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Purification and Characterization of Antibacterial Compounds from *Thuja arborvitae*

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Abstract

T. arborvitae has been employed for its medicinal efficacy, especially for the treatment of bacterial infections. In this study, the aim was to isolate and analyze the antibacterial activity of bioactive materials from Thuja leaf extract. Both Gram-positive *Staphylococcus aureus* and *Streptococcus mutans*, as well as Gram-negative Acinetobacter baumannii, *Escherichia coli*, and *Pseudomonas aeruginosa*, were inhibited by the methanol extract. During the purification process, three distinct bioactive compounds P1, P2, P3 and P4 were separated. The purified compounds exhibited antibacterial activity with MICs between 10-50 µg/ml. All P1, P2, and P3 were remarkable against the multi-drug resistant strain Acinetobacter baumannii. These results support the effectiveness of the compounds from *T. arborvitae* proving their value in developing new therapeutic agents.

Keywords: *T. arborvitae* • Antibiotics • Bacteria • *Staphylococcus aureus* • Gram-positive • Gram-negative

Introduction

Resolving the problem of antibiotic-resistant bacterial infections is a mounting global public health challenge. A major portion of the population has been diagnosed with Multivariate-Resistant (MDR) pathogens due to the rampant misuse of antibiotics which has resulted in harsher mortality rates [1]. This has highly improved the need to search for new antibacterial medicine that has a different method of functioning. Nature and more specifically, plants have always served as a backbone in combat against infectious agents and have been a key source of antibiotics [2]. Bioactive secondary metabolites produced by medicinal plants possess therapeutic uses, making them important in plant-based medicines. Traditionally *T. arborvitae*, a North American and East Asian evergreen tree, has been used in folk medicine due to its antimicrobial, anti-inflammatory and immunomodulatory properties [3]. However, little has been done to isolate and assess the purified bioactive compounds for antibacterial action, which is quite astonishing given its widespread application.

Biological activity of herbal and medicinal plants is largely due to the isolates active against microbes and extracts from Thuja leaves are one of the best examples of such activity [4]. But in order to use them effectively, we will need to study and purify specific compounds. Specific plant extracts of *T. arborvitae* leaves were purified to determine their bioactive properties against both Gram-positive and Gram-negative bacteria, along with MDR strains. Through purification assisted by bioactivity-guided fractionation, three unique antibacterial compounds were identified and named P1, P2 P3 and P4.

All clinical isolates and their purified compounds were chosen to test for antibacterial efficacy against the most common *S. aureus* and *S. mutans* clinical isolates pathogens. The tested bacterial strains also included Acinetobacter baumannii, Gram negative known for its multi-drug-resistance, and *E. coli* and *P. aeruginosa* [5]. The multi-drug-resistant *Acinetobacter baumannii* is an emerging opportunistic pathogen responsible for severe nosocomial infections and thus remains an important target for new antibacterial agents [6]. Skin infections, dental caries, pneumonia, and bloodstream infections have made these bacterial species clinically significant [7].

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The P1, P2, P3 and P4 fractions obtained from Extracts of Thuja leaves were analyzed for the antibacterial action by determining the Minimum Inhibitory Concentration (MIC). The purpose of determining the MIC value of extract was to know the least amount of concentration that could inhibit bacterial growth [8]. They disclosed that P1, P2, P3 and P4 had their powerful antibacterial activity expressed, which was evidenced by the MIC values of 10 to 50 µg/ml [9]. Additionally, all three compounds had potent inhibitory activity towards Acinetobacter baumannii which raises the possibility of them being used as gold standard novel therapeutic agents for MDR bacterial infections [10].

These results illustrate the great necessity to study *T. arborvitae* in order to identify the source of new antibacterial. The ability to purify bioactive compounds and demonstrate their action against drug resistant pathogens indicates that there are great prospects for this line of research in deeper understanding the compounds, enhancing their efficiency, and considering using them in the clinic [11]. As worldwide society is facing an increased threat of antimicrobial resistance, the investigation of the antimicrobial extracts from plants gives hope in the creation of new treatment options [12].

Materials and Methods

Sample collection and plant extract preparation

Foeniculum vulgare and *Zanthoxylum americanum* were purchased from a local grocery store while the leaves of *Magnolia grandiflora*, *Toxicodendron radicans*, *T. arborvitae* and *Lycium barbarum* berries were collected from Northwestern Atlanta, GA. The plant samples were dried at room temperature and blended to powder using a blender. 10 g of each powdered sample was put in a 50ml plastic tube and treated with 20 ml of the solvent. Extraction was done at room temperature for 24 hours on a rotator. The extract was then filtered and lyophilized to obtain dry powder. The lyophilized extract was kept at -20 degrees Celsius until further analysis.

