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Anthraquinones from leaves of *Tectona grandis*: A detailed study on its antibacterial activity and other biological properties

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Abstract

The search for new molecules against pathogenic species continues unabated due to drug resistance. *Tectona grandis*, commonly known as teak, is a widespread woody plant with lot of biological properties. In the present study, attempts were made to isolate antibacterial compounds from *Tectona grandis* against Staphylococcus aureus, Klebsiella pneumoniae, Salmonella paratyphi and Proteus mirabilis at different concentration. Antimycobacterial activity was checked against Mycobacterium tuberculosis. Cytotoxicity of isolated compounds was evaluated. As part of activity studies, antioxidant potential of both compounds was also checked. Antibacterial activity was checked by disc diffusion and microplate dilution method. Cytotoxicity of pure compounds was evaluated by MTT assay. Antioxidant activity was checked against DPPH and $ABTS^+$ free radicals. Two compounds isolated from chloroform extract of leaf showed activity against S. aureus (Compound 1: MIC – 2.5µg/ml, IC_{50} - 72µg/ml ; Compound 2: MIC - 5 µg/ml, IC_{50} - 98 µg/ml) and K. pneumoniae (Compound 2: MIC – 6.2 µg/ml, IC_{50} – 113.5 µg/ml). These compounds failed to show antimycobacterial activity on testing against M. tuberculosis. On cytotoxicity analysis of both compounds against chick embryo fibroblast (CEF), HEK293, HCT119 and L929 cells, compound 2 showed activity against HEK293 (IC_{50} - 2 µg/ml). Antioxidant activity of these compounds was very low and was able to scavenge only 10% of free radicals even at the highest concentration (1000 µg/ml) tested. Purity of compounds was confirmed by HPLC analysis and structural characterization was carried out based on IR and NMR spectral data with supporting phytochemical results.

Keywords: Anti-bacterial, Anthraquinones, MIC, MTT assay, Antioxidant, Anti-mycobacterial, HPLC profile, Structural studies.

Introduction

Infectious disease is considered as the second leading cause of death in developing countries and stands third in developed countries [1,2]. A recent survey about High Thoroughput Screening (HTS) by pharmaceutical companies and academic laboratories showed that not even a single anti-bacterial drug was able to get screened from those with activities defined. However, most of the large pharmaceutical companies ceased their antibacterial screening procedures by HTS and drug discovery and consequently lost their natural product libraries [3]. The uses of traditional medicinal plants for primary health care have steadily increased worldwide in recent years. Most of the antibacterial agents currently in use are natural products or potent semi-synthetic variations thereof [4, 5]. Of 90

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antibacterial drugs that became commercially available worldwide, about 79% are natural product origin [3]. Intense use of antibiotics has resulted in development of resistant strains all over the world, especially in developing countries. Several reports has mentioned about the increasing rate of drug (oxazolidinones, CTX-M β-lactams, sulfonamides, polyketides, carbapenems etc) resistant microorganisms in developed and developing countries [6,7]. The search for new molecules against pathogenic species continues unabated due to drug resistance. Worldwide, industries are in search of plant secondary metabolites that possess anti bacterial and antifungal activities.

In the present study, we tried to isolate compounds with antibacterial from different parts of *Tectona grandis*, commonly known as teak. Even though commonly seen, teak has not been explored much on pharmacological aspects than for its timber value. The durability and strength of teak wood implies its efficiency to fight against pests and micro organisms. According to Ayurveda, wood is acrid, colling, laxative, sedative to gravid uterus and useful in treatment of piles, leucoderma and dysentery. It allays thirst and also possesses anthelmintic and expectorant properties. Teak wood is considered as anti-inflammatory and effective internally against dermatitis or as vermifuge [8,9]. Lot of chemicals, with and without biological activity has been isolated from teak. Many anthra- and naphtho-quinones isolated from teak is reported to have biological activity. Some are 2-hydroxymethylanthraquinone, 3'-OH-deoxyisolapachol, hemitectol, 5-hydroxyl-1-4-naphthalenedione, lapachol, deoxylapachol, tectoquinone etc [10-14].

