

Bioprospecting the antimicrobial, antibiofilm and antiproliferative activity of *Symplocos racemosa* Roxb. Bark phytoconstituents along with their biosafety evaluation and detection of antimicrobial components by GC-MS

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Abstract

Background: The increased occurrence of the antibiotic resistance has added up to the misery of mankind. There is an immediate need to search alternatives and *Symplocos racemosa* is one such potent medicinal plant. The aim of the study was to explore various activities of its phytoconstituents, confirm their biosafety and identify the active components.

Methods: The ethylacetate extract and the major group of phytoconstituents were tested for minimal inhibitory concentration, viable cell count studies, Post antibiotic effect, activity against 34 multi-drug resistant microorganisms, anti-biofilm and anti proliferative potential. The biosafety was evaluated by Ames and MTT assay. In vivo safety of flavonoids was determined by acute oral toxicity test using Swiss albino mice. The antimicrobial components in flavonoids were also identified by using TLC and GC-MS analysis.

Results: Ethyl acetate was the best organic extractant. The phytochemical analyses revealed the presence of flavonoids, cardiac glycosides, saponins, tannins, triterpenes and Phytosterols. Flavonoids exhibited the highest antimicrobial potential whereas tannins, triterpenes and phytosterols were completely inactive. Their Minimum Inhibitory Concentration ranged from 0.5-10 mg/ml; exhibited a bactericidal mode of action and a prolonged post antibiotic effect. The phytoconstituents displayed a significant antibiofilm potential by inhibiting the initial cell attachment, disrupting the pre-formed biofilms and reducing the metabolic activity of biofilms. The phytoconstituents were significantly active against the drug-resistant strains of *E.coli*, MRSA and *Salmonella* spp. Flavonoids showed significant cytotoxic effect against the RD, L20B and Hep2 cell lines (IC 50 361-494 µg/ml). All the test extracts were biosafe as depicted by the Ames test, MTT assay. Also, flavonoids did not induce any abnormality in biochemical parameters and Organs' histopathology of the Swiss albino mice during the in vivo acute oral toxicity studies. The flavonoids were resolved into 4 bands (S1-S4) using TLC, where S3 was the most active and its GC-MS analysis revealed the presence of Bicyclo [2.2.1]heptan-2-one,1,7,7-trimethyl-, (1S)- at an RT of 11.14 as the major compound.

Conclusions: These findings suggest that *Symplocos racemosa* bark could be a potential source of antimicrobial metabolites, which could substantially contribute to the arsenal of existing natural drugs for combating the antimicrobial resistance.

Background

Antibiotics have revolutionized the medical field and have helped us to live a healthy life. However, incidences of multiple resistances in human pathogenic microorganisms are on the rise and the problem has now become a global concern. The scientists are studying every aspect of antibiotic resistance [1] to come forward with possible ways out.

To meet out this objective, scientists are searching for new antimicrobials from various sources, where natural products still remain as one of the best reservoir for new compounds. The use of medicinal herbs is an age-old tradition and the recent progress in modern therapeutics has stimulated for further exploitation [2]. Numerous studies have revealed the potential of herbs as sources of drugs and have subsequently identified natural plant-based antimicrobial compounds [3–6]. The activity of these plants against different bacteria, fungi and parasites might be due to the presence of a wide variety of active secondary metabolites such as flavonoids, phenolic acids, coumarins, terpenoids and sterols [7–11].

In addition to being a potential source of novel antimicrobials, plants also play an important role in chemotherapy and represent a promising source of anticancer agents. However, the use of herbal products should be based on scientific validation as the traditional use of any plant for medicinal purposes, by no means, guarantees the safety of the plant or treatment. Toxicity testing can identify any such risk that may be associated with herbal usage, therefore avoiding their potential short term or long term negative effects when used as medicine [12].

One such potential medicinal plant is *Symplocos racemosa* Roxb. (also known as Lodhra) from the family Symplocaceae, which is an evergreen tree or shrub found in the plains and lower hills throughout North and East India, ascending in the Himalayas up to an elevation of 1400 m [13]. Traditionally, bark is given in menorrhagia and other uterine disorders. It is a potent remedy for inflammation and cleaning uterus [14]. Since the potential of its aqueous extract has been reported earlier in Sood et al. [15], the present study has thus been further extended to evaluate the best organic solvent and hence the major groups of bioactive phytoconstituents. The organic extract and active phytoconstituents have been tested for their various bioactivities such as antimicrobial [minimal inhibitory concentration (MIC), viable cell count (VCC) studies, Post antibiotic effect (PAE), antimicrobial efficacy against clinical isolates of multi-drug resistant microorganisms], anti-biofilm and anti proliferative potential against the cancerous cell lines (RD, L20B and Hep 2). The biosafety of all the test extracts was evaluated by Ames and MTT assay. The in vivo biosafety of the flavonoids [at a single dose (5000 mg/ kg)] has also been determined by acute oral toxicity test using Swiss albino mice (males and females) weighing between 25 g to 35 g and aged 8 to 10 weeks [12, 16–18].

Methods

Plant material

The plant *Symplocos racemosa* bark was procured from a local market of Amritsar, Punjab, India; which has been identified and deposited in the Department of Botanical and Environmental Sciences, Guru Nanak Dev University, Amritsar wide accession no. 766-768 Bot.& Env. Sc. dated 02/07/15. The plant material was sterilized as described earlier in Sood et al. [15].

Test microorganisms and Inoculum preparation

Reference strains: *Enterococcus faecalis* (MTCC 439), *Staphylococcus aureus* (MTCC 740) and *Staphylococcus epidermidis* (MTCC 435) as Gram positive bacteria; *Escherichia coli* (MTCC 119), *Klebsiella pneumoniae* 1 (MTCC 109), *Klebsiella pneumoniae* 2 (MTCC 530), *Pseudomonas aeruginosa* (MTCC 741), *Salmonella typhimurium* 1 (MTCC 98), *Salmonella typhimurium* 2 (MTCC 1251) and *Shigella flexneri* (MTCC 1457) as Gram negative bacteria and yeast strains such as *Candida albicans* (MTCC 227) and *Candida tropicalis* (MTCC 230) were obtained from Microbial Type Culture Collection (MTCC), Institute of Microbial Technology (IMTECH), Chandigarh, India. A clinical isolate (MRSA) obtained from PGIMER, Chandigarh, India was also used in the study. Their glycerol stocks were preserved at -80°C.

Drug resistant strains- The drug resistant strains used in the study were clinical isolates: MRSA (DSECI 1-11), *Enterococcus* sp. (DSECI 12) (procured from Shri Guru Ram Das medical College & Hospital, Amritsar (Pb.), India; MDR *Escherichia coli* (CRIRS 1-12) and MDR *Salmonella* spp. (CRIRS 13-22) obtained from Central Research Institute, Kasauli (H.P.), India.

The inoculum of these organisms was prepared by 4h activation in a suitable broth followed by its standardization upto 0.5 McFarland standards [15].

Antimicrobial Screening of organic extracts of *Symplocos racemosa* bark

Different organic solvents were screened to work out the best extractant. The organic extracts were prepared by using solvent-solvent extraction of the aqueous extract with an equal volume of the given organic solvent for three times independently. The pooled organic layers were concentrated; the residue was reconstituted in 30% DMSO and then screened for antimicrobial activity against the test microorganisms by Agar Diffusion Assay (ADA), where 30% DMSO served as a control.

Qualitative and Quantitative phytochemical profiling of *Symplocos racemosa*

Qualitative analysis for the major groups of phytoconstituents in the powdered plant material was carried out by standard methods as (Additional file 1). The qualitatively detected phytoconstituents were then quantified as described previously [19, 20] and screened for their antimicrobial potential, which was then compared with standard antibiotics (Additional file 1).