Bacterial strains and maintenance of bacteria

ATCC provided the following bacterial strains: *S. aureus* (49775 and #12600), *Acinetobacter baumannii* (19606), *P. aeruginosa* (#10145), *S. mutans* (#25175) and *E. coli* (#33694). Bacterial cultures were kept in 20% glycerol which was stored at -80 ℃. Activated bacterial strains were obtained by streaking the stored culture on LB (Lysogeny Broth) agar plates and incubated overnight at 37 ℃. One colony from each plate was picked and inoculated into LB broth and incubated overnight at 37 ℃ prior to use. *P. aeruginosa* was kept and grown in Brain Heart Infusion Broth (CM1135B, OXOID).

Zone of Inhibition assay

In order to assess the antibacterial activity of the crude plant extracts as

well as purified compounds, the zone of inhibition assay was performed. The bacterial strains used in the experiment were grown overnight on LB broth and were then plated on agar LB plates. The agar was punched with a sterile disposable glass pipette tip, resulting in the creation of wells which were around 7mm in round shape and had a depth of 3 mm. Next, the wells were covered with 50µl of the sample and marked. After, the petri dishes were inverted. The control group for this sample was the solvent used to dissolve the sample. The positive control was ampicillin (100 µg/ml) and chloramphenicol (25 µg/ml). Next, the petri dishes were placed in an incubator for 18 hours at 37 degrees Celsius. The inhibition zones were evaluated using a ruler. Any sample with at least a 10mm diameter inhibition zone was deemed positive.

Minimum inhibition concentration

The Minimum Inhibition Concentration (MIC) was established according to the procedures by the European Committee on Antimicrobial Susceptibility Testing (EUCAST) (www.eucast.org). Bacteria grown in LB medium overnight were centrifuged for 5 min at room temperature at 4000 RPM (850 g). The bacterial pellet was collected and suspended in fresh LB medium to a final concentration of 1 × 105 CFU/ml and dispensed 0.1 ml of the diluted bacterial culture into each well of a 96-well microliter plate. A serial 2X dilution of the test samples were done in sterile tubes or 96 well micro plates, beginning with the highest concentration which was prepared and subsequently diluted to achieve the necessary concentration range. Control wells containing ampicillin (100 µg/ml) or chloramphenicol (25 µg/ml) were included as positive controls. Negative control comprised solvent used to prepare the sample. The micro plates were incubated at 37 degrees Celsius for 18 hours after which the bacteria were re suspended and the absorbance values at 600 nm were read using an automatic microplate reader (Synergy Hl, BioTek, USA). The minimum concentration (µg/ml) that completely inhibited the growth of the bacterial strain was calculated.

Extraction of antibacterial compounds from *T. arborvitae*

The leaves of *T. arborvitae* were dried and then methanol extraction was performed at normal temperature for 24 h. After this period, the mixture was filtered through a funnel containing filter paper. Then most of the methanol was removed using rotavapor, and the residue was then concentrated in a freezer drier to get crude methanol extract (140 g). The crude extract was further diluted in methanol and then passed through the alumina column. The column was eluted with ethyl acetate and methanol (1:1) and fractions were collected (10 ml each) with a fraction collector. A portion of each fraction was concentrated and sent for zone inhibition assay to measure antibacterial activity. The active fractions were then combined and dried under vacuum. The dried powder was then reconstituted in methanol and passed through silica column. The column was eluted with ethyl acetate (800 ml) followed by methanol. The pooled fractions were dried and tested using zone inhibition assay. Lastly, the pooled fractions that exhibited antibacterial activity were combined and dried.

Dried material was dissolved in methanol and processed through an HPLC C18 column (Xterra, 19 × 100 mm, Water). A linear gradient from 10 to 100% acetonitrile in water was used to elute the column. The peaks were collected at absorbance of 280 nm, dried and then underwent zone inhibition assay. Antibacterial activity was noted in four peaks designated P1, P2, P3, and P4, with retention times of 45, 46, 47, and 71 minutes, respectively. From 800 g dry leaves, Pl 12.7 mg, P2 16.8 mg, P3 7.7 mg, P4 41.4 mg was obtained.

Bacterial colony-formation assay

The inoculum of bacteria (*S. aureus,* 1 × 106 CFU) was transferred to an LB solution (3ml) and kept for 1 hour at 37 ℃. After 1 hour of growth, the culture (39 µl) was spread on an LB agar plate (35 mm) which already had different dilutions for the tested compound premixed in it. The plates were then placed in an incubator for 16 hours at 37 ℃, and the colonies were counted.

Bacteria viability assay

For both the viable and non-viable bacterial counts, the LIVE/DEAD®BacLight™ Bacterial Viability Kit from Life Technologies Carlsbad was employed. S. aureus aliquots (1 × 106 CFU/ml) were added in 100 µl rest of S. Aureus was in LB broth without or with the compound of interest (one compound was used in the experiment) and then added to all wells of 96 well plates. The plates were then incubated at 37 degrees Celsius for 18 hours. Reactions with dyes were done according to the manufacturer's instructions. Measurements for fluorescence intensity were taken using a fluorophotometer Gemini XPS, Molecular Devices. The optical filters were set at 530 nm and 630 nm, for the emission and excitation wavelengths, respectively, of 485 nm. Measurements of the ratio of the sample fluorescence ratios of 530 and 630 nm allows detection of the ratio of live cells to dead cells. For the microscopic analysis S. aureus (1 × 109 CFU/ml) in LB medium was incubated with the compound for an hour, then underdoes, and the pre-stained samples were viewed on AXl0, ZEISS fluorescent microscope with 100X lens.

