ala 602 Val 399	Val 605, Cys 300, Thr 352, Ala 400, Leu 601, Gly 301, Leu 484, Glu 488
Ciprofloxacin (Standard Drug)		7.7	3	2.89 3.06 3.14	Ser 303 Ser 303 Ser 401	Val 605, Cys 300, Thr 352, Gln 348, Thr 302, Ala 602, Ala 400, Val 399, Leu 601, Glu 488

Table 4: Docking of Amentoflavone with mevalonate 5-diphosphate decarboxylase (1FI4), 1,2-mannosyltransferase (1S4N) and exocyst complex component SEC3 (3A58).

Ligands	Target Protein	Affinity (kcal/mol)	H-Bonds	H-Bond With	Hydrohphobic Interactions
Amentoflavone	1FI4	10.6	4	Ser 153 Ser 155 Ala 119 Asn 306	Tyr 19, Trp 20, Lys 18, Gly 117, Asp 302, Leu 118, Gly 304, Ala 303, Ser 208, Ser 121
Amphotericin B (Standard Drug)		9.6	10	Ser 153 Ser 153 Ser 155 Lys 22 Asp 302 Leu 118 Leu 118 Asn 306 Asn 306 Ser 208	Tyr 19, Gly 117, Val 308, Ala 119, Gly 304, Ile 14, Ala 303, Thr 209, Asn 72
Amentoflavone	1S4N	12.8	7	Glu 133 Gln 417 Leu 128 Glu 247 Ser 326 Ala 362 Trp 325	Tyr 214, Pro 248, Ser 219, Arg 130, Trp 190, Met 223, Arg 245, Asp 361, Tyr 220, Asp 327, Ser 217
Amphotericin B (Standard Drug)		8.8	4	Lys 142 Tyr 143 Lys 251 Lys 251	Lys 251, Gly 407, Ser 139, Lys 135, Tyr 253, Tyr 143, Lys 142, Ser 139, Ala 175, Leu 138, Ala 171
Amentoflavone	3A58	9.7	2	Pro 41 Val 43	Lys 136, Glu 45, Arg 113, Tyr 47, Glu 158, Val 140, Ile 28, Ser 160, Pro 36, Asn 159, Thr 42, Phe 44
Amphotericin B (Standard Drug)		9.0	6	Lys 136 Gly 158 Arg 157 Gly 33 Gly 33 Gly 162	Lys 134, Glu 132, Tyr 47, Gly 45, Asn 46, Ile 28, Glu 34, Lys 32, Asp 161, Phe 44

4. Conclusion

The study showed that the methanol extract and their phytoconstituent Amentoflavone exhibited broad spectrum of antimicrobial activity.

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Conflict of interest

The author's declares none.

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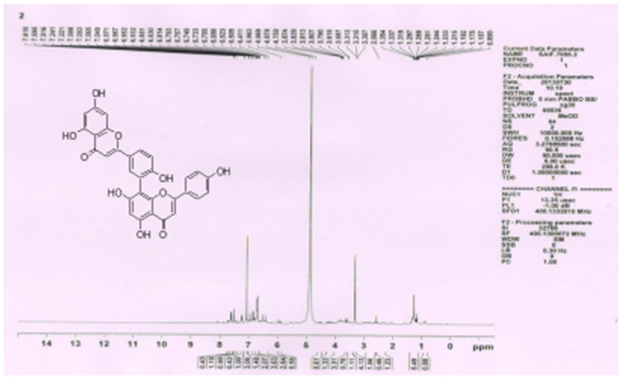
Supplementary Materials

Table S1: Minimal inhibitory concentrations of the *S. anacardium* stem bark extracts and amentoflavone against bacterial pathogens

