ORIGINAL ARTICLE

Antimicrobial Activity and Molecular Docking Studies of a Sesquiterpenoid Alcohol from Leaf Solvent Extracts of *Juniperus communis* L.

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Abstract

Aim: The present investigations were focused on isolation, characterization and antimicrobial evaluation and molecular docking of phytocompounds from *Juniperus communis*. **Materials and Methods:** α-Cadinol together with several other phytocompounds such as α-pinene, β-pinene, decanal, and α-terpineol were isolated from leaves of *J. communis* L using Soxhlet extraction with petroleum ether, ethanol, methanol, and aqueous solvents and identified through gas chromatography-mass spectrometry were then evaluated for the antimicrobial potential against *Bacillus subtilis* and *Escherichia coli* under *in vitro* conditions at different concentrations, namely 20, 40, 80, 100, 200, and 300 ppm. Furthermore, molecular docking analysis of α-Cadinol was performed against common antibiotic drug target proteins, namely penicillin-binding protein 2X (PBP), DNA gyrase Subunit B and topoisomerase IV using AutoDock4 (version 4.2) with the Lamarckian genetic algorithm. **Results:** Among these, maximum growth inhibition was observed at 300 ppm. Docking studies proclaim that α-Cadinol, a current phytochemical of *J. communis* L., has the most elevated wellness (fitness) score and more specificity toward the PBP than other protein targets and the bioactivity might be a result of these compounds exhibited in leaf extracts.

Key words: Antimicrobial activity, gas chromatography-mass spectrometry, leaf solvent extracts, molecular docking, zone of inhibition

INTRODUCTION

We are continuously exposed to enormous microbes in the environment in which we are living, and most of them are harmless and do not cause any kind of infectious disease in human populations. However, among them, a fewer number of microorganisms are perilous and responsible for various kind of diseases that can deteriorate human immune health and might even prove lethal and cause fatal diseases such as tuberculosis, diphtheria, cholera, diarrhea, leprosy, tetanus, pneumonia, and typhoid.[1]

Antibiotics are the drugs, developed against harmful organisms to stop their infection and reduce the chances of disease occurrence in populations. Antibiotics recognize specific enzymes or proteins where they usually bind and inhibit the metabolic processes in the microbial cells. These specific enzymes and proteins are referred to as antimicrobial drug targets. Bacterial cells can mutate at a very fast rate which may lead to the development of resistance against the antibiotics. Hence, there is a continuous demand for the development of

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new antibiotic drugs which need extensive research facilities, skilled personals, and very high investments.

However, antibiotics are also responsible for the killing of non-target microorganisms such beneficial gut flora which may lead to the development of other health problems in human beings. Hence, there is an immediate need for the development of drugs which should be specific in action, overcome existing drawbacks and also do not have any kind of side effects on human health. Moreover, herbal remedies are of great importance in the primary health care of individuals and communities in many developing countries.[2] Medicinal plants have been used as traditional treatments for numerous human and plant diseases for thousands of years in many parts of the world. Plants are a good source of biologically active compounds and contain potent biochemical components of phytomedicines, and since time immemorial, man is able to obtain a marvelous assortment of industrial chemicals and disease control agents from plants. Such natural constituents can be derived from any part of the plant such as bark, leaves, flowers, roots, fruits, and seeds containing active ingredients.

The plant under present studies is *Juniperus communis* L. which has been reported as antimicrobial, aphrodisiac, and styptic in nature and is useful in the treatment of asthma, chronic bronchitis, and diseases of liver and spleen. It yields 0.25% of essential oil, resin, juniperin, and organic acids.[3] Despite enormous antimicrobial potential of this plant, still much work has not been done to exploit its antimicrobial compounds and to develop herbal formulation against common bacterial infections.[4]

Juniper berries and leaves have long been used as medicine by many cultures including the Navajo people.[5] Western American tribes combined the leaves and berries of *J. communis* with Barberry is root bark in herbal tea. Native Americans also used juniper berries as a female contraceptive.[6]