Cell cytotoxicity assay

Normal human primary epidermal keratinocytes (PCS-200-010-010) and prostate cancer PC3 cells (CRL-1435) were obtained from ATCC and grown in dermal cell basal medium (ATCC) and RCMI1640 medium supplemented with 10% fetal bovine serum, respectively. The cells were seeded in 24-well plates (l × 104 cells in each well) and exposed to the purified compounds for 96 h. The exposed cells were tracked with an Incucyte.

Results

Antibacterial activity of the extracts from plants screening

The seeds of *Foeniculum vulgare*, *Ginkgo biloba*, *Pimpinella anisum*, *Zanthoxylum americanum* and of leaves *Magnolia grandiflora*, *Toxicodendron radicans* and *T. arborvitae* as well as berries of *Lycium barbarum* were gathered. The screening was done with four species of bacteria, two Gram positive and two Gram negative, that are primarily associated with typical infections. The crude extracts of the various species of plants were prepared using methanol for screening purposes. The extraction of methanol from *M. grandiflora*, *T. arborvitae* and *T. radicans* was found to be effective against the bacterial strains *S. aureus* Figure 1a (Table 1). According to the literature, *P. aeruginosa* is known to have natural resistance to a range of antibiotics like penicillin and chloramphenicol due to its unique structure and biochemistry [13]. The extract of *T. arborvitae* and *T. radicans* did show some activity against the ampicillin and chloramphenicol resistant *P. aeruginosa* (Figure 1). Out of the tree species tested, *T. arbovitae* Figure 2a was more effective than *T.*

Table 1. The antibacterial activity of different plant extracts against gram-positive bacteria S. aureus+.

Zone of Inhibition Assay of Different Plant Extracts		
Plant	***S. aureas***	***P. aeruginosa***
F. vulgare	NA	NA
G. biloba	NA	NA
L. barbarum	NA	NA
M. grandiflora	2.0 ± 0.2	NA
P. anisum	NA	NA
T. arborvitae	1.9 ± 0.1	1.1 ± 0.1
T. radicans	1.7 ± 0.1	1.7 ± 0.2
Z. americamum	NA	NA