Previously, studies have been carried out by authors on antibacterial activity of hexane, chloroform, ethyl acetate and methanol extract of leaves, bark and wood of teak [15]. Based on the results, chloroform extract of leaves found to possess potent antibacterial activity and was selected for isolation of active ingredient(s). Our systematic search of antibacterial compounds from teak was divided in to two stages. 1) The extract with antibacterial activity was selected for separation and purification of active components followed by its chemical characterization and confirmation of activity. 2) The second stage of work consisted of evaluating anti-mycobacterial, cytotoxic and free radical scavenging potential of isolated compounds.

Materials and Methods

General

UV-Vis spectrum was taken with spectrophotometer (UV1700, Shimadzu, Japan), FT-IR spectrum recorded with Spectrum 100 (Perkin Elmer, Beaconsfield, UK). H-NMR spectrum of compounds recorded with 500MHz spectrometer (Avance^{II} 500, Bruker, USA).

Plant material and extraction

Leaf, bark and wood of *Tectona grandis* were selected for the study and collected from Trivandrum in Kerala, India during the month of August 2008. Plant material was compared with deposited specimen (No. 5616, 5617) at Institute herbarium, Kerala Forest Research Institute (KFRI), Kerala, India by Dr. Valsala devi (Reader and Curator, Dept. of Botany, University of Kerala). A voucher specimen deposited at herbarium of Department of Botany, University of Kerala. Plant materials after collection were washed, shade dried, powdered and stored in moisture free condition until use. 200g of each plant material was extracted serially with hexane, chloroform, ethyl acetate and methanol of 300ml each using soxhlet apparatus. Residue was collected and weighed after evaporating solvents under reduced pressure. Dried extracts were stored at -20°C till the extraction of all parts got completed. Extracts at different concentrations were prepared from a master mix of 10mg/ml for equal distribution of components. All solvents used for extraction were of analytical grade and purchased from Merck specialties (India). For extraction and separation studies, solvents were further purified by distillation.

Isolation of active compounds

Chloroform extract (6.2g) was selected based on preliminary studies and fractionated with hexane. The hexane fraction (2 mg) was re-dissolved in chloroform and separated by silica column chromatography (Silica gel 60-120, Merck, India). Column packed and elution started with chloroform to ethyl acetate (100:0 - 0:100), progressing to ethyl acetate – methanol (100:0 – 1:1) with 5% gradient increase in polarity. Collected fractions (15 ml each) were dried in vacuum and checked by thin layer chromatography (Silica G, Merck, India). Positive fractions were pooled (500 mg) and did re-column under above mentioned conditions. Active fractions (4.6%, 23 mg) were separated by preparative TLC and purity checked by HPLC (600 series, Waters, USA) in µ-Porasil column at a flow rate of 1ml/min, UV detector – 248 nm. Phytochemical analysis and chemical characterization of isolated pure compounds (will be denoted as **C-1** and **C-2** here after) were carried out.

Biological studies

Disc diffusion method

Antibacterial activity of Anthraquinones C-1 and C-2 was checked by disc diffusion method as described by Darias et al. [16]. Briefly, paper discs of 5mm diameter were impregnated with 5µg of compound and solvent allowed to evaporate under reduced pressure. 200µl of bacterial culture (McFarland standard 0.5) was spread on sterile nutrient agar plate before placing compound impregnated discs and allowed to incubate overnight in 37 ± 2°C. Carrier solvent containing discs served as control. Result expressed as diameter of inhibition zone and compared with standard antibiotic ciprofloxacin.

MIC and IC_{50} values

Minimum Inhibitory Concentration (MIC) and IC_{50} values of **C-1** and **C-2** against sensitive organisms were determined by microdilution method [17]. Overnight grown culture was diluted with sterile distilled water to McFarland standard 0.5 and loaded in 96-well plate. Compounds were tested at different amounts from 100ng - 25 µg. Ciprofloxacin served as standard and was given at same concentrations. The plate was set to incubation at 37ºC overnight. Absorbance read at 600nm with microplate reader (Model 680, Bio-Rad, USA).