The gas chromatography-mass spectrophotometry (GC-MS) analysis of *J. communis* L. leaf, stem, bark, roots, and berries extracts revealed the presence of terpene, namely alpha and beta pinene, myrcene, sabinene, thujone, and limonene which are known biologically active metabolites and have multiple uses. The essential oils from Juniper also possess secondary metabolites like sesquiterpene which includes caryophyllene, cadinene, elemene, and terpene alcohols, for example, terpinen-4-ol.[7-9]

Moreover, present studies focused on the isolation of essential oils from *J. communis* L., biochemical characterization of plant extracts using GCMS, *in vitro* antimicrobial activity of against *Bacillus subtilis* (MTCC no. 121) and *Escherichia coli* (MTCC no. 118) and the interaction of phytocompounds with antibacterial drug targets using *in silico* techniques. *B. subtilis* (Gram +ve), best-studied microbe so far and well-thought-out to be a model organism for explaining various metabolic processes such as prokaryotic replication and cell differentiation.[10] *B. subtilis* is not a pathogenic bacterium and can be taken as probiotic in case of healthy individuals but sometimes may report to cause disease in severely immunocompromised patients.[11] It rarely causes food poisoning[12] but can be associated with the development of autoimmune diseases at times.

Similarly, *E. coli*, a Gram-negative, facultative, anaerobic bacterium, seldom be a root of any disease but at the same time may cause gastroenteritis, urinary tract infections (UTI), and neonatal meningitis infections which are accompanied by common symptoms such as severe abdominal cramps, and bloody diarrhea within 24 h and fever. These types of infections and disease symptoms are generally reported to be caused by some of its virulent strains. In some rare cases, these strains may be also responsible for bowel necrosis (tissue death) and perforation without progressing to hemolytic-uremic syndrome, peritonitis, mastitis, septicemia, and Gram-negative pneumonia.[13]

METHODS

Media and chemicals

Media used in present study were nutrient agar media from HiMedia, India, with a final pH adjusted at 7.3 ± 0.2 at 25°C and used for the determination of zone of inhibition and antibacterial activity of leaf extracts against B. subtilis and *E. coli*. Tetracycline (50 µg/disk) and ampicillin disks (50 µg/disk) were procured from HiMedia to be used as standards for determining minimum inhibitory zone.

Collection of plant material

Plant material used

It is a shade-dried leaves of *J. communis* L.

Time of sample collection

Samples were collected in the active growth season, i.e., month of March–April

Site of collection

Chitkul village in Sangla Valley located in Kinnaur district of Himachal Pradesh, India, and the samples were identified and verified by Dr. Ravinder Raina Principal Scientist, Department of Forest Products, College of Forestry, Dr. Y.S Parmar University of Horticulture and Forestry, Nauni, Solan, H.P., India.

Extraction procedure

Leaves were washed thoroughly and dried under shade at the room temperature (20 ± 2°C). The dried leaves were then

ground to a fine powder in an electric grinder. Stock solutions of the extract were prepared by adding ground leaf powder to 200 ml of each solvent (w/v, 50 g/200 ml). Different solvents used for extraction according to polarity were petroleum ether, methanol, ethanol, and water. Extracts were then shaken for at least 6 h for homogenous mixing of ground leaf powder in the solvent. After that, each extract was passed through Whatman filter paper no.1. Final filtrate was then concentrated to 25% crude extract on a rotary evaporator or Soxhlet extraction apparatus under vacuum at 20°C and was utilized for the experiments.

Disc diffusion method

Antibacterial tests of selected microorganisms were carried out using disc-diffusion method.[14] Nutrient agar plates (90 mm size) were prepared and cooled down at room temperature (20 ± 2°C). A small sterile cotton swab was dipped into the 24 h old culture of bacteria and was inoculated by streaking the swab over the entire agar surface. This process was repeated by streaking the swab two or more times rotating the plates approximately 60° each time to ensure even distribution of inoculum. After inoculation, the plates were allowed to dry at room temperature (20 ± 2°C) for 15 minutes in the laminar chamber for settle down of inoculum. The filter paper discs (5 mm) loaded with 40 µl of extract were placed on the surface of the bacteria seeded agar plates and it was allowed to diffuse for 5 min then these plates were incubated at 37 ± 1°C for 24 h.