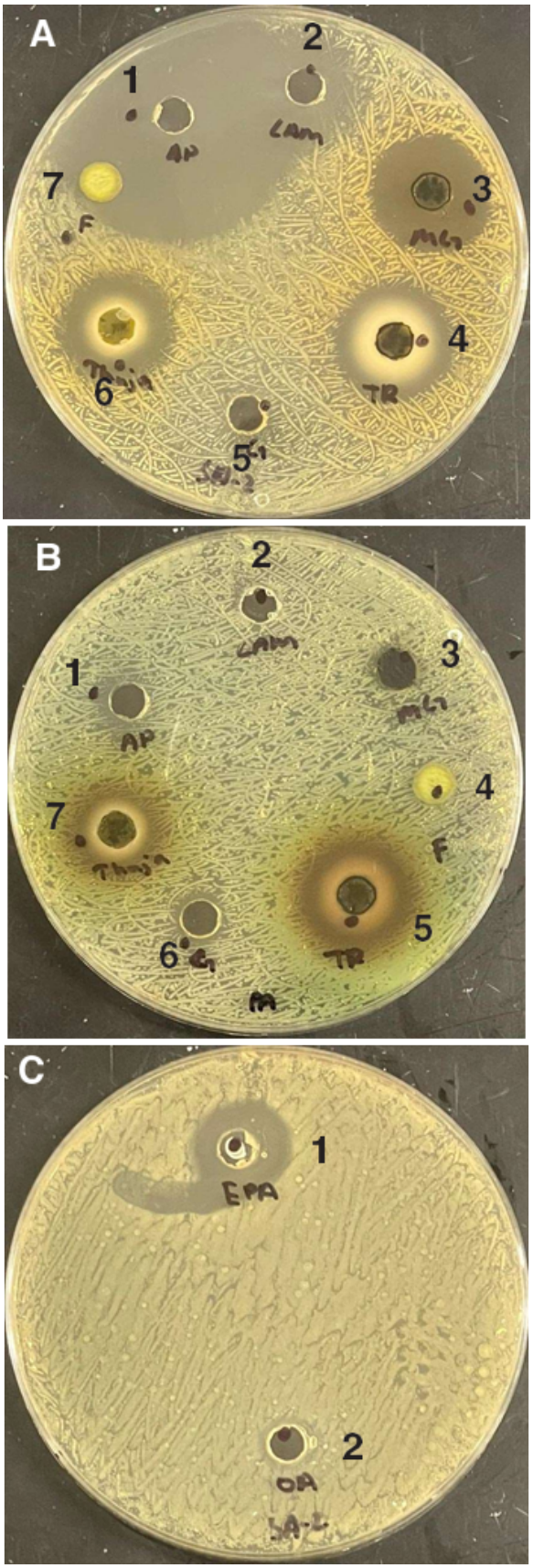


Figure 1. Zone of inhibition assay to detect the antibacterial activity. **A)** Zone of inhibition assay was performed with plant extracts against *S. aureus* (ATCC 49775) (a, c) and *P. aeruginosa*. **B)** 1, ampicillin (5 µg); 2, chloramphenicol (1.25 µg); 3, *M. grandiflora* extract (5 mg); 4, *T. radicans* extract (5 mg); 5, *G. biloba extract*, (5 mg) 6, *T. arborvitae* extract (5 mg); and 7, *F. vulgare* extract (5 mg). **C)** Zone of inhibition assay was performed with Eicosapentaenoic Acid (EPA) (0.5 mg) and Oleic acid (SA) (0.5 mg) against *S. aureus* (ATCC 4977.

radicans (Figure 2). at inhibiting the growth of all four bacterial strains. Extracts of *S. aureus* and *A. baumannii*, including *S. mutans*, inhibited the activity of *P. aeruginosa* more than any other infection.

In addition, extract of *T. arbovitae* had the greatest out of 4,000 µg/ml, completely restricting *P. aeruginosa. T. arbovitae* leaf extract restrains the ability of *S. aureus* bacteria to form colonies in a dependent dosage fashion (Figure 3). At a concentration of 125 µg/ml, the formation of colonies was totally prevented. The antibacterial properties in Toxicodendron as well as in Thuja have been noted before. Unfortunately, the antibacterial compounds responsible for the said activity have not been located in these plants. A *M. grandiflora* analysis discovered several antibacterial, but the focus of this study was on T. arbovitae whose leaves and their antibacterial properties are the most notable. Due to the leaves of *T. arbovitae* having the best antibacterial activity and being considerably more accessible, we have decided to use them as the material for purification and chemical identification of the compounds responsible for the antibacterial activity.

Extraction of active antibacterial substances from *T. Arborvitae*

The leaves of *T. Arborvitae* were dried and soaked in methanol, ethyl acetate, or water for 12 hours. Only the methanol and ethyl acetate extracts possessed a certain degree of inhibition against bacteria, *S. aureus*. Table 2 from which it may be inferred that the antibacterial compounds are not soluble in water. The diagram shows the purification steps. (Figure 4). The ethanol extract was chromatographed on an alumina column using ethyl acetate and methanol (1:1) as the mobile phase. For the determination of the antibacterial activity of column fractions, the zone inhibition assay was applied. The suspensions with detected antibacterial activity were concentrated and freeze-dried under a vacuum. The obtained dissolved freeze-dried material was dissolved in ethyl acetate and applied to silica column. Ethyl acetate was used to elute the column, and bound materials were subsequently eluted with methanol. The active antibacterial compound was only found in the methanol fraction out of the previously separated fractions. The rest of the compounds that are used for the cell were further separated using HPLC C1s reversal phase chromatography with a linear gradient of water-acetonitrile 10-100%. 23 peaks were gathered and put in a freezer drier. The inhibition zone assay showed that the antibacterial activity was concentrated in the four peaks labeled as P1, P2, P3, and P4 which had retention times of 45, 46, 47, and 71 minutes respectively. These results imply that T. arborvitae is most likely comprised of several additional antibacterial components. The peak P4 was scaled up with a linear gradient from 10 to 100 percent methanol in water-ethyl acetate on C18 reverse phase HPLC Symmetry columns. From 800 g of dried leaves of T. arborvitae, we obtained approximately 12.7 mg of constituents. The purified compounds showed MIC against *S. aureus* ranging from 10 to 50 ug/ml (Table 3).

Bacteria viability assay

The LIVE/DEAD viability kit was employed to assess the impact of purified compounds on the bacterial cells' viability. It was revealed that the purified compounds P3 and P4 significantly triggered cell death of the bacterial strain *S. aureus* at concentrations equal to or higher than 5 µg/ml (Figure 5). The effectiveness of the purified compounds was compared by measuring the ratio of alive and dead the higher values mean more live bacteria; lower values mean more dead bacteria. The different concentrations of P3 (blue bars) and P4 (red bars) were tested over 18 hours depicted in Figure 5b. When the concentration of P3 and P4 was set above 2.5 µg/ml, the red fluorescence's intensity was significantly greater, suggesting that the bacteria were dead. Little red fluorescence was present at 2.5 µg/ml or lower concentration of P3 or P4. The purified antibacterial compounds were shown to effectively induce cell death of *S. aureus* Figure 5b.