Anti-mycobacterial activity

Anti-mycobacterial activity of both **C-**1 and **C-2** were checked as described previously (Banfi et al., 2003). In brief, M. tuberculosis H37Rv was grown in Middlebrook 7H9 broth (Difco BBL, USA) supplemented with 10% OADC (Becton Dickinson, USA). The culture was diluted to McFarland 1 standard with the same medium. From this, 50µl culture was added to 150µl of fresh medium in 96 well microtitre plates. Stock solutions (2 mg/ml) of the test compounds were prepared in dimethyl formamide (DMF). The compounds were tested at 1, 10 and 100 µg/ml, concentrations. Control wells had the same volumes of DMF without any compound. Rifampicin (0.5µg/ml) served as positive control. After incubation at 37ºC for 7 days, 15µl of 0.01% Resazurin (Sigma, St. Louis, MO, USA) in water was added to each well. The compounds which prevented the change of colour of the dye were considered to be inhibitory to M. tuberculosis.

Cytotoxicity studies

Toxicity of **C-1** and **C-2** were checked against four cell lines. Chick embryo fibroblast (CEF), HEK 293 (kidney), HCT119 (colon) and L929 (fibroblast) cells were selected for the study and maintained in DMEM (PAA laboratories, GmbH) with 10% FBS (Gibco BRL, NZ). Chick fibroblast cells were isolated from 10-11 days incubated embryo. Cells after removal were washed in PBS and trypsinized. After centrifugation at 1500 rpm for 2 minutes, detached cells were pipette out and cultured in MEM with 20% serum. Growth conditions were set at 37ºC and 5% CO_2. 2 x 10^4 cells were seeded per well at log phase in 96-well plate and compounds given at 0.5, 5, 12.5 and 50 µg/ml concentration. Plate was incubated for 24h and

effect of compound on cell viability checked by MTT assay [18]. Absorbance measured at 570 nm using microplate reader (Model 680, Bio-Rad, USA). The values were estimated by plotting drug concentration (µg/ml) against percentage of cell viability. Plasticwares used for cell culture were purchased from Greiner Labortechnik, GmbH, Germany.

Antioxidant activity

Antioxidant activity of isolated compound was checked by estimating its potential to scavenge DPPH and $ABTS^+$ free radical. DPPH assay was carried out as previously described protocol (Lee et al., 2002) and activity at 5-1000 µg quantities were checked and compared with quercetin. Effect of compounds on $ABTS^+$ decolourization was checked according to previously described procedure (Re et al., 1999). Activity at different quantities (1, 10, 100 and 1000 µg) and time (1-6 min) and SC_{50} (minimum concentration needed to scavenge 50% free radical) values were calculated based on equation 1. All experiments were repeated three times and standard deviation calculated.

$$\% \text{ inhibition} = 100(A_{blank} - A_{sample})/A_{blank} \quad \text{(Eq. 1)}$$

Spectral details

C-1: Orange powder, **UV (λ max)**: 239, 265, 275, 475 nm; **IR (cm^{-1})**: 3400, 2920, 2850, 1730, 1600, 1463; **Fab^+- MS (m/z)**: 391[M+H]; 295, 279 [295 - CH_2], 239 [279 - CH-OH], 219, 167, 149; **^{1}H-NMR ($CDCl_3$, 500MHz)**: δ 1.25(1H), 1.85(1H), 3.28(1H), 3.29(1H), 3.72-3.76 (10H), 4.19(1H), 4.2(2H), 4.22(1H), 4.29(1H), 4.90(3H), 4.92(3H), 4.95-4.96(2H), 4.99(2H), 5.16-5.17(3H), 5.76(1H), 5.78(1H), 5.79(1H), 5.81(1H), 6.56(1H), 6.57(1H), 7.14(1H), 7.16(1H), 7.26(1H), 7.28(1H), 7.51-7.53(4H), 7.68-7.69(2H), 7.69-7.70(2H), 11.48(1H), 12.82(1H). (Supplementary file 1)

C-2: Reddish orange powder, **UV (λ max)**: 211, 248, 492 nm; **IR (cm^{-1})**: 3444, 2925, 2854, 1736, 1608, 1455; **Fab^+- MS (m/z)**: 385[MH+]; 337, 279, 191, 149, 109; **^{1}H-NMR ($CDCl_3$, 500MHz)**: 0.88-0.89(2H), 1.25(1H), 1.28-1.29(2H), 1.33(1H), 1.37(1H), 1.41-1.42(2H), 1.60(1H), 2.11(1H), 3.49(1H), 4.07-4.1(2H), 4.19-4.21(2H), 4.91-4.93(6H), 4.97(2H), 5.0-5.01(2H), 5.78-5.84(4H), 6.71(1H), 6.74(1H), 6.94(1H), 6.97(2H), 7.09(1H), 7.2(2H), 7.26(1H), 12.52(1H), 12.72(1H). (Supplementary file 2)