GC-MS analysis

GC-MS analysis of *J. communis* was carried out simultaneously on a Finnigan Thermo Quest Trace GC with a dual split/splitless injector, an FID detector, and a Finnigan Automass quadrupole mass spectrometer. One inlet was connected to a 50 m × 0.25 mm × 1.0 µm SE-54 fused silica column (CS Chromatographie Service, Langerwehe, Germany). The other injector was coupled to a 60 m ×0.25 mm × 0.25 µm Carbowax 20 M column. The two columns were connected at the outlet with a quartz Y connector and the combined effluents of the columns were split simultaneously to the FID and MS detectors with a short (ca. 50 cm) 0.1 mm ID fused silica restrictor column as a GC/MS interface. The carrier gas was helium 5.0 with a constant flow rate of 1.5 mL/min; injector temperature was 230°C, FID detector temperature 250°C, GC/MS interface heating 250°C, ion source at 150°C, EI mode at 70 eV, and scan range 40–300 amu. The following temperature program was used: 46°C for 1 min; 46°C–100°C at a rate of 5°C/min; 100°C–230°C at 2°C/min; and 230°C for 13.2 min. GCMS was performed using GC-MS Shimadzu Model QP-2010 mass spectrometer under the following conditions: DB-Polyethylene glycol coated fused silica capillary column (30 m length × 0.25 mm ID × 0.25 µm film thickness): Helium carrier gas (1.34 ml/min); 250°C injector temperature; 240°C interface temperature; and 200°C on source temperature. Column temperature was programmed at 60°C with 10°C/min rise to 230°C. For GC-MS detection ionization energy of 70ev was used.[15]

Molecular docking analysis

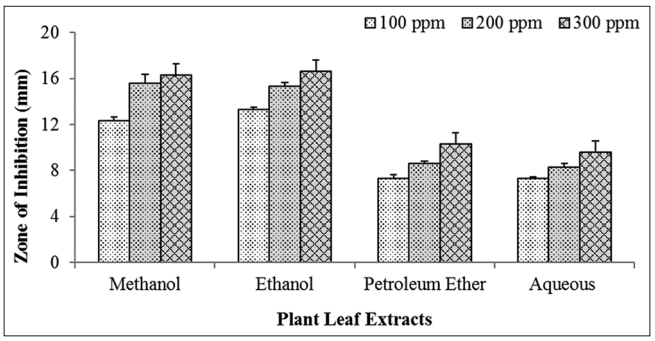
Virtual screening of all the compounds was performed against penicillin-binding protein 1a (PBP1a), alanine racemase (Alr), D-alanyl-D-alanine synthase, isoleucyl-tRNA synthase, DNA gyrase Subunit B, topoisomerase IV (TopoIV), dihydropteroate synthase (DHPS), and dihydrofolate reductase using AutoDock4. All the predicted docking poses presented a root mean square deviation (RMSD) lower than 2 Å when compared to the experimental cocrystallized binding pose. This is a strong evidence that AutoDock4 can predict docking poses accurately, as 2 Å is usually considered a good threshold value for RMSD. To validate scoring predictability, E (free energy used for binding, Kcal/Mol) values estimated by AutoDock4 or X-Score were compared with experimental E values. Therefore, X-Score may be a better scoring function for the protein structures studied when compared to AutoDock4.

RESULTS

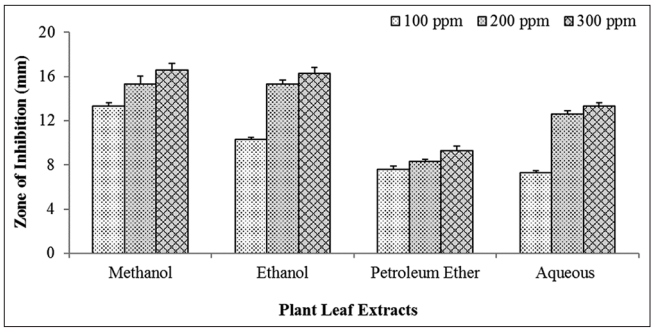
Antibacterial studies were carried out using plant leaf solvent extracts at various concentrations (20, 40, 60, 80, 100, 200, and 300 ppm) for an incubation period of 24 h. The maximum zone of inhibition against *B. subtilis* was 16.6 mm recorded at 300 ppm in case of ethanol extract minimum zone of inhibition, i.e., 7.3 mm was displayed by petroleum ether and aqueous extract at 100 ppm [Graph 1 and Plate 1].