Minimum Inhibitory Concentration (MIC)

The ethyl acetate extract and the active phytoconstituents were tested for their MIC against test organisms as per protocol followed in Arora and Sood [21]. The values were then compared with that of the standard antibiotics.

Viable Cell Count (VCC) studies and Post Antibiotic effect (PAE)

Time kill assay of the test extracts was done so as to ascertain the microbicidal/static nature of the compounds. A stock solution was prepared and the study was performed according to Arora and Onsare [22]. A comparison was done with standard antibiotics and the experiment was performed in duplicate.

The PAE of the ethyl acetate extract, flavonoids and cardiac glycosides was performed according to [23]. The diluted suspension of the test organism was exposed to the test compounds for 2 h at 37°C under shaking. The drug activity was stopped by diluting the suspension in a suitable double strength broth. Hundred microlitre, *i.e.*, 0.1 ml aliquot from each set was plated at an interval of two hours upto 24 h. The PAE was calculated as follows: $PAE = T - C$ (T= time required for the colony count in the test to increase by 1 log₁₀CFU/ml; C= the time required in case of untreated control).

Antibiofilm potential of the phytoconstituents of *Symplocos racemosa* bark

The antibiofilm potential was tested against four organisms, *i.e.*, *Escherichia coli*, *Staphylococcus aureus*, *Klebsiella pneumoniae* 1 and a yeast *Candida albicans* using protocol detailed in Additional File 2.

Screening for biofilm formation

The biofilm formation by test pathogens was screened out by the microtitre plate according to Stepanovic et al. [24] and Costa et al. [25], where the organisms were grown in suitable broth for 24 h. The 200 µl aliquot of the activated cultures were dispensed into microtitre plates and further incubated at for 24 h, following which the wells were decanted off and stained with 0.1% crystal violet solution for 15 min. The wells were destained with 150 µl of 95% ethanol and the intensity of biofilm formation was affirmed in terms of Optical density (OD) of stained adherent

biofilm by using an ELISA reader (Biorad 680-XR, Japan) at 590 nm. The results are interpreted by calculating the cut-off value (OD_c), which separates biofilm producers from the non-biofilm producing strains, as follows:

Optical density cut-off value (OD_c) = Average OD of the negative control + [3×standard deviation (SD) of negative control].

Upon confirmation the following assays were carried out to establish the antibiofilm potential of most active phytoconstituents.

Inhibition of initial cell attachment

The inhibitory potential of the partially purified phytoconstituents was carried out according to Jadhav et al. [26] and Onsare and Arora [27]. One hundred microlitres of the flavonoids and cardiac glycosides was added to the 96-well microtitre plates, to which an equal volume of the cultures was then added. The plates were incubated at suitable temperature for 24 h. Gentamicin and amphotericin B were used as positive control. Following incubation, the inhibition potential was established using crystal violet assay (described later).

Screening of phytoconstituents for their disruptive potential of preformed biofilms

It was performed as described earlier [25, 27] with slight modifications. One hundred microlitre aliquot of each of the 4h activated cultures were dispensed into a 96-well microtitre plate and were incubated at 37°C/24 h to allow biofilm formation and was treated with equal volume of the phytoconstituents. The plates were further incubated at suitable temperature and biomass content was estimated after 24 h by the crystal violet assay.

Estimation of biofilm biomass by Crystal Violet (CV) assay

The estimation of biofilm biomass was assessed by Crystal Violet (CV) assay [27-29]. Following the treatment of the test organisms, the culture medium from each well was discarded and plates were then washed with sterile distilled water and then dried, which were then stained with 100 µl of 0.1% crystal violet for 15 min. The plates were then repeatedly washed several times with sterile distilled water to remove unabsorbed stain. Absolute ethanol (125 µl) was then added to destain the wells and the absorbance was determined at 590 nm to quantitatively estimate the biofilm density and the percentage inhibition was calculated using the formulae:

Percentage inhibition= $100 - \left\{ \frac{\text{OD}_{590\text{nm}} \text{ test well}}{\text{OD}_{590\text{nm}} \text{ negative control well without antimicrobial agent}} \times 100 \right\}$.

Estimation of metabolic activity by XTT assay

Post exposure to the flavonoids, cardiac glycosides and standard antibiotics, the metabolic activity of the treated biofilms was assessed using the modified {2, 3-bis [2-methoxy-4-nitro-5-sulfophenyl]-2H-tetrazolium-5-carboxanilide} (XTT) reduction assay [27, 30]. After incubation of the biofilm with test extracts for 24 h, 200 µl of menadione–XTT working solution was added in respective well and further incubated in dark for 2 h at the suitable temperature. Upon incubation, the quantification of color was done using microtitre plate reader (Bio-Rad 680-XR, Tokyo, Japan) at 490 nm. The mean absorbance of test wells was determined in comparison to that of negative control.

Antimicrobial potential of the phytoconstituents against clinical isolates of MRSA, some drug-resistant strains of *Escherichia coli* and *Salmonella* spp.

The Flavonoids and cardiac glycosides were tested for their antimicrobial efficacy by ADA against different strains of MRSA, drug-resistant *Escherichia coli*, *Salmonella* spp. and *Enterococcus* sp. The MIC of the most sensitive organisms was also worked out by broth dilution method [31]. The lowest concentration with no visible growth was defined as the minimum inhibitory concentration (MIC). To determine the MBC, the aliquots from the wells showing no visible growth were swabbed onto the nutrient agar plates and incubated at 37°C for 24 h. The concentration corresponding to no growth on the plates was taken as MBC.

In vitro cytotoxicity against cancerous cell lines RD (Human Rhabdomyosarcoma), L20B (Diploid mouse lung cell line) and Hep 2 (Human epithelioma of larynx) by MTT assay

The cytotoxic effect of the most active phytoconstituent, *i.e.*, flavonoids was studied against these three cell lines obtained from Central Research Institute (C.R.I), Kasauli, Himachal Pradesh, India by MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide] assay as described previously [32-34] with slight modifications. The experiment was performed in triplicates as per protocol detailed in Additional file 3. A stock solution (10 mg/ml) was prepared and subjected to two fold serial dilutions ranging from 10 mg/ml to 0.039 mg/ml for the experimentation. The percent growth inhibition was calculated with respect to untreated control. The IC₅₀ of the flavonoids was calculated from the dose-response curve generated for each cell line.

Biosafety evaluation of *Symplocos racemosa* bark

Ames mutagenicity test

The test extracts were subjected to Ames test [21]. The overnight activated inoculum of *Salmonella typhimurium* (MTCC 1251, IMTECH, Chandigarh) was serially diluted and eventually mixed with equal volume of the extract (MIC concentration) and was then added to the top agar containing a histidine–biotin mixture (1:1 ratio). Here, sodium azide was used as a positive control.

MTT toxicity assay

In order to ascertain the cellular toxicity of the test extracts, MTT [3-(4, 5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide] assay was performed as described in Eren and Ozata [35] with slight modifications. The wells with untreated cells served as control and percent viability was thus calculated.

Acute Oral Toxicity study of *Symplocos racemosa* flavonoids in Swiss albino mice

In order to validate the non-toxicity of the compound in animal models, acute oral toxicity was studied as described previously [12, 16] with slight modifications. The experiment was conducted at Central Research Institute, Kasauli, Himachal Pradesh, India as per the protocol detailed in Additional file 4. Healthy Swiss albino mice (males and females) weighing between 25 g to 35 g and aged 8 to 10 weeks were obtained from the Animal house, Central Research Institute. The mice were randomly divided into 2 test groups (6 male; 6 female) and 2 control groups (6 male; 6 female), where the test groups were exposed to a single dose (5000mg/kg) of the flavonoids by oral route. *This study was carried out in accordance with the principles of the Basel Declaration and recommendations of Organization of Economic Co-operation and Development (OECD) guideline 420 for testing of chemicals. The protocol was approved by the Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA), New Delhi, India (No. CPCSEA/IAEC/CRI/14-114-2016). On the 15th day, the final weight of mice was noted and was anaesthetized in the laboratory using xylaxine and ketamine (5mg/kg and 2.5mg/kg b.wt. respectively). The blood samples were collected via cardiac puncture in non-heparinized tubes. The serum samples,*

thus collected, were analyzed for determination of alanine aminotransferase (ALT), alkaline phosphatase (ALP), aspartate aminotransferase (AST), total bilirubin (TBIL), urea and creatinine levels. Following blood collection, all the animals were sacrificed by overdose of anesthesia. The vital organs mainly liver, kidney and heart were removed, cleaned with saline and subjected to histopathological analysis.