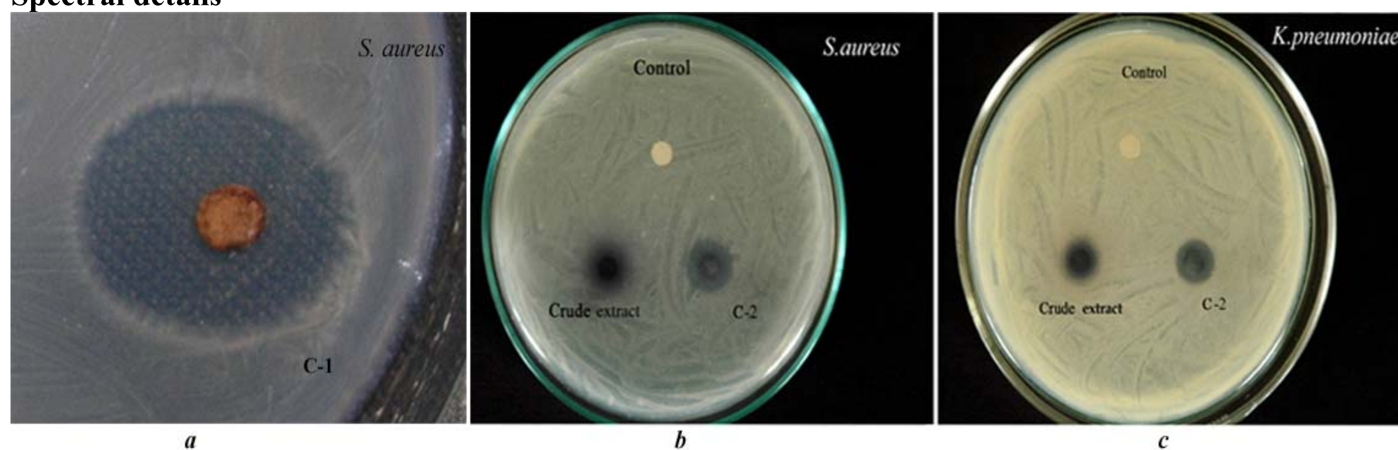

Fig 1: Antibacterial activity of isolated compounds against bacterial strains. a) C-1 against S. aureus. b) C-2 against S. aureus and c) K. pneumoniae

Table 1: Result of phytochemical tests to identify the compounds C-1 and C-2.

Phytochemical Tests	C-1	C-2
Frothing test for **saponins**	-	-
Colour test for **Flavanoids**	-	-
Borntrager's test for **Anthraquinones**	+	+
Test for **alkaloids**		
Dragendorff's test	-	-
Wagner's test	-	-
Hager's test	-	-
Ferric chloride test for **tannins**	-	-
Lieberman-Burchard and Salkowski test for **terpenoids**	-	-
Baljet reagent test for **sesquiterpene lactones** and **cardiac glycosides**	-	-

Results

Antibacterial activity and chemical characterization

Activity guided fractionation of chloroform extract of leaf yielded two anthraquinones and was checked by disc diffusion assay (5µg/disc) against Staphylococcus aureus, Klebsiella pneumoniae, Salmonella paratyphi and Proteus mirabilis. **C-1** exhibited activity against S.aureus with inhibition zone of 25mm diameter. Compound **C-2** showed antibacterial against S.aureus (Zone diameter - 24mm) and K. pneumoniae (Zone diameter - 17mm) (Fig 1). Activity of both compounds against S.aureus was more than ciprofloxacin (Zone diameter – 22.2mm).