Cell toxicity of purified compounds on human cells

In this study, purified P3 and P4 compounds were evaluated for cytotoxicity against prostate cancer PC3 cell line and normal epidermal keratinocyte cells using lx MIC and 2x MIC doses. No toxicity and proliferation were recorded on PC3 cells (Figure 6a). On the other hand, P3 at lx MIC 50 µg/ml was able

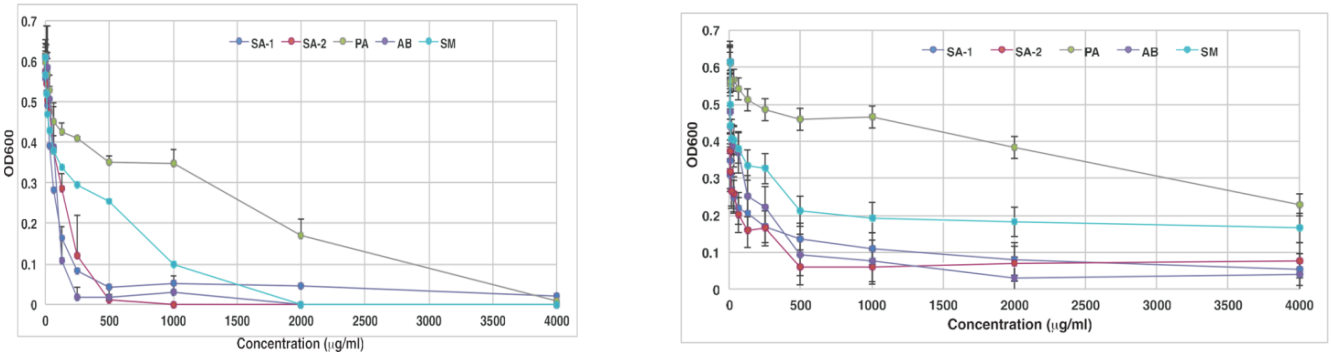


Figure 2. Effects of **a)** *T. arborvitae* and **b)** *T. Radicans* extracts on bacterial growth. Growth curves were determined by culturing bacteria for 18 h in the presence of various concentrations of plant extracts. The y-axis shows the turbidity of the bacterial culture measured by spectrometry. OD600: absorbance at 600 nm. The x-axis shows the concentration of the extract. SA-1 (ATCC 49775) and SA-2 (ATCC 12600): *S. aureus*: PA: *P. aeruginosa*: (ATCC 10145); AB: *A. baumannii* (ATCC 19606); SA: *S. mutans* (ATCC 25175). The values are plotted as mean ± SD obtained from the experiment performed in triplicate.

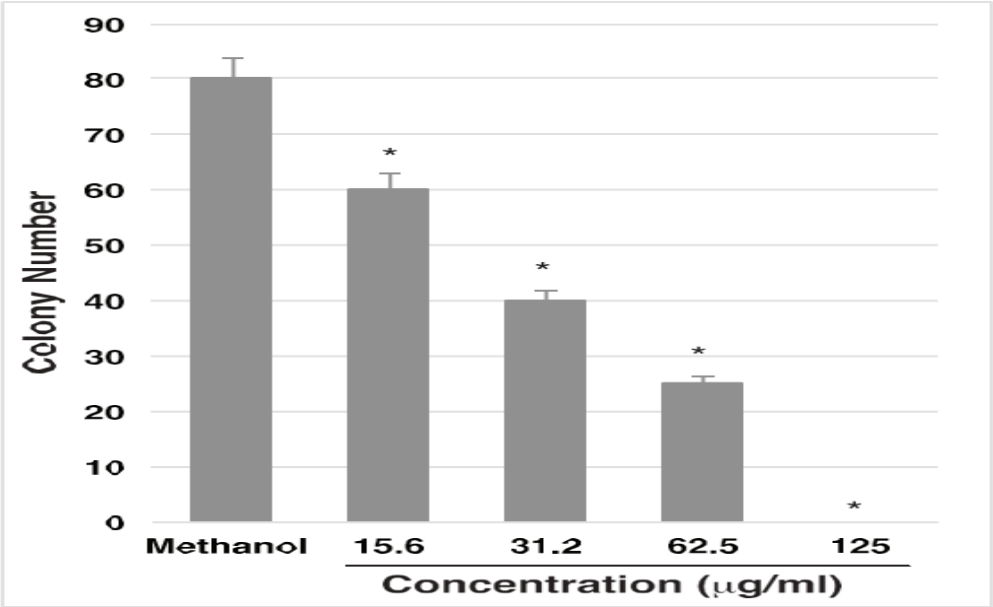


Figure 3. Antibacterial effects of *T. arborvitae* extract on the colony-forming ability. The y axis shows the colony numbers of the bacteria *S. aureus* (ATCC 49775). The x-axis shows the concentrations of *T. arborvitae* extract. Methanol was used as the solvent to dissolve the extract. Data are represented as mean ± SD of three independent tests. *Significantly different ($p<0.05$) compared to the methanol control.

Table 2. The antibacterial activity of different solvent extracts of Thuja arborvitae testing for antibiotic compounds against gram-positive bacteria *S. aureus*+.

Solvents	MIC (µg/ml)
Ethyl acetate	1028 ± 41
Methanol	500 ± 20
Water	>2000 ± 80

Table 3. The minimum inhibition concentration assay from purified compounds against gram-positive bacteria *S. aureus*+.