Identification of the bioactive components of the flavonoids

In order to find out the component responsible for antimicrobial activity of the most active phytoconstituent, *i.e.*, flavonoids, various techniques such as TLC, Preparative-TLC and Gas Chromatography-Mass Spectrometry (GC-MS) analysis were performed.

Thin Layer Chromatography (TLC) analysis

To work out the best solvent system, the quantitatively isolated flavonoids were subjected to TLC on the pre-coated Silica gel F₂₅₄ plates using different solvent systems. The developed chromatograms were allowed to air dry and were visualized using natural light, UV light (254nm, 365nm) and iodine vapors so as to assess the degree of separation of bands and the Retention factor (R_f) were calculated for the bands resolved in the most suitable solvent system. The best worked out solvent system was taken up for quantitatively separating the bands using Preparative TLC (P-TLC) method.

Quantitative separation of the bands using Preparative TLC (P-TLC) method and their antimicrobial screening

The bands were resolved and were separated in the selected solvent system using powdered Silica gel F₂₅₄. Once developed, the separated bands were scrapped off carefully into separate vials, which were eluted overnight in methanol. The solvent containing the eluted band was decanted off carefully, which was evaporated to dryness to obtain a constant weight. The separated bands were dissolved in a minimum known volume of methanol and were screened for their antimicrobial activity against *Staphylococcus aureus*, *Staphylococcus epidermidis* (Gram positive) and *Klebsiella pneumoniae* 1, *Shigella flexneri* (Gram negative) and a yeast *Candida albicans* using Disc diffusion method, where the filter paper discs were impregnated with 20 μ l of this suspension, while methanol alone acted as a negative control. The band/s showing the most significant antimicrobial activity was then subjected to GC-MS analysis.

Gas Chromatography-Mass Spectroscopy (GC-MS) analysis

The GC-MS analysis of the most active band/s was carried out using Thermo Trace 1300GC coupled with Thermo TSQ 800 Triple Quadrupole MS with column BP 5MS (30m X 0.25mm, 0.25 μ m). The instrument was set to an initial temperature of 60°C, and maintained at this temperature for 3 min. At the end of this period the oven temperature was raised to 280 °C, at an increase rate of 15 °C/ min and maintained for 19 min. Injection port temperature was ensured as 260 °C and Helium flow rate as 1 ml /min. The ionization voltage was 70eV. The samples were injected in split mode as 10:1. Mass spectral scan range was set at 50-650 (m/z). Using computer searches on a NIST Ver.2.1 MS data library and by comparing the spectrum obtained through GC-MS, compounds present in the flavonoids were identified.

Data analysis

The experiments were performed in duplicate and repeated thrice. The statistical analysis was done at 5% level of significance by IBM SPSS Statistics Data editor Version 20.

Results

Antimicrobial activity of organic extracts

Ethyl acetate extracted out maximum antimicrobial metabolites and demonstrated an average inhibition zone of 19.4 mm, closely followed by butanol extract. Hexane extract was active against only against *Candida albicans* and *Klebsiella pneumoniae* 1, whereas chloroform extract was completely inactive.

Qualitative and Quantitative phytochemical evaluation

Phytochemical groups such as flavonoids, cardiac glycosides, saponins, tannins, triterpenes and phytosterols were detected (Table 1); however, alkaloids, diterpenes and coumarins were absent. Among the isolated phytoconstituents, cardiac glycosides were the most abundant, *i.e.*, 50.25%/g plant powder, followed by flavonoids (10.39% /g) while triterpenes (1.193% /g) were present in least amount (Fig. A1 in Additional file 5).

Flavonoids were the most effective (Table 2), with inhibition zone ranging from 16.6mm to 25.66 mm, but were ineffective against *Enterococcus faecalis*. However, *Candida albicans* (25.66 mm) and *Candida tropicalis* (17.3 mm) were quite sensitive to it. Cardiac glycosides, with an inhibition zone ranging from 12.3 mm to 25.66 mm, where *Klebsiella pneumoniae* 1 (25.66 mm) and MRSA (17.66 mm) were most sensitive among Gram negative and Gram positive organisms, respectively. *Salmonella typhimurium* 1, *Escherichia coli*, *Shigella flexneri* and yeast *Candida tropicalis* were resistant to it. Saponins were effective only against *Klebsiella pneumoniae* 1 and *Candida albicans*. Triterpenes, tannins and phytosterols were completely inactive against all the test organisms. The efficiency of flavonoids was comparable to both the antibiotics in case of *Staphylococcus epidermidis* whereas it was comparable to chloramphenicol in case of *Escherichia coli*, *Klebsiella pneumoniae* 2 and *Pseudomonas aeruginosa*.

Minimum Inhibitory Concentration (MIC)

MIC of ethyl acetate extract, flavonoids and cardiac glycosides was quite variable (Table 3). Ethyl acetate extract was found to be highly potent, showing the lowest against *Salmonella typhimurium* 2 and *Pseudomonas aeruginosa*. MIC for cardiac glycosides and flavonoids ranged from 0.5-10 mg/ml and 0.7-10 mg/ml, respectively. For *Pseudomonas aeruginosa* and MRSA, cardiac glycosides showed higher MIC than the organic extract and flavonoids.

Viable Cell Count (VCC) study and Post Antibiotic Effect (PAE)

A variable killing time was observed for different test extracts against different test pathogens (Fig.A2 in Additional file 5). *Pseudomonas aeruginosa* and *Candida albicans* were killed instantaneously upon incubation with ethyl acetate extract whereas *Salmonella typhimurium* 1 took a maximum 24 h incubation period to achieve complete killing. In case of *Shigella flexneri* and MRSA, the killing time was similar to that of standard antibiotic. Flavonoids were quite effective where *Candida albicans* was killed instantaneously. *Escherichia coli* and *Pseudomonas aeruginosa* were killed completely in 2 h, whereas *Salmonella typhimurium* 1 and *Salmonella typhimurium* 2 took 6 h for complete killing. Their Killing time was lesser than Gentamicin in case of organisms like *Staphylococcus aureus*, *Klebsiella pneumoniae* 2, *Salmonella typhimurium* 2, *Pseudomonas aeruginosa* and *Candida albicans*. Cardiac glycosides had a kill time falling between 2-8 h, where 2 h incubation was sufficient to kill *Klebsiella pneumoniae* 1 and *Candida albicans*. They were better in case of organisms like *Klebsiella pneumoniae* 1 and

Klebsiella pneumoniae 2. All the test extracts were highly effective against *Escherichia coli* (2 h), *Pseudomonas aeruginosa* (0-4 h) and *Candida albicans* (0-2 h).

Flavonoids and cardiac glycosides showed a Post Antibiotic Effect (PAE) of 2-4 h and 2-6 h, respectively. Flavonoids were equally effective (4 h) against *Salmonella typhimurium* 2 and *Klebsiella pneumoniae* 2, whereas cardiac glycosides had a maximum effect against *Candida albicans* (6 h). The organic extract also showed a similar PAE of 2-6 h. Among all the test extracts, the longest effectivity was observed against *Candida albicans* (4-6 h), whereas for organisms like *Staphylococcus aureus*, *Salmonella typhimurium* 2 and *Klebsiella pneumoniae* 2, their effect lasted for 2-4 h only (Fig. A3 in Additional file 5).