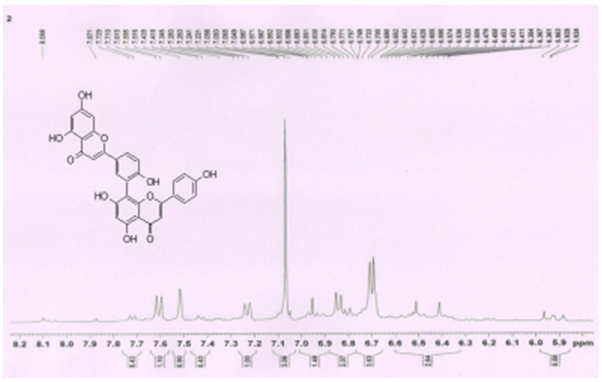
Sl. No.	Drugs tested	*P.vulgaris* (µg/ml)	*S. aureus* (µg/ml)	*S .typhi* (µg/ml)	*S. paratyphi* (µg/ml)	*K. pneumo-niae*(µg/ml)	*P. aeruginosa* (µg/ml)
01	Pet. ether	300	350	300	300	200	200
02	Chloro-form	500	350	350	350	400	350
03	Methanol	150	100	100	150	100	100
04	Amento-flavone	250	300	300	350	250	300

Table S2: Minimal inhibitory concentrations of the *S. anacardium* stem bark extracts and amentoflavone against fungal pathogens

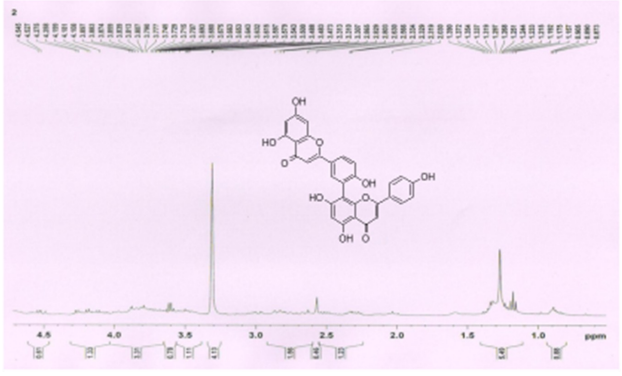
Sl. No.	Drugs tested	*Aspergillus niger* (µg/ml)	*Aspergillus fumigatus* (µg/ml)	*Aspergillus flavus* (µg/ml)	*Candida albicans* (µg/ml)
01	Pet. ether	500	500	500	450
02	Chloroform	350	350	450	300
03	Methanol	100	150	200	50
04	Amentofla-vone	450	450	450	150



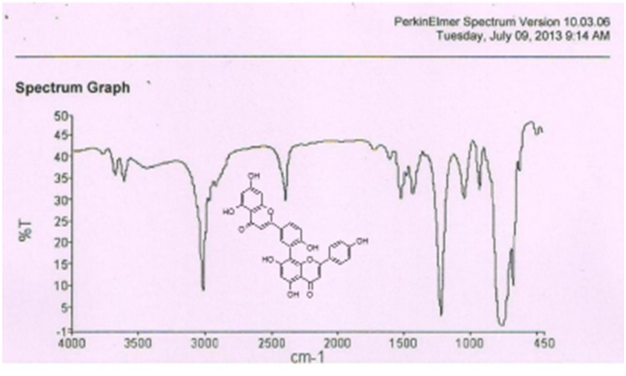
S1A: ^{1}H NMR spectra of compound Amentoflavone



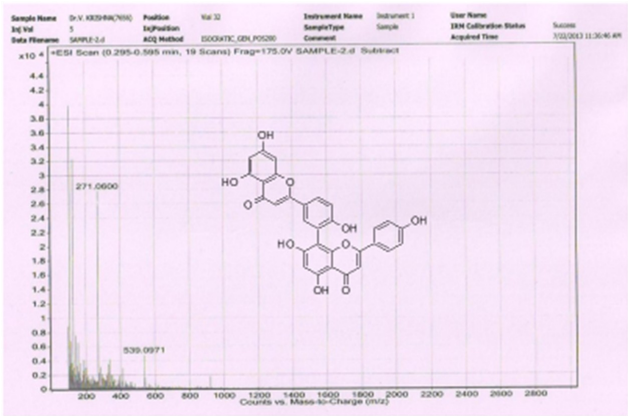
S1B: ^{1}H NMR spectra of compound Amentoflavone