300 ppm of ethanol extract was most inhibitory against *E. coli,* the diameter for inhibition zone was recorded to be 21.30 mm while least inhibitory (7.10 mm) concentration was 100 ppm of petroleum ether extract as revealed from Graph 2 to Plate 2.

GC-MS investigation demonstrated the presence of a comparative number of mixes in the leaf concentrates/extracts [Figures 1-3]. The phytocompounds having crest



Graph 1: Graph showing zone of inhibition of *Juniperus communis* L. leaf extracts against *Escherichia coli*



Graph 2: Graph showing zone of inhibition of *Juniperus communis* L. leaf extracts against *Bacillus subtilis*

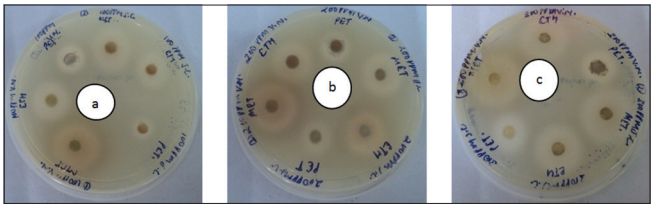


Plate 1: (a-c) Zone of inhibition of *Juniperus communis* against *Bacillus subtilis*

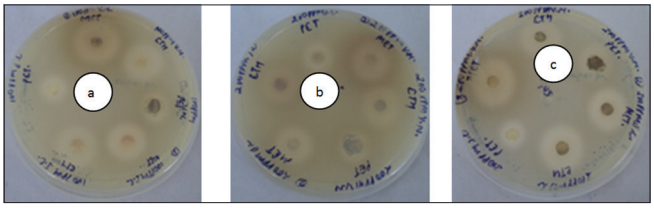


Plate 2: Zone of inhibition of *Juniperus communis* against *E. coli*. *(a) 100 ppm, (b) 200 (c) 300 for methanol, ethanol, petroleum ether, and aqueous extracts. **Met - methanol, Eth - Ethanol, Pet - Petroleum Ether

zone more than 5% were displayed in Table 1. Out of these phytocompounds, Phenanthrene carboxylic corrosive was the significant compound (11.44%) trailed by Cedrol (9.12 %), 8-Beta cedron-8-ol (7.28%), and Alpha-Cadinol (7.04%) while other staying substance mixes were believed to be available in trace sums. The significant components and their maintenance times are outlined in Table 2.

The test bacteria have specific receptor sites (protein targets) which specifically attract and bind to the ligands (bioactive groups of phytocompounds), available in the compounds obtained from the test plants. Based on literature review studies, it is observed that among all the phytocompounds, α-Cadinol is having very high bioactivity against bacteria and therefore was selected to be tested against antibiotic drug target proteins using computational biology analysis (Molecular Docking). The results produced from molecular docking studies revealed and confirmed the bioactivity of α-Cadinol against all the tested antimicrobial drug target proteins including DNA gyrase Subunit B protein, TopoIV Subunit B (Topo IV), and PBP 2x. The maximum value of X-Score corresponding to free energy of binding between

Table 1: Major phytocompounds of *Juniperus communis* L. essential oil (GC-MS analysis)

Sr. No.	Compound	R. Time	Area %
1	Trans-Z-alph a-Bisabolene epoxide	18.77	6.13
2	8 Beta, H-cedron-8-ol	19.054	7.28
3	(-) Globulol	20.843	6.36
4	Alpha-Cadinol	18.761	7.04
5	Cedrol	19.044	9.12
6	Limonene dioxide	20.821	5.87
7	Ferruginol	33.694	5.9
8	Selina-6-ene-4-ol	15.335	5.71
9	1-Phenanthrene carboxylic acid	19.02	11.44
10	Stigmast-4-en-3-one	20.785	7.85
11	Benzoic acid	33.673	8.24
12	Dodecanoic acid	49.923	6.4