Antibiofilm potential of the phytoconstituents isolated from *Symplocos racemosa* bark

The most active phytoconstituents, *i.e.*, flavonoids and cardiac glycosides were assessed for their antibiofilm potential against the test pathogens. The Optical Density cut-off value (OD_c) of the negative control was 0.097. All the test organisms were found to be strong biofilm-formers as their average OD values were higher than 4×OD_c (0.388), *viz.*, 0.649 (*Klebsiella pneumoniae* 1), 0.720 (*Staphylococcus aureus*), 0.892 (*Candida albicans*) and 0.557 (*Escherichia coli*).

Inhibition of initial cell attachment

The flavonoids and cardiac glycosides effectively inhibited the attachment of the planktonic cells of these organisms (Fig.1a). The inhibitory potential of the phytoconstituents and antibiotic (amphotericin B) was comparable in case of *Candida albicans*. Flavonoids (61.01-69.33%) were found to be more active than cardiac glycosides (61.64-66.66%) against all the test organisms. The maximum % inhibition was seen against *Candida albicans* and *Klebsiella pneumoniae* 1. The inhibitory potential of phytoconstituents was in close proximity to the respective antibiotics (gentamicin and amphotericin B).

Disruptive potential on the pre-formed biofilms

The test extracts exhibited varying efficacy towards the pre-formed biofilms of the test organisms (Fig.1b). Flavonoids were more effective to disrupt the pre-formed biofilms than the cardiac glycosides. In case of *Candida albicans*, the inhibitory potential of flavonoids (61.85%) and cardiac glycosides (57.73%) was comparable to amphotericin B (65.97%). A comparative effect was also exhibited by the cardiac glycosides (60.96%) and gentamicin (68.42%) against *Klebsiella pneumoniae* 1.

Metabolic activity of the treated pre-formed biofilms by using XTT assay

The inhibitory effect of flavonoids on the metabolic activity of the biofilms ranged from 50-64.73%, whereas for cardiac glycoside it was 45.88-62.11% (Fig.1c). The test extracts considerably reduced the cell viability, indicated by the reduced metabolic activity (low OD values). In case of *Candida albicans*, flavonoids (0.298) were more effective than cardiac glycosides (0.342), however, both of them had an effect comparable to amphotericin B (0.279). Cardiac glycosides reduced the metabolic activity of *Klebsiella pneumoniae* 1 biofilm more efficiently than the flavonoids, whereas, the effect of flavonoids and cardiac glycosides was almost similar in case of *Staphylococcus aureus*. These were found to be good biofilms inhibitors by efficiently reducing the metabolically active cells.

Antimicrobial potential of the phytoconstituents against the resistant clinical isolates

The most active phytoconstituents, when tested against the drug resistant clinical isolates of *E.coli* and *Salmomella* spp. (CRIRS1-22) as well as MRSA (DSECI01-12), showed a good antimicrobial potential (Table 4). Flavonoids were better as compared to cardiac glycosides and were most effective against CRIRS7 and CRIRS17. Cardiac glycosides were active only against 6 organisms (CRIRS 4, 5, 6, 8, 10, 13) with a relatively smaller Inhibition Zone (IZ). Flavonoids were effective against 11 out of 12 strains of MRSA with IZ ranging from 20-28.33 mm. DSECI04 was the most sensitive with IZ of 28.33 mm, while DSECI11 was completely resistant. Cardiac glycosides were effective against 8 out of 12 strains, where the inhibition zone ranged from 14-18 mm. DSECI04 was the most sensitive (18 mm), whereas the strains DSECI07, DSECI09, DSECI10 and DSECI11 were completely resistant. The *Enterococcus* sp. was susceptible to both flavonoids and cardiac glycosides. Because of their effectiveness, the flavonoids were further tested against the 9 most susceptible strains (CRIRS7, CRIRS8, CRIRS13, CRIRS17, DSECI03, DSECI04, DSECI07, DSECI09 and DSECI10) for their MIC and MBC. The MIC values ranged from 15-35 mg/ml, lowest being against CRIRS7, DSECI07 and DSECI10 (15 mg/ml). The corresponding MBC values ranged from 20-40 mg/ml (Table T1 in Additional file 5).

***In vitro* cytotoxicity studies by MTT assay against RD, L20B and Hep 2 cell lines**

The Flavonoids were further evaluated for their cytotoxicity against some cancerous cell lines. A considerably good effect was seen with IC₅₀ ranging from 361-494 µg/ml. The lowest IC₅₀ (361 µg/ml) was seen against Hep 2 cell line, whereas the highest IC₅₀ was observed against RD cell line. IC₅₀ for L20B cell line was 448 µg/ml (Fig. A4 in Additional file 5). In case of RD, complete rounding off of the cells was seen upto the concentration of 2.5 mg/ml, and thereafter the inhibitory effect gradually reduced down the concentration gradient. For Hep 2 and L20B, rounding off of the cells could be seen upto 1.25 µg/ml and 0.625 µg/ml, respectively.

Biosafety evaluation

All the test extracts, *i.e.*, ethyl acetate extract, flavonoids and cardiac glycosides found to be non-cytotoxic and non-mutagenic in nature as assessed by Ames test and MTT assay, respectively. No revertant colonies were seen in Ames test as compared to the positive control, *i.e.*, sodium azide (856), while in MTT assay, the test extracts showed a cell viability of >92%.

***In-vivo* acute oral toxicity studies**

The flavonoids, further tested for their toxicity in mice, did not show any toxic effects during the whole experimentation period as evident by the normal behavior and sleep patterns, salivation, breathing rate as well as the absence of any abnormalities on the skin, fur, eyes etc. No significant difference was seen between the weights of the test (6/6) and the control group (6/6) (Fig.A5 in Additional file 5). The absolute and relative weight of the vital organs such as liver, kidney and heart of the test groups (male and female) (6/6) showed no statistical difference from the control group (Table 5). The kidney and liver functioning remained normal in the test group (6/6) was completely normal since no statistical difference was seen in the serum levels of Urea, Creatinine, total bilirubin, AST, ALT and ALP for the treated groups in comparison to the control (Table 6). Further, the general and histopathological examination of the three vital organs revealed no abnormalities in terms of color, texture or structural organization. (Fig.2). The organs' sections showed normal architecture and cellular details of the myocardium, glomeruli-tubules and hepatocytes.

Identification of the bioactive components in *Symplocos racemosa* flavonoids

The bioactive compounds, which may be responsible for the antimicrobial efficacy of *Symplocos racemosa* flavonoids, were detected by separation using Preparative-Thin Layer Chromatography followed by their antimicrobial screening against two Gram positive, two Gram negative and an yeast strain. The most active band was then subjected to Gas Chromatography-Mass spectrometry (GC-MS) to identify the antimicrobial components present in it.

Thin Layer Chromatography (TLC) analysis

The flavonoids were best resolved using the solvent system consisting of Butanol, Ethanol and water in the ratio 4:1:2. This solvent system separated the flavonoids into 4 bands having a Retention Factor (R_f) in the range of 0.151-0.769. When observed under natural light, only 2 Bands were visible. Band 1 was seen as a dark brown spot while Band 2 was a light brown spot (Fig.3a). All the four bands were visible when observed in iodine vapors and UV short wavelength (264nm). Upon staining with iodine vapors, Band 1 was seen as the intensely brown spot and the other three bands showed light-brown intensity (Fig.3b), whereas these bands could be seen as blue spots under UV short wavelength (264nm). Here, Band 1 was intense in comparison to the other three bands (Fig.3c). When observed under long wavelength UV at 365nm, only Band 1 could be seen as a dark blue spot against the blue background while the other bands were not visible (Fig.3d).