Purified Compounds	MIC (µg/ml)
Crude	500 ± 20
P1	20 ± 0.8
P2	10 ± 0.4
P3	50 ± 2
P4	10 ± 0.4

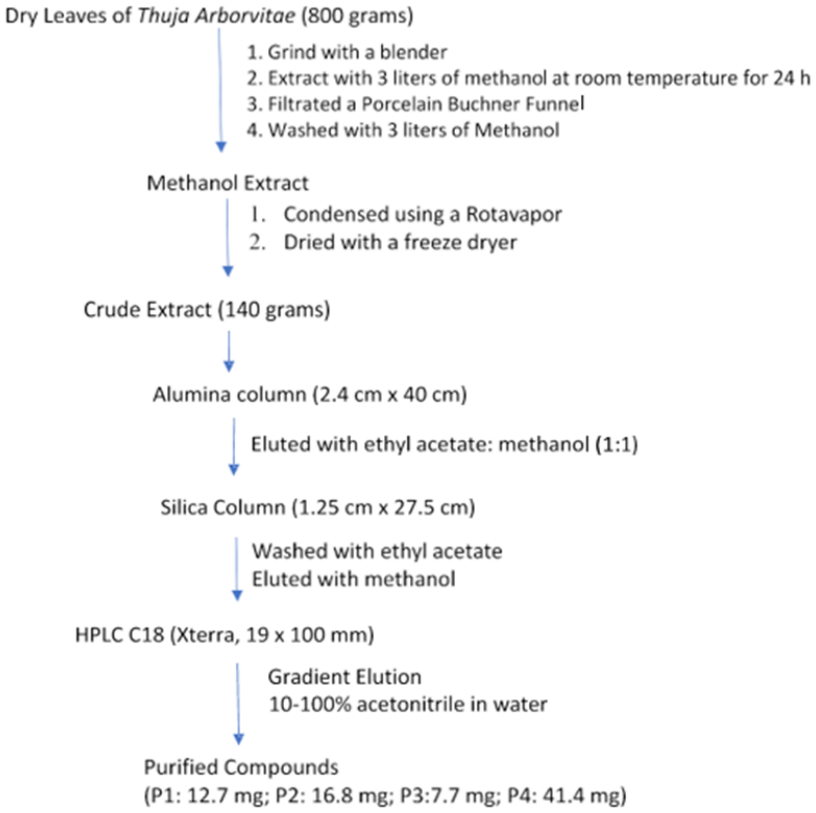


Figure 4. The scheme to purify antibacterial compounds from the *T. arborvitae* extract.

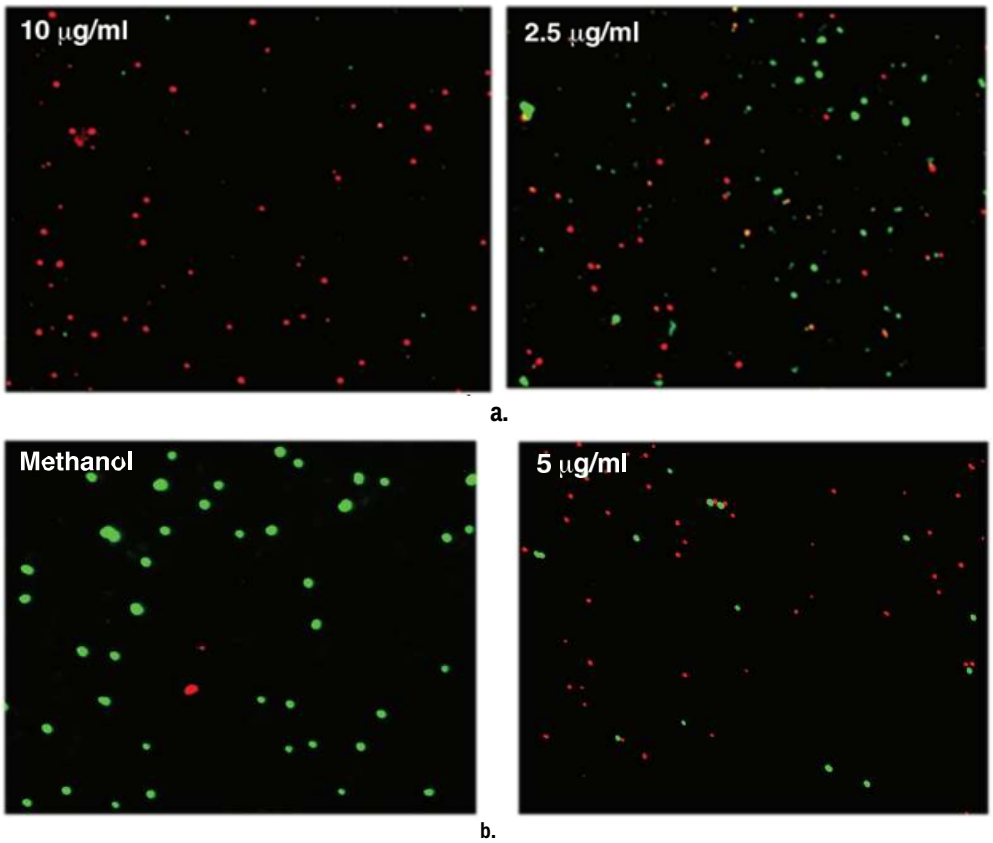
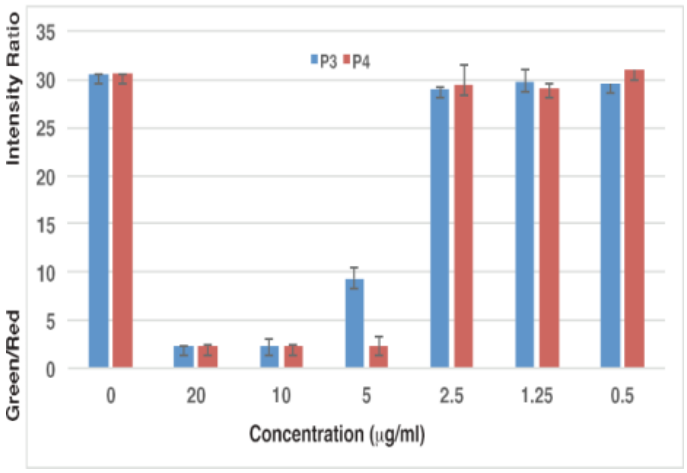