GC-MS: Gas chromatography-mass spectrophotometry

phytocompound and drug target was found to be highest in case of PBP 2x (ΔG = 6.6) followed by DNA gyrase Subunit B (ΔG = 5.8) and least for TopoIV Subunit B (ΔG =5.0). Therefore, it can be deciphered from above results that α-Cadinol is mainly involved in inhibition of cell wall synthesis as the maximum binding capacity was in case of PBP which is a common drug target for antibiotic penicillin and involved in peptidoglycan biosynthesis of the cell wall in bacteria. Furthermore, DNA gyrase Subunit B and TopoIV Subunit B are the enzymes subunits which are major components of gyrase and topoisomerase enzymes, involved in prokaryotic DNA replication. Hence, α-Cadinol also supposed to inhibit replication and thus can be related to inhibition of bacterial growth which was observed during *in vitro* antibacterial studies [Table 2, Figures 4-6].

DISCUSSION

The leaves and berries of *J. communis* are widely used as an anti-inflammatory, treatment of wounds and kidney and stomach disorders.[5,16] Earlier studies on Juniper have also revealed the antimicrobial activity of leaf extracts against a variety of plant and animal pathogenic bacteria.[17] The antimicrobial assay of present studies showed that growth inhibition was consistently observed against *B. subtilis* and *E. coli* by all the leaf extracts including methanol, ethanol, petroleum ether, and aqueous as well. However, it is noteworthy that all these observations were recorded for leaf solvent extracts only and similar results have also been obtained against a plant pathogenic bacterium, i.e., *Xanthomonas axonopodis*.[18]

In agreement with the previous antimicrobial assays, we observed maximum growth inhibitory activity for the ethanolic

Table 2: X-Scores from molecular docking analysis of phytochemicals against antibacterial drug target proteins

S. No.	Phytocompound	Protein	PDB entry	Resolution (Å)	X-Score(ΔG)
1	Alpha-Cadinol	DNA gyrase Subunit B	1AJ6	2.3	–5.8
2	Alpha-Cadinol	Penicillin-binding protein 2x	1QMF	2.8	–6.6
3	Alpha-Cadinol	DNA topoisomerase 4 Subunit B	3FV5	1.8	–5.0

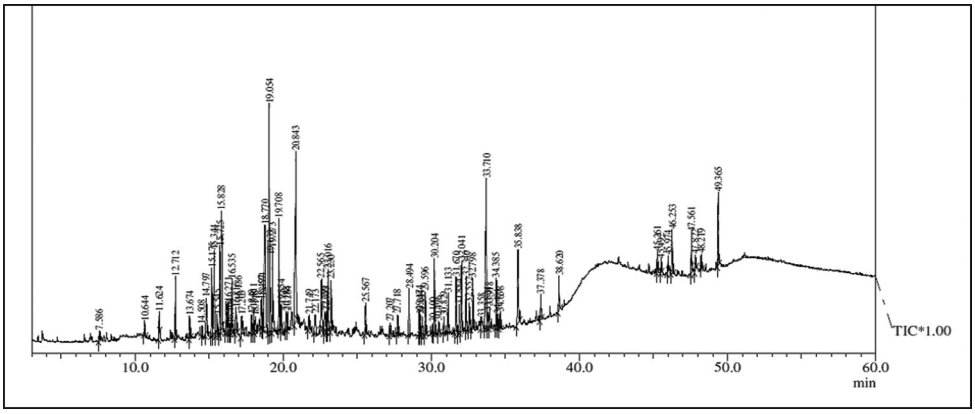


Figure 1: Gas chromatography-mass spectrophotometry chromatogram of Methanol extract of *Juniperus communis* L.