Purity of both compounds were confirmed by HPLC (Fig 2 &3) and identified as anthraquinone derivatives with the help of various spectroscopic data, MS and chemical tests (Table 1). The structural characterization of compounds was based on various spectral data such as UV-Visible, IR and ^{1}H-NMR. In UV spectrum, λ_{max} at 239 and 423 nm for C-1 and 248 and 475 nm for C-2 denotes the presence of quinone ring. IR analysis of C-1 showed peaks at 3400 (hydroxyl), 1730 (non-chelated carbonyl), 1600 cm^{-1} (ester) and C-2 showed peaks at 3444 (hydroxyl), 1736(non-chelated carbonyl), 1608 cm^{-1} (ester). Peaks denoting C-H stretch (2920 and 2850 cm^{-1} for C-1, 2925 and 2854 cm^{-1} for C-2) and C-C bonds (1450 cm^{-1} for C-1, 1455 cm^{-1} for C-2) confirmed the compounds to be anthraquinone derivative.

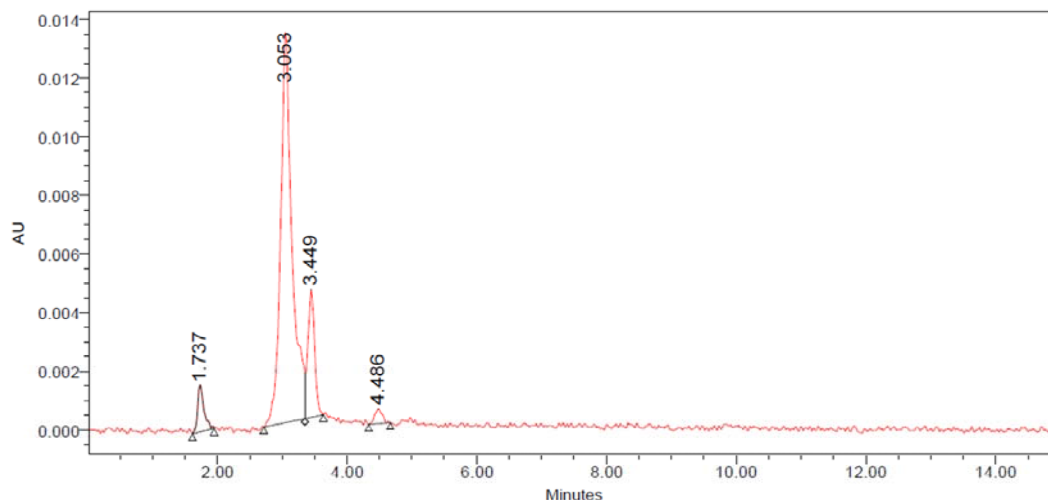

Fig 2: HPLC chromatogram of compound C-1.