Quantitative separation of the bands using Preparative- TLC (P-TLC) method and their antimicrobial screening

In case of *Symplocos racemosa*, 76.66mg dry wt. of the flavonoids, when subjected to P-TLC, resulted in the separation of 4 bands (S1-S4) which were obtained with a dry wt. of : S1 (5mg), S2 (8mg), S3 (12mg), S4 (9mg). These were dissolved in methanol and a 20 μ l aliquot was tested for their antimicrobial potential. Band S3 was significantly active against all the five test organisms. Band S1 was weakly effective against *Klebsiella pneumoniae* 1 and *Candida albicans* but was quite active against *Staphylococcus epidermidis*. Band S2 was negligibly active against *Staphylococcus aureus* and *Candida albicans*, but did not show any activity against *Klebsiella pneumoniae* 1, *Staphylococcus epidermidis* and *Shigella flexneri*. Band S4 was only active against *Staphylococcus epidermidis*. Since, Band S3 was significantly active against the test organisms; it was taken up further for spectroscopic studies.

Identification of the compounds present in the active band 3 (S3) by Gas Chromatography-Mass spectroscopy (GC-MS) analysis

Symplocos racemosa flavonoids, when subjected to P-TLC analysis revealed the presence of 4 bands, out of which Band 3 (S3) was found to be antimicrobially most active, when screened against various test organisms. Its GC-MS analysis revealed the presence of mixture of a number of compounds at RTs falling within the range of 3.22- 34.40 (Fig. 4). The library search led to detection of a number of compounds at each RT (Table 7). Based on the abundance in terms of area %, compounds at three RTs were found to be major compounds in this active band, which were identified as Bicyclo [2.2.1]heptan-2-one,1,7,7-trimethyl-, (1S)- at an RT of 11.14; Silane, Dimethyl(dimethyl(dimethyl(2-isopropylphenoxy)silyloxy)silyloxy)(2-isopropylphenoxy)- at an RT of 13.47 and 7,11b-Dihydro-6H-indeno[2,1-c] chromene-3,4,6a,9,10-pentol pentakis (trimethylsilyl)ether at an RT of 34.40.

Discussion

Antibiotics have always been bliss for the human civilization; however, their irrational and irresponsible use has significantly contributed to antimicrobial resistance. There is a promising future for the medicinal plants' usage and their hidden potential could be decisive in changing the course of medical history [36]. Keeping this in mind, the present study aimed at scientifically exploring the bioactive potential of *Symplocos racemosa*. It has been observed that most of the antimicrobial compounds identified in plants are mostly aromatic or saturated organic molecules, which can easily be solubilized in organic solvents [37] and in the present study, ethyl acetate was found to be the best organic extractant followed by butanol and hexane. The better antimicrobial potential of the ethyl acetate extract may be attributed to the better solubility of such residues in ethyl acetate [38]. The presence of major groups like flavonoids, cardiac glycosides, saponins, tannins, triterpenes and phytosterols are in consonance with other reports on this plant [39, 40] and other medicinal plants [41, 17]. Cardiac glycosides were the most abundant followed by flavonoids whereas triterpenes were present in least quantity. Quite encouragingly, flavonoids and cardiac glycosides were effective against 12 and 9 strains respectively and our results are well supported by the previous observations for various plant parts of *Moringa oleifera* [42, 43], where these were quite effective against the Gram positive, Gram negative and the yeast strains. The significance of the study can be gauged from the fact that these phytoconstituents were highly effective against resistant microbes e.g., MRSA and *Candida albicans*, which cause severe infections in hospitals/healthcare settings, which is in line with another study on *Eugenia jambolana* seed extracts which was effective against MDR human pathogens [44]. The antimicrobial potential of phytoconstituents is well-supported by the fact that these substances serve as plant defense mechanisms against predation by microorganisms and that the wide array of such microbial infections could have triggered the synthesis of such vast variety of phytoconstituents with a wide and variable spectrum [37]. The results obtained from ADA and MIC is supportive of each other. MIC determination is an effective method for evaluating the efficacy of an antimicrobial [45], where all the extracts were quite effective against the nosocomial resistant pathogens MRSA, *Candida albicans*, *Pseudomonas aeruginosa* and *Escherichia coli*, which highlights the importance of the study. The MIC values obtained in this study (0.5–10 mg/ml) were comparable to or lower than the values obtained for other medicinal plants' extracts, viz., 0.4-4 mg/ml [22]; 2.5-5 mg/ml [46], 5–20 mg/ml [47]. VCC studies give an assessment of the bacteristatic/cidal effect of the compound, which further endorsed the potential of this medicinal plant, as it showed a significant bactericidal effect against MRSA, *Escherichia coli*, *Pseudomonas aeruginosa*, *Staphylococcus aureus*, *Staphylococcus epidermidis* etc. The PAE gives an idea of the time period upto which the compound is effective post-exposure and hence could be a decisive factor for development of any natural compound into a useful drug. The PAE in this study ranged from 2–6 h.

It is a well known fact that the biofilms-formers are a leading cause of the rising cases of the chronic infectious diseases and recurrent infections. Therefore, bioactive compounds which are able to modulate/ disrupt the biofilm forming ability, have an exceptional importance in the field of drug development [48]. The promising antibiofilm potential of flavonoids and cardiac glycosides highlight the importance of the study, where the former were more effective [49]. This could be justified by the fact that the plant metabolites such as flavonoids, phenolic acids etc reportedly possess antibiofilm activity [50, 51]. The potency was also tested against some drug-resistant strains of *E.coli*, *Salmonella* spp. and MRSA, where flavonoids and cardiac glycosides showed a broad spectrum activity, thus, justifying its candidature as a potential antimicrobial and antibiofilm drug of future. To add up on the bioactive potential of the plant, the flavonoids were screened for their cytotoxicity against three cancerous cell lines and our results are in consonance with those obtained for compounds of *Acanthus hirsutus* Boiss (11.17–700 µg/ml), *Euphorbia hirta* (625 µg/ml) [52, 53] and some naturally occurring quinones [54].

The in vitro biosafety evaluation of the extracts by Ames and MTT assay proved extracts to be non-mutagenic and non-cytotoxic, respectively. To further strengthen the biosafety aspect of this medicinal plant, acute oral toxicity of its flavonoids was evaluated in mice. Toxicological evaluation is an important aspect of pharmacology for assessing any negative effect of the bioactive substance on living organisms prior to its clinical usage by humans. This aspect is crucially important as toxicity results from animals, especially mice and rats, help in judging the safety of the compounds, since mice and rats have an anatomy close to that of human body and any effect on the animal model would clearly indicate towards its possible toxic effects. Abundant literature is available validating the ethno-medical knowledge on the significance of medicinal plants in prevention and treatment of various diseases. However, the medicinal plants contain a complex mixture of many bioactive phytochemical groups with diverse mode of action, which may possibly show any adverse effects upon interaction with the human/animal cells. Thus, it is of utmost importance to investigate the safety and biological properties of the medicinal plants prior to their usage. The biosafety of the flavonoids was thus confirmed since no signs of abnormality, illness or biochemical and pathological changes were observed in the test group of animals, indicating a normal metabolism and growth. These results are in consonance with other studies where plant extracts were found to be non toxic [12, 16–18].

So as to establish the active compounds which impart a significant and broad spectrum antimicrobial potential to the most active groups of phytoconstituents (flavonoids), the chromatographic and spectroscopic techniques such as TLC and GC-MS methods were employed. The chromatogram revealed the presence of several bands (S1-S4) with varying R_f values. The number of separated bands, corresponding to the number of separated compounds, strictly depended on the solvent system used. It means that the separation of bands depends on the nature and interaction of the compounds being separated with the solvents of the used system. The bands obtained were quantitatively separated using preparative-TLC method, where one band i.e., S3 was found to be the active with a broad spectrum. Hence, it was further subjected to GC-MS analysis so as to identify the antimicrobial components in this active band. Interestingly, the presence of compound Bicyclo[2.2.1]heptan-2-one,1,7,7-trimethyl-, (1S)- as one of the major compounds in band S3 corresponded well with a report on the GC-MS analysis of the methanolic extract of *Coriandrum sativum* leaves, where this compound was identified as one of the major bioactive phytochemical compounds and possessed antimicrobial activity, which provided further credence to the study [55]. Its presence as a major phytochemical compound was also in concordance with other studies on chloroformic extract of *Acacia karoo* root and *Artemisia lavandulaefolia* essential oil, where Bicyclo[2.2.1]heptan-2-one,1,7,7-trimethyl-, (1S)- was present as a major bioactive phytochemical constituent [56–57].