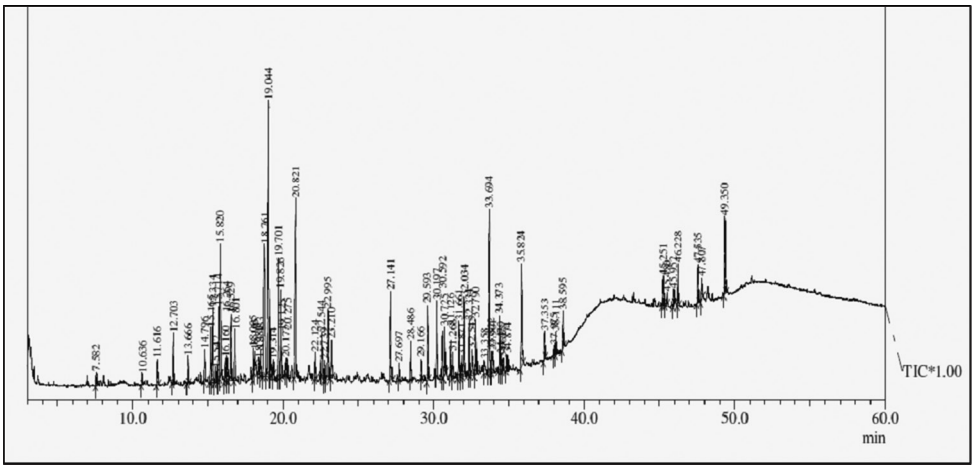


Figure 2: Gas chromatography-mass spectrophotometry chromatogram of Ethanol extract of *Juniperus communis* L.

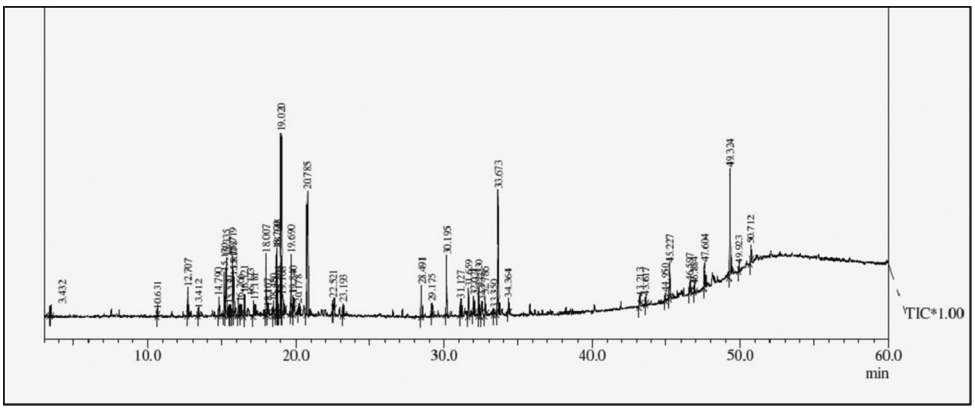


Figure 3: Gas chromatography-mass spectrophotometry chromatogram of Petroleum ether extract of *Juniperus communis* L.

extract against the tested bacterial species at 300 ppm. The bacterial species tested in present studies, i.e., *E. coli* and *B. subtilis* are moderate pathogenic bacteria and do not directly in kind of disease response in healthy individuals, but they have been reported to trigger inflammatory immune responses for stomach, intestinal, and UTI.[19] In 2001, it has

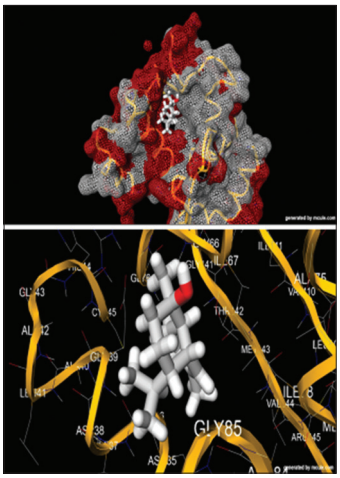


Figure 4: Molecular interactions of alpha-Cadinol molecule against DNA gyrase Subunit B protein