Figure 5. Antibacterial effects of purified P3 and P4 on bacterial viability. **a)** Fluorescence microscopic analysis was performed using the bacteria *S. aureus* incubated in P3 or P4 for 1 h and stained with SYTO 9 (green: viable cells); and propidium iodide (red; dead cells). **b)** Fluorophotometric measurement was performed using the bacterial *S. aureus* treated with several concentrations of P3 or P4 for 18 h. The y-axis shows the ratio of green/red fluorescence as indicated by the intensity at the wavelength of 530 nm divided by that of 630 nm and the horizontal axis shows the concentration of P3 or P4 compound. Data represents mean ± SD of three independent tests. *Significantly different ($p<0.05$) compared to untreated control.



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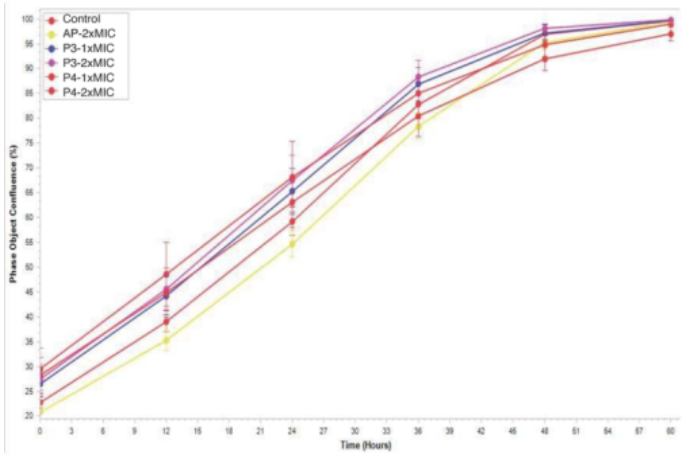

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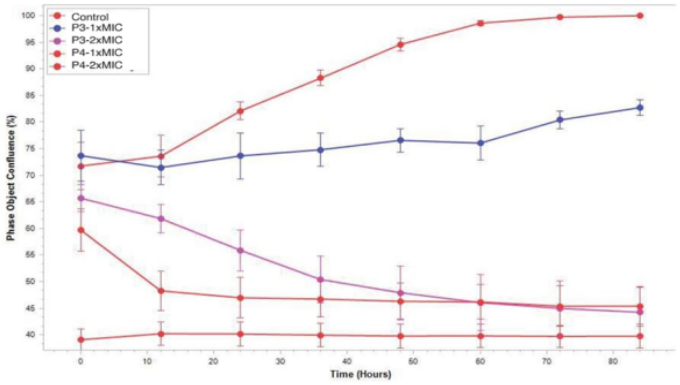