Conclusion

The present study thus provides a scientific validation to the medicinal value of *Symplocos racemosa* bark and its isolated phytoconstituents, as demonstrated by the broad ranged zone of inhibitions, low MIC values, lesser killing time and extended post antibiotic effect. These compounds showed a significant antibiofilm potential, were potent against drug resistant strains and were cytotoxic against the cancerous cell lines, which further add credence to the reported antimicrobial spectrum. The extracts and phytoconstituents had a biosafe profile as seen in Ames test, MTT assay and acute oral toxicity studies. This study holds importance in the sense that it has revealed the various bioactivities of this plant, which strengthens its candidature for development into potent antimicrobial drugs.

Abbreviations

ADA- Agar Diffusion Assay, **MIC**- Minimum Inhibitory Concentration, **MTT**- [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide], **MTCC**- Microbial Type Culture Collection, **MRSA**- Methicillin-Resistant *Staphylococcus aureus*, **PPPs**- Partially Purified Phytoconstituents, **TAP**- Total Activity Potency, **VCC**- Viable Cell Count Study, **PAE**: Post Antibiotic effect.

Declarations

Ethics approval and consent to participate

All procedures performed in studies involving animals were in accordance with the ethical standards of the institution or practice at which the studies were conducted. Ethics approval has been provided by the competent body of the institute vide no. CPCSEA/IAEC/CRI/14-114-2016. The written informed consent to use the animals in this study was obtained from the Animal house, Central Research Institute, Kasauli (H.P.).

Consent to publish

Not applicable

Availability of data and materials

Not applicable

Competing interests

The authors declare that they have no conflict of interest, *i.e.*, the submitted work was not carried out in the presence of any personal, professional or financial relationships.

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Authors' contributions

DSA: As a Principle Investigator (PI), contributed substantially in interpretation of data. **HS:** Performed the experimental work. Both the authors contributed in designing of the experiments and drafting of the manuscript. **YK:** Designed and guided through the *in vivo* experiment. **VKG:** Carried out the histopathological examination and interpreted the results. All authors have read and approved the manuscript.

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Supplemental Information Note

Additional File 1 (.docx): Qualitative and Quantitative analysis for the detection of major group of phytoconstituents.

Additional File 2 (.docx): Antibiofilm potential of the phytoconstituents of *Symplocos racemosa* bark

Additional File 3 (.docx): *In vitro* cytotoxicity studies by MTT assay against RD, L20B and Hep 2 cell lines.

Additional File 4 (.docx): Acute Oral Toxicity study of *Symplocos racemosa* flavonoids in Swiss albino mice.

Additional File 5 (.docx): Figures A1-A5 and Table TI of the main manuscript.

Additional file 6 (.docx): Completed "The ARRIVE Guidelines Checklist" for reporting animal data in this manuscript.

Tables

Table 1: Qualitative detection and antimicrobial activity of *Symplocos racemosa* bark

Phytoconstituents	Detected group	Stock solution (mg/ml)	Antimicrobial activity
Alkaloids		NA	NA
Mayer's reagent test	- ^a		
Hager's reagent test	-		
Wagner reagent test	-		
Flavonoids		54.75	+++ ^c
Shinoda test (Magnesium turnings)	+ ^b		
Zinc-hydrochloride reduction test	+		
Lead acetate test	+		
Ferric chloride reagent test	-		
Saponins		41.6	+ ^d
Froth test	+		
Tannins		100.5	+
Ferric chloride reagent test	+		
Lead acetate test	+		
Cardiac glycosides		59.5	++ ^e
Keller- killiani test	+		
Terpenoids			
Triterpenes (Salkowski's test)	+	12	- ^f
Diterpenes (Copper acetate test)	-	NA	NA
		ND	ND
Anthranol glycosides			
Borntrager's test	+		
Phytosterols		38	-
Libermann Burchard's test	+		
Salkowski's test	+		
Coumarins		ND	-

*a- absent; b- present; c- most active; d- least active; e- active; f- not active

**NA - Not applicable; ND- Not done

Table 2: Antimicrobial activity of phytoconstituents isolated from *Symplocos racemosa* bark

Test Organisms ^b	Average zone of inhibition (mm)**				
	Flavonoids	Saponins	Cardiac Glycosides	Gentamicin	Chloramphenicol
SA	19.66 ± 0.333	-*	15.66 ± 0.333	34.5 ± 0.50	26 ± 1.00
SE	22.33 ± 0.333	-	16.33 ± 0.333	26.5 ± 0.50	28.5 ± 0.50
EC	23 ± 1	-	-	31 ± 1.00	25 ± 0
EF	-	-	12.33 ± 0.333	27.5 ± 0.50	26.5 ± 0.50
KP1	20.33 ± 0.333	14.33 ± 0.333	25.66 ± 0.333	40.5 ± 0.50	38 ± 1.00
KP2	25.66 ± 0.333	-	19.33 ± 0.333	37.5 ± 1.50	26.5 ± 0.50
SF	16.66 ± 0.333	-	-	30.5 ± 0.50	27.5 ± 0.50
ST1	16 ± 0	-	-	35 ± 0	23 ± 0
ST2	24 ± 0.577	-	17 ± 0	43 ± 1.00	40.5 ± 0.50
PA	21.66 ± 0.333	-	15.66 ± 0.333	40.5 ± 0.50	28.5 ± 0.50
CA	25.66 ± 0.333	15.33 ± 0.333	25 ± 0	36.5 ± 0.50 ^a	ND
CT	17.33 ± 0.333	-	-	27.5 ± 0.50 ^a	ND
MRSA	24.33 ± 0.666	-	17.66 ± 0.333	42 ± 0	39.5 ± 0.50

*No activity; ** Values are expressed as Mean ± SEM of three determinations; ND- not done

^aAmphotericin B; ^b Organisms- SA- *Staphylococcus aureus*; SE- *Staphylococcus epidermidis*; EC- *Escherichia coli*; KP1- *Klebsiella pneumoniae* 1; KP2- *Klebsiella pneumoniae* 2; SF- *Shigella flexneri*; ST1- *Salmonella typhimurium* 1; ST2- *Salmonella typhimurium* 2; PA- *Pseudomonas aeruginosa*; CA- *Candida albicans*; CT- *Candida tropicalis*; MRSA- Methicillin- Resistant *Staphylococcus aureus*

Table 3: Minimum inhibitory concentration (MIC) of organic extract and isolated phytoconstituents of *Symplocos racemosa* bark

Organisms [§]	MIC (mg/ml)				
	EA **	Flavonoids	Cardiac glycosides	Gentamicin	Chloramphenicol
SA	0.7	3	1	0.0002	0.01
SE	1	3	1	0.01	0.01
EC	1	3	ND	0.005	0.01
EF	ND*	ND	ND	0.03	0.3
KP1	0.5	0.7	0.5	0.0002	0.01
KP2	ND	1	10	0.0005	0.001
SF	1	3	ND	0.005	0.01
ST1	3	1	ND	0.005	0.1
ST2	0.5	3	3	0.0003	0.001
PA	0.5	0.7	10	0.005	0.7
CA	0.7	0.7	0.5	0.0003 [¥]	ND
CT	ND	10	ND	0.1 [¥]	ND
MRSA	1	1	5	0.005	0.01