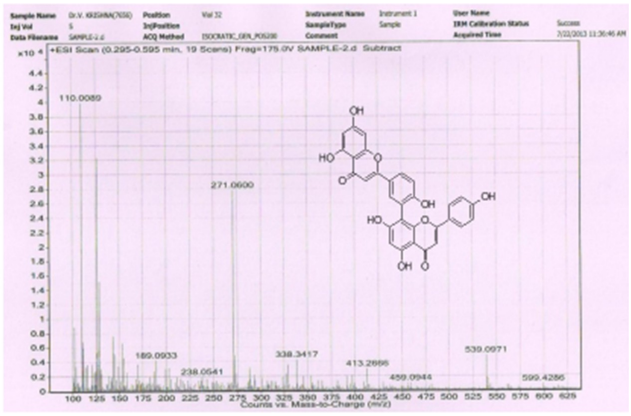
S1C: ^{1}H NMR spectra of compound Amentoflavone



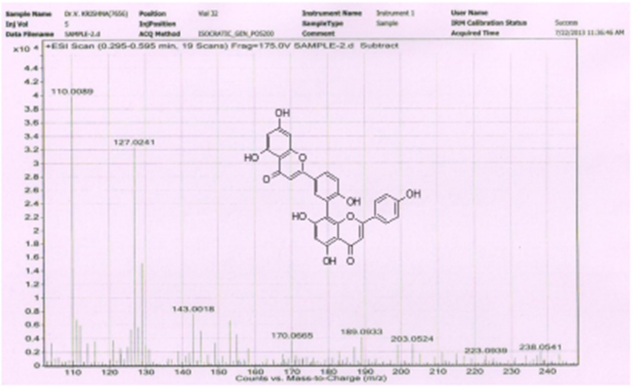
S2: IR spectra of compound Amentoflavone



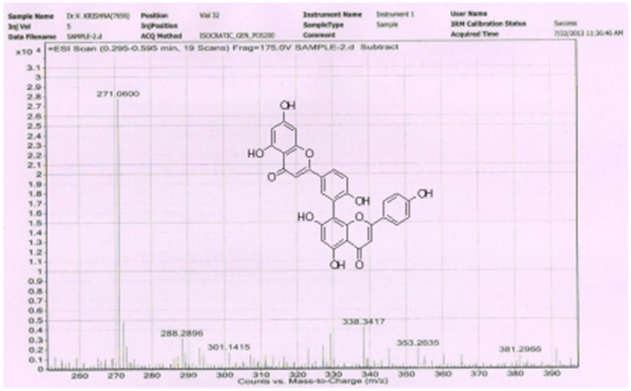
S3A: ^{1}H NMR spectra of compound Amentoflavone



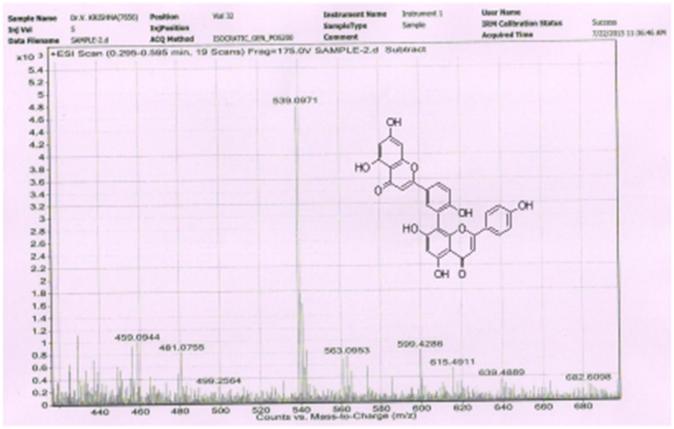
S3B: ^{1}H NMR spectra of compound Amentoflavone



S3C: ^{1}H NMR spectra of compound Amentoflavone



S3D: ^{1}H NMR spectra of compound Amentoflavone



S3E: ^{1}H NMR spectra of compound Amentoflavone