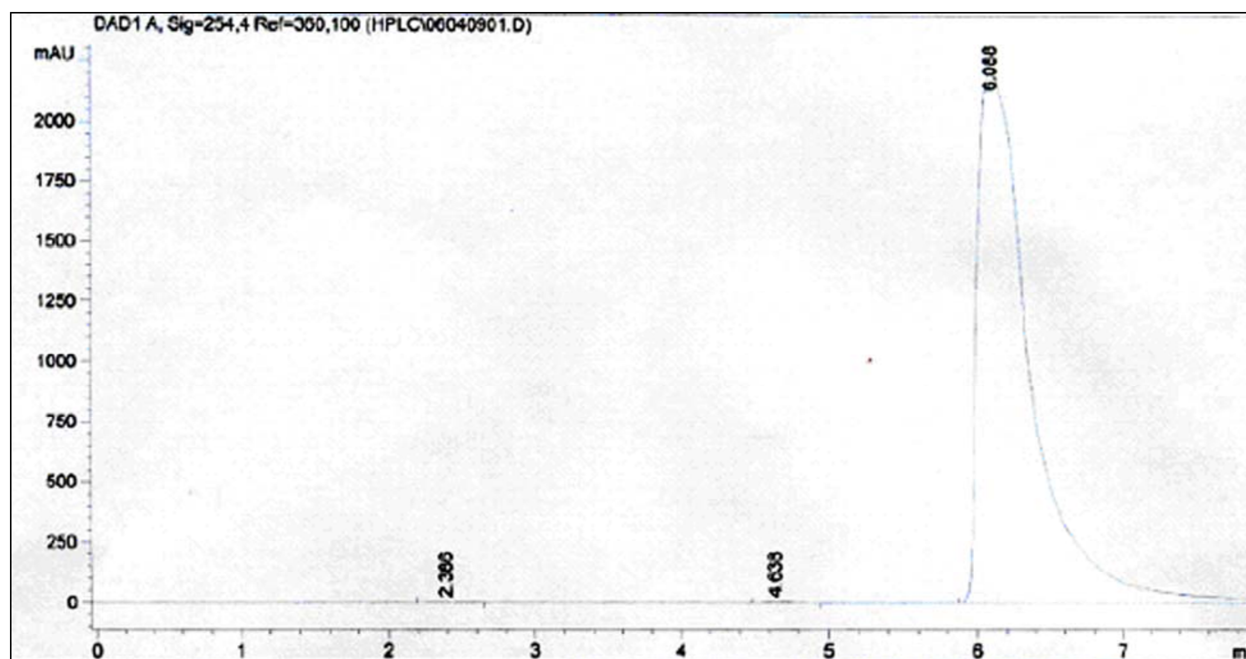

Fig 3: HPLC chromatogram of compound C-2.

MIC and IC_{50} values of C-1 and C-2

Minimum inhibitory concentration of C-1 as tested by microdilution plate method is 2.5 µg/ml and IC_{50} at 72µg/ml against S. aureus and that of **C-2** is 5µg/ml and 98µg/ml respectively against S. aureus. Against K. pneumoniae, **C-2** exhibited MIC of 6.2µg/ml and IC_{50} at 113.5µg/ml. Control antibiotic ciprofloxacin showed 0.15µg/ml against S.aureus and 62µg/ml against K. pneumoniae.

Other biological activities of C-1 and C-2

Anti-mycobacterial activity: **C-1** and **C-2** at 1, 10 and 100 µg/ml concentration showed that both were not active against M. tuberculosis. The study was based on change in colour of redox dye resazurin.

Cytotoxicity: Cytotoxic potential of **C-1** and **C-2** against cell lines (chick embryo fibroblast (CEF), HEK 293, HCT119 and L929) were determined by MTT assay (Fig 2). The potential to inhibit growth of cells were determined based on IC_{50} values. Results showed that **C-1** was not able to elicit significant toxicity against CEF, HEK293, HCT119 and L929 cells even at the highest concentration tested (50µg/ml). The second compound **C-2** showed high toxicity against HEK293 (IC_{50} - 2 µg/ml) while other cell lines remain unaffected.

Antioxidant activity: Analysis of antioxidant potential of **C-1** and **C-2** proved that they are ineffective in scavenging DPPH and $ABTS^+$ free radicals. Even at the highest concentration tested (1000 µg) both compounds was not able to scavenge even 10% DPPH and was very low compared to quercetin (SC_{50} - 20µg/ml) and the increase in activity was not dose-dependent. The condition was same in $ABTS^+$ assay with decolourisation of free radical less than 10% at highest concentration tested (1000µg) and was very low compared to Trolox (SC_{50} 4µg/ml) . Previous studies proved that radical scavenging potential of anthraquinones is less relevant compared to standard compounds [19-21].