*Not determined; ** Ethyl acetate extract; [¥]Amphotericin B[§]SA- *Staphylococcus aureus*; SE- *Staphylococcus epidermidis*; EC- *Escherichia coli*; KP1- *Klebsiella pneumoniae* 1; KP2- *Klebsiella pneumoniae* 2; SF- *Shigella flexneri*; ST1- *Salmonella typhimurium* 1; ST2- *Salmonella typhimurium* 2; PA- *Pseudomonas aeruginosa*; CA- *Candida albicans*; CT- *Candida tropicalis*; MRSA- Methicillin- Resistant *Staphylococcus aureus*

Table 4: Antimicrobial potential of the most active phytoconstituents of *Symplocos racemosa* bark against drug- resistant strains and some clinical isolates of MRSA

Microorganisms	Average zone of inhibition (mm)*	
	Flavonoids	Cardiac glycosides
CRIRS 1	15.33±0.881	-
CRIRS 2	15.66±0.333	-
CRIRS 3	16±0.577	-
CRIRS 4	17.33±0.333	14.66±0.333
CRIRS 5	17±0.577	12.33±0.333
CRIRS 6	16.66±0.333	13±0
CRIRS 7	22.66±0.666	-
CRIRS 8	17.66±0.333	13.66±0.333
CRIRS 9	14.66±0.881	-
CRIRS 10	15.33±0.881	14.33±0.333
CRIRS 11	15±0.577	-
CRIRS 12	15.66±0.666	-
CRIRS 13	19.66±0.333	14±0
CRIRS 14	18.66±0.333	-
CRIRS 15	14.66±0.333	-
CRIRS 16	16.33±0.881	-
CRIRS 17	20±0	-
CRIRS 18	19.33±0.333	-
CRIRS 19	16±0	-
CRIRS 20	15.33±0.333	-
CRIRS 21	17.66±0.333	-
CRIRS 22	18.66±0.333	-
DSECI 01	21.33±0.333	14±0
DSECI 02	20±0	17.33±0.666
DSECI 03	27.66±0.333	16.66±0.881
DSECI 04	28.33±0.333	18±0
DSECI 05	24.33±0.666	15±0
DSECI 06	22.33±0.333	14.33±0.333
DSECI 07	26.33±0.666	-
DSECI 08	22±0.577	14.33±0.666
DSECI 09	25.66±0.333	-
DSECI 10	27.33±0.333	-
DSECI 11	-	-
DSECI 12	23.33±0.333	15.66±0.333

*Values are expressed as Mean ± Standard error of means (SEM) of three determinations

Table 5: Absolute and relative organ weight of control and treated mice (male and female) in the acute toxicity study of *Symplocos racemosa* flavonoids

Organs	Absolute organ weight (g)			Relative organ weight (%)		
	Control	Treated	p-value	Control	Treated	p-value
MALE						
Liver	0.724 ± 0.031	0.692 ± 0.013	0.365	2.453 ± 0.031	2.441 ± 0.007	0.715
Kidneys	0.401 ± 0.022	0.379 ± 0.015	0.437	1.355 ± 0.035	1.334 ± 0.026	0.638
Heart	0.165 ± 0.007	0.157 ± 0.005	0.390	0.558 ± 0.008	0.577 ± 0.008	0.137
FEMALE						
Liver	0.827 ± 0.044	0.783 ± 0.029	0.435	2.613 ± 0.055	2.653 ± 0.060	0.638
Kidneys	0.410 ± 0.023	0.368 ± 0.006	0.131	1.297 ± 0.031	1.248 ± 0.006	0.179
Heart	0.166 ± 0.0118	0.150 ± 0.004	0.254	0.524 ± 0.0195	0.509 ± 0.009	0.651

*Data indicate Mean ± SEM (n=6 for each group). There was no significant difference between the test and control groups as indicated by Post hoc Tukey's t-test.

Table 6: Effect of *Symplocos racemosa* flavonoids on biochemical parameters in acute oral toxicity study

Parameters	Male			Female		
	Control	Treated	p-value	Control	Treated	p-value
Urea (Mmol/L)	42.633 ± 0.839	42.460 ± 0.553	0.867	40.13 ± 0.991	40.183 ± 0.539	0.963
Creatinine (µmol/L)	0.774 ± 0.026	0.765 ± 0.019	0.799	0.618 ± 0.024	0.601 ± 0.012	0.535
Total bilirubin (µmol/L)	2.376 ± 0.093	2.371 ± 0.063	0.882	1.76 ± 0.025	1.772 ± 0.014	0.677
Aspartate aminotransferase (AST)(U/L)	208.96 ± 4.192	209.11 ± 2.312	0.976	160.66 ± 2.708	159.76 ± 1.574	0.780
Alanine aminotransferase (ALT) (U/L)	79.95 ± 2.095	81.698 ± 0.591	0.441	61.33 ± 2.677	61.501 ± 1.338	0.955
Alkaline phosphatase (ALP) (U/L)	258.3 ± 2.510	256.89 ± 1.344	0.633	183.33 ± 1.612	181.733 ± 0.794	0.403

*Values are expressed as mean ± SEM (n=6 for each group). There was no significant difference (p>0.05) between the test and control groups as indicated by one way ANOVA followed by Post hoc Tukey's t-test.

Table 7: The compounds identified in the active band S3 obtained from the flavonoids of *Symplocos racemosabark* using GC-MS

S.No.	RT (min)	Compounds identified	Molecular formula	Molecular weight (g/mol)
1.	3.22	3,5-Dithiahexanol 5,5-dioxide	C ₄ H ₁₀ O ₃ S ₂	170.25
2.	4.25	3-Amino-4-[4-hydroxyphenyl]butanol	C ₁₀ H ₁₅ NO ₂	181.23
3.	5.55	Propane, 2-chloro-	C ₃ H ₇ Cl	78.54
4.	9.22	9,12,15-Octadecatrienoic acid,2-[(trimethylsilyl)oxy]-1 [[(trimethylsilyl)oxy]methyl]ethyl ester, (Z,Z,Z)-	C ₂₇ H ₅₂ O ₄ Si ₂	496.90
5.	9.63	2-Butanol, 3-benzyloxy-	C ₁₁ H ₁₆ O ₂	180.24
6.	9.84	Propane, 1,1,3-triethoxy-	C ₉ H ₂₀ O ₃	176.25
7.	10.46	Bicyclo[2.2.1]heptane-2,5-diol, 1,7,7-trimethyl-(2-endo, 5-exo)-	C ₁₀ H ₁₈ O ₂	170.25
8.	10.94	3,4-Dihydroxymandelic acid, ethyl ester, tri-TMS	C ₁₉ H ₃₆ O ₅ Si ₃	428.70
9.	11.14	Bicyclo[2.2.1]heptan-2-one,1,7,7-trimethyl-, (1S)-	C ₁₀ H ₁₆ O	152.23
10.	11.93	2,6,11-trimethyldodecane	C ₁₅ H ₃₂	212.41
11.	13.47	Silane, dimethyl(dimethyl(dimethyl(2-isopropylphenoxy)silyloxy)silyloxy)(2-isopropylphenoxy)-	C ₂₄ H ₄₀ O ₄ Si ₃	402.67
12.	14.78	Nonane, 3-methyl-5-propyl-	C ₁₃ H ₂₈	184.36
13.	15.72	Cycloheptasiloxane, tetradecamethyl-	C ₁₄ H ₄₂ O ₇ Si ₇	519.07
14.	17.29	Dodecane, 2,6,11-trimethyl-	C ₁₅ H ₃₂	212.41
15.	17.72	Benzeneethanamine,N-[(pentafluorophenyl)methylene]-.beta.,3,4-tris[(trimethylsilyl)oxy]-	C ₂₄ H ₃₄ F ₅ NO ₃ Si ₃	563.80
16.	21.66	Phthalic acid, butyl hept-4-yl ester	C ₁₉ H ₂₈ O ₄	320.42
17.	34.40	7,11b-Dihydro-6H-indeno[2,1-c] chromene-3,4,6a, 9, 10-pentol pentakis(trimethylsilyl) ether	C ₃₁ H ₅₄ O ₆ Si ₅	663.18