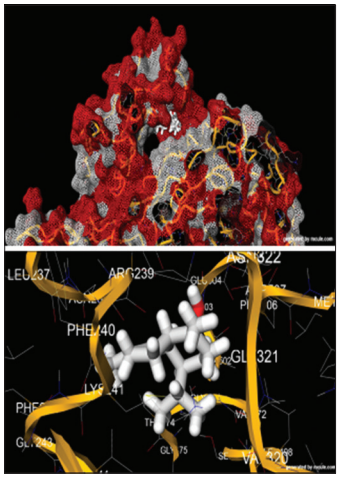


Figure 5: Molecular interactions of alpha-Cadinol molecule against penicillin-binding protein

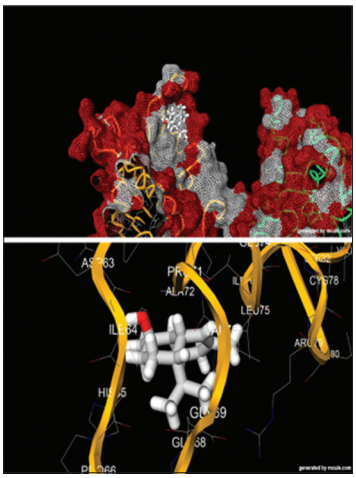


Figure 6: Molecular interactions of alpha-Cadinol molecule against topoisomerase Subunit B protein

been revealed that innate immune response to Uropathogenic *E. coli* involves IL-17A (innate - adaptive immunomodulator cytokine) causing multiple sclerosis in a murine model of UTI.[20] Therefore, it can be suggested that leaf extracts which are showing growth inhibitory activities against tested microorganisms may prove to be useful in decreasing the extent of multiple sclerosis in susceptible individuals. Indeed, *J. communis* leaf extracts may be a better therapeutic option for preventing multiple sclerosis and effective growth inhibitor for both, i.e. *E. coli* and *B. subtilis*.

The phytochemical characterization of *J. communis* leaf extracts through GC-MS has focused on terpenoid contents mainly.[21] A number of phytocompounds, namely Phenanthrene carboxylic acid, Cedrol, 8- Beta cedron-8-ol, Alpha-Cadinol, and Dodecanoic acid were identified.

Many of these compounds reported to have broad-spectrum antimicrobial activity.[22,23] Therefore, these may contribute to the bacterial growth inhibitory activity in the present studies as well. Oxygenated sesquiterpene alpha-Cadinol is a potent antifungal, possible remedy for drug-resistant tuberculosis.[4,25] and hepatoprotective in action. Hence, based on these previous studies it has been selected for further testing using *in silico* methods. AutoDock 4.0 was used to simulate the interaction of alpha-Cadinol against three antibacterial target proteins, namely topisomerase IV Subunit B, DNA gyrase Subunit B, and PBP which is common in most of the bacterial species. The free energy scores or X-Scores which are generated as a result of docking analysis suggest that alpha-Cadinol is showing binding affinity for all the docked antimicrobial drug targets but having a maximum binding affinity for Penicillin-binding protein 2x.

Comparable outcomes were referred when researchers have completed the docking of antibacterial compounds from mushrooms (to be specific, enokipodins, ganomycins, and austrocortiluteins) and principle mechanism of the activity was the restraint of cell wall generation, Alr and Ddl being the likely protein targets.263] Other researchers have also carried out the docking of terpene compounds (specifically diterpenoids) against a variety of microorganisms which are also in line with present investigations where different terpenoids have been identified to be potential ligands against specific protein targets in microorganisms.[27-29]

Neogrifolin (Sesquiterpenes) and p-hydroxybenzoic acid (phenolic subsidiary of benzoic acid) were superimposed to the protein structure utilized as a part of the present examinations (DHPS). The place where neogrifolin, PABA and p-hydroxybenzoic acid intervene with DHPS is thought to be at an indistinguishable site as for sulfonamides or sulfa drugs, which are PABA (p-aminobenzoic acid) analogs and go about as interchange substrates for DHPS.[30]

From these observations, it is, therefore, concluded that the test bacteria have specific receptor sites (protein targets) which specifically attract and bind to the ligands (bioactive groups of phytocompounds), available in the compounds obtained from the *J. communis* L. However, the results are yet to be authenticated

through wet-lab experimentations for each biomolecule before going ahead for potential drug development.

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