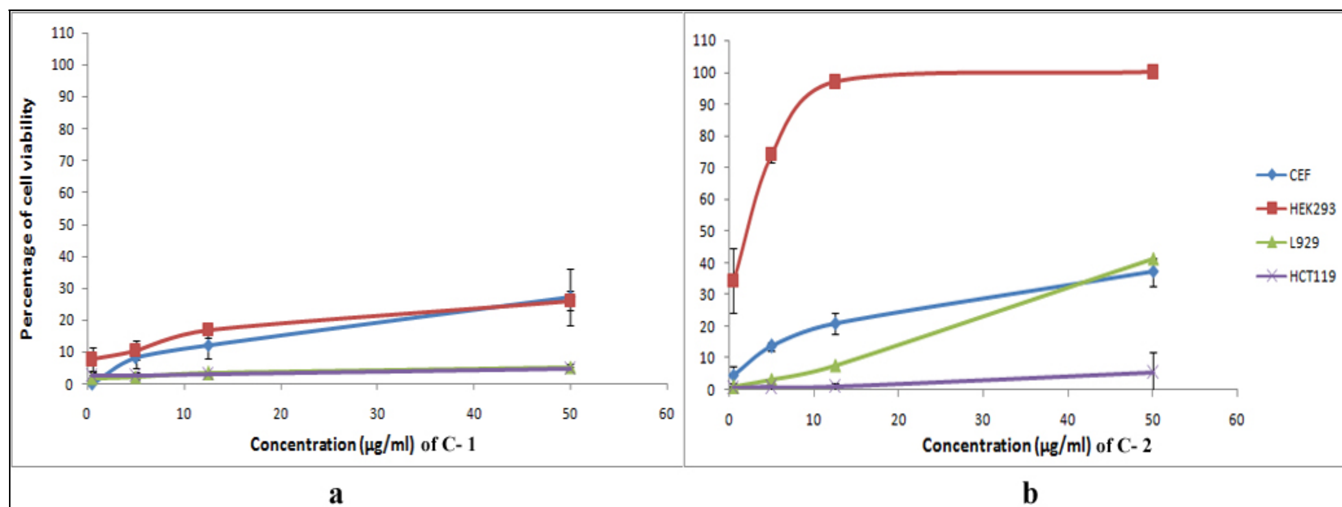

Fig 4: Cytotoxic effect of antibacterial compounds at different concentrations against various cell lines a) showing activity of C-1 b) showing activity of C-2

Discussion

Aim of the present study was to characterize antibacterial potential of *Tectona grandis* and to isolate active compounds. Chemical characterization of compounds and its further biological activities were also checked as a part. Preliminary study on antibacterial activity of crude extract from leaf, bark and wood showed that chloroform extract of leaf is most promising. Out of the four cultures tested, it showed good activity against *S. aureus* and *K. pneumoniae* at minimum concentration. The result supports previously reported data on antimicrobial activity of aqueous extract of teak [15,22].

From the literature survey, tectoquinone, an anthraquinone from teak sawdust, possess antifungal activity [10]. Naphthoquinones like juglone, lapachol and deoxylapachol reported in teak also possess antimicrobial activity [11,12]. MIC of some of the anthraquinones with anti-staphylococcal activity include emodin (3.9 µg/ml), parietin (320 µg/ml), fallacinol (20 µg/ml), fallacinal (160 µg/ml), parietinic acid (80 µg/ml), emodin (320 µg/ml). Anthraquinones with reported anti-Helicobacter pylori activity are 2-(Hydroxymethyl) anthraquinone (2 µg/ml) and anthraquinone-2-carboxylic acid (8 µg/ml) [23-25]. Some of the anthraquinones were able to inhibit Gram-negative bacteria (Pseudomonas fluorescence) with high MIC values [23]. But none of them are able to inhibit growth of K. pneumoniae even at higher concentrations. On comparison with reference data, we are able to say that compound **2** is exhibiting fairly high antibacterial activity against Gram positive and negative species. Many of the antibacterial compounds isolated from natural sources are able to inhibit Gram positive species but inactive against Gram negative. The anthraquinones isolated in the present study has not been reported earlier from *Tectona grandis*.

The studies on other biological activities of the isolated compounds C-1 and C-2 demonstrated the effectiveness of C-2 in inhibiting the uncontrolled growth of HEK293, a transformed cell line. Even though C-1 and C-2 were not able to inhibit the growth of Mycobacterium tuberculosis, it should be stated that no work has been carried out so far on checking antimycobacterial activity of either crude extracts or compounds from *Tectona grandis*. Both compounds were also not able to show any reduction in free radicals such as DPPH and $ABTS^+$ at any concentrations tested. On comparison, the values recorded for the isolated compounds were much less than that of standards.

These results clearly emphasis and supports the need of these two compounds to enter the next

level of pharmacological trials and to develop synthetic varieties of these compounds with more virulence and wide action spectrum.

Conclusion

In conclusion, the antibacterial activity of *Tectona grandis* and its active ingredient was investigated. With spectral and phytochemical data, the compounds were classified as anthraquinone. Its activity against S.aureus and K. pneumoniae was confirmed with MIC and IC_{50} values. A detailed structural analysis is needed for elucidating the mode of action of the compound. As the compound is non-toxic to normal cells selected for present study, it can be considered for further steps in drug development.

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