Figures

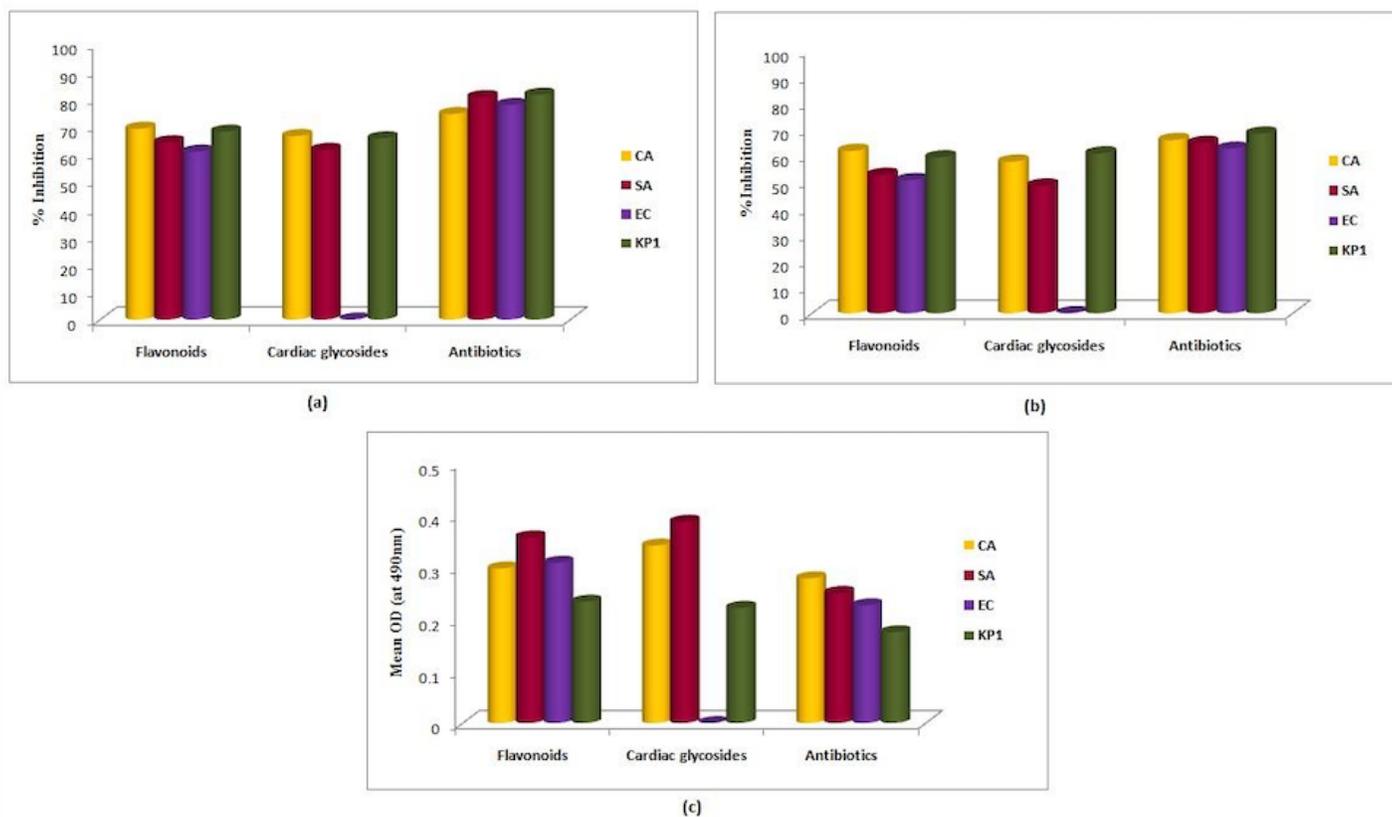


Figure 1

Inhibitory action of the Partially Purified Phytoconstituents of *Symplocos racemosa* bark on the (a) Initial cell attachment (b) Pre-formed biofilms of the test organisms (c) Metabolic activity of the biofilms.*EC-Escherichia coli; KP1- Klebsiella pneumoniae 1, CA- Candida albicans, SA- Staphylococcus aureus.



Figure 2

Photomicrographs of the tissue sections of (a) Heart (b) Kidney and (c) Liver taken from mice treated with *Symplocos racemosa* flavonoids for 14 days for assessment of acute oral toxicity in comparison with untreated control.

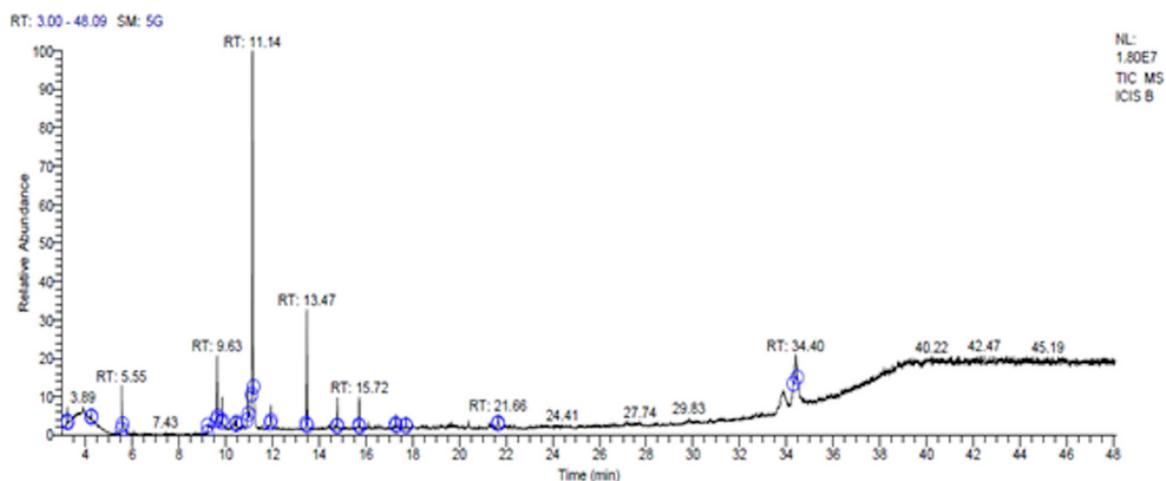


Figure 3

TLC chromatograms of the *Symplocos racemosa* flavonoids revealing the presence of various bands under (a) Natural light (b) Stained with Iodine vapors (c) UV light (264nm) (d) UV light (365nm).

Sample Header

Data File: B
 Original Data Path: C:\GCMS-DATA\YEAR 2019JUNE\21
 Sample Type: Unknown
 Sample ID: B



Qual Peak Table

RT	Peak Area	Area %	Peak Height
3.22	721881.07	1.14	693994.73
4.25	160089.03	0.25	230579.60
5.55	2907723.75	4.59	2019768.89
9.22	141733.43	0.22	240523.84
9.63	4524195.64	7.14	2949956.82
9.84	1478517.41	2.33	1151840.21
10.46	321694.31	0.51	316816.30
10.94	2564603.95	4.05	1537426.31
11.14	28588424.25	45.13	15951170.33
11.93	1291139.96	2.04	794475.39
13.47	8128156.37	12.83	5408657.63
14.78	1931709.86	3.05	1328515.79
15.72	1837936.22	2.90	1351520.63
17.29	529468.59	0.84	487257.30
17.72	397361.77	0.63	391171.61
21.66	546416.71	0.86	376776.36
34.40	7279061.62	11.49	1201517.65

Figure 4

The GC-MS Chromatogram of the active band S3 obtained from the flavonoids of *Symplocos racemosa* bark

Supplementary Files

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