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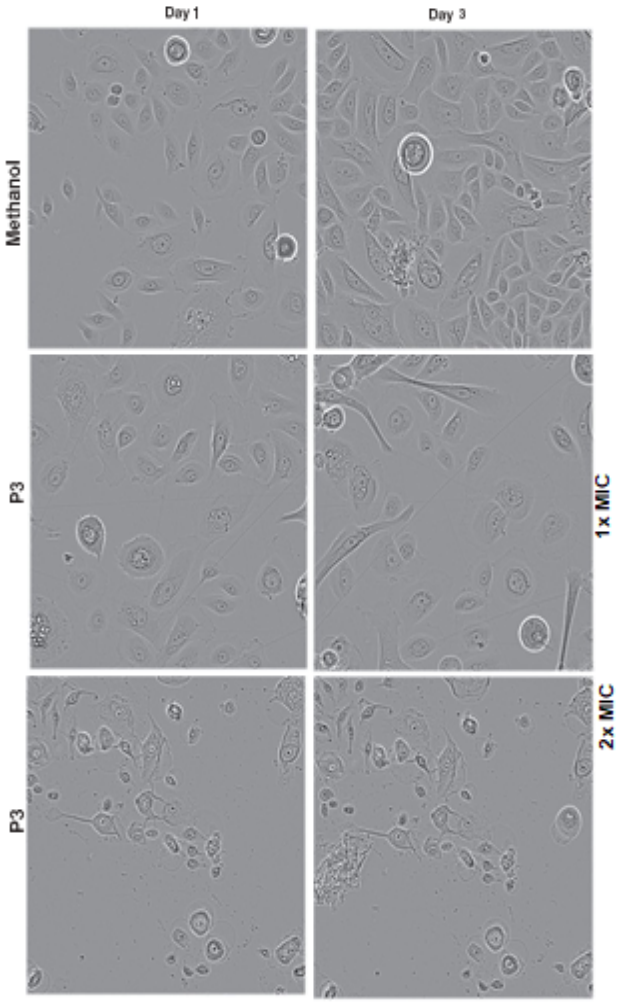

Figure 6c

Figure 6. Cytotoxicity of P3 and P4 compounds in human prostate cancer PC3 and primary keratinocytes. Methanol was used as the solvent to dissolve compounds and as the control. Cells were monitored with Incocyte for about three days. **a)**. P3 and P4 did not affect growth of PC3 cells **b)**. P3 at 1X MIC (50 µg/ml) inhibited growth of keratinocytes. c). P3 at 1X MIC did not lead to death of keratinocytes. Human keratinocytes were treated with methanol (top) or P3 at the concentrations of 1X MIC (middle) or 2X MIC (100 µg/ml) (bottom).

to block the growth of epidermal keratinocytes (Figure 6) without causing cell death (6c middle). At P3's 2x MIC dose, death of cells was recorded after 24 hours of treatment (6c bottom). P4 as well as more pronounced death of epidermal keratinocytes at lX and 2X MIC concentrations after 24 hours treatment (Figure 6c).

Discussion

The rise in the prevalence of bacterial infections that are resistant to antibiotics poses a great burden to a country's health care system. The challenge of finding effective treatment for Multidrug Resistant (MDR) pathogens suggests a need for innovative efforts towards the extraction of new antibacterial agents from natural resources [13]. In this research, bioactive substances P1, P2, P3 and P4 were isolated from the leaf extracts of *T. arborvitae* and tested for their antibacterial activity against important pathogenic bacteria. The results obtained showed that Gram-positive (*S. aureus* and *S. mutans*) and Gram-negative (*Acinetobacter baumannii*, *E. coli*, and *P. aeruginosa*) bacterial pathogens were highly sensitive to the purified compounds. Moreover, *A. baumannii*, an example of an MDR bacterium, was especially inhibited by these purified compounds [4,14].

Extracts of thuja have shown antibacterial activity in previous studies, where crude extracts were shown to have an effect on different strains of bacteria [3,6,15]. But it could not be identified which specific compounds were responsible for this activity. This study sought to fill that gap by isolating P1, P2, P3 and P4 and testing them against some common bacterial pathogens. The results verified the hypotheses which stated that these compounds are responsible for the antibacterial activity found in thuja extract. It was also found that P1, P2, P3, and P4 did exhibit some inhibitory activity in the range between 10 to 50. Such concentrations could make those compounds extremely useful to have, especially in medicines, as geraniums are known to pose potent antibacterial properties [8].

Most noteworthy was the ability of P1, P2, and P3 to suppress the growth of one of the most problematic drug-resistant bacteria; *A. baumannii*. This gram-negative bacillus is recognized for its multiple resistance mechanisms including efflux pumps, enzymatic breakdown and biofilm creation and, therefore, has very limited treatment options [9,16]. The ability of these thuja compounds to inhibit the growth of *A. baumannii* provides an optimistic outlook on developing these constituents and extracts for combatting MDR infections.

All three purified compounds showcased antibacterial activities, but their effectiveness was distinct for each bacterial strain. P1, P2, P3 and P4 were capable of inhibiting the growth of *S. aureus*, *S. mutans* and *E. coli*, while *P. aeruginosa* was the least susceptible. This is consistent with the previous reports on the resistance of *P. aeruginosa* which is associated with its very impermeable outer membrane and other intrinsic resistance mechanisms [17]. However, the broad-spectrum antibacterial activities of P1, P2, P3 and P4 against other clinically important pathogens does bear out their broad therapeutic potential.

The clearest and strongest implication is that *T. arborvitae* could yield novel antibacterial compounds. The purification of P1, P2, P3 and P4 and their demonstration of activity against numerous MDR bacterial strains sets the stage for more work to be done. Future work should be aimed at the modification of these compounds, their pharmacokinetics, and possible clinical uses for resistant bacterial infections [18]. The constant investigation of phytochemicals with antimicrobial properties remains one of the most effective ways of mitigating the danger posed by antibiotic resistance in public health today [2].

Acknowledgement

None.

Conflict of Interest

None.

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