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In vitro antituberculosis activities of the constituents isolated from *Haloxylon salicornicum*

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ABSTRACT

In vitro antituberculosis activities of fractions and pure compounds (**1**–**20**) including seven triterpenes, two alkaloids, two cycloheximide derivatives, two coumarins six sterol derivatives and a long chain alcohol, respectively, isolated from *Haloxylon salicornicum* were determined against *Mycobecterium tuberculosis* H37Rv. Actively growing cultures were tested by rapid colorimetric method while the stationary phase cultures were tested by drug exposure methods for bactericidal activity. The MIC values were found to be 50 μg/ml for compounds **15**, **19** and **20** where as rest of the compounds invariably showed MIC value of 100 μg/ml against the logarithmic phase culture. These were compare to Isoniazid as a control drug. The compounds exhibited no activity against the stationary phase culture of *M. tuberculosis* H37Rv up to 200 μg/ml. Further studies are required to investigate the in vivo efficacies and activities of the compounds in combination with antimicrobials that are already being used for TB therapy.

Tuberculosis is a well known disease that has afflicted human being since antiquity.[1,2]

According to estimation by the World Health Organization (WHO), approximately one third of the world's population is infected with *Mycobacterium tuberculosis* (the causative agent of TB), eight million people develop tuberculosis disease annually, while two million people die and another three million new cases are being added each year.[3] In 1993, TB was declared a global health emergency by the WHO.[4,5] Pakistan ranks 8th in TB among the high burden countries with 270,000 new cases and an incidence of 181 cases/100,000/year.[6] Development of drug resistance is a big threat to tuberculosis control as DOTS (directly observed treatment short course) has become ineffective in treating the MDR-TB and requires extensive chemotherapy, which is quite expensive and is also more toxic to patients. The alarming increase of multidrug-resistant TB (MDR-TB) cases calls for an urgent need to develop new, more effective and safer anti-TB drugs especially for the treatment of the MDR-TB cases.[7] Medicinal plants have long been used in traditional healthcare systems as remedy of various ailments including tuberculosis and related conditions such as ulcer and lung infections.[8] Natural plant products have provided an alternative source for the development of antimicrobial drugs.[9,10]

Haloxylon salicornicum belongs to the family Chenopodiaceae which comprises 100 genera and 1200 species. Most of the members of this family are weedy and grow in waste and unfertile areas of soil. In Pakistan, this family is represented by 35 genera. Only five species of genus *Haloxylon* are found in Pakistan. *H. salicornicum* is a diffuse shrub, pale, much branched, almost leafless, 25–60 cm tall, with woody stem. It is widely distributed in Egypt, Palestine, Jordan, Iraq, Kuwait, Iran, and Pakistan. The plant is traditionally reported for its toxicity and applied externally on insect stings.[11–14] The ash of the plant is used for internal ulcers. As part of our study of anti-infective properties of medicinal plants easily available in Pakistan and have been reported to be used historically for the treatment of different ailments, present study was carried out to determine antituberculosis activities of four fractions and pure compounds isolated from *H. salicornium* (Fig. 1).

The whole plant of *H. salicornicum* Bunge ex Boiss was collected from Cholistan desert near district Bahawalpur (Punjab), Pakistan in October, 2003. Further identification was carried out at Cholistan Institute of Desert Studies, Islamia University Bahawalpur, where a voucher specimen has been deposited.

The whole plant of *H. salicornicum* (20 kg) was shade-dried, ground, and extracted with methanol (3 × 50 L). The combined methanolic extract (900 g) was partitioned between *n*-hexane

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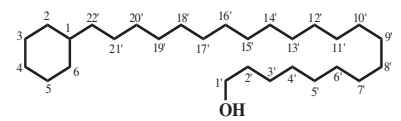
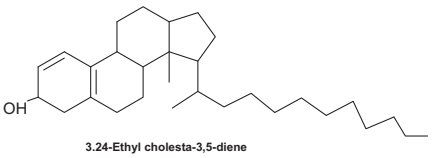
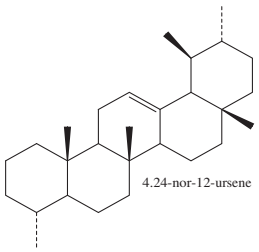


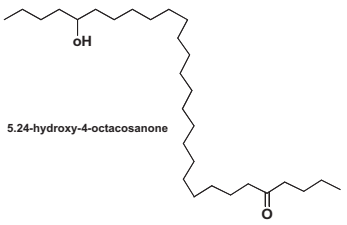
1.1'-Hydroxytetracosanyl cyclohexane n=22
2.1'-Hydroxydocasanyl cyclohexane n=20



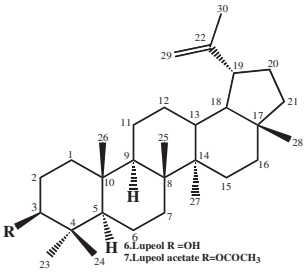
3.24-Ethyl cholesta-3,5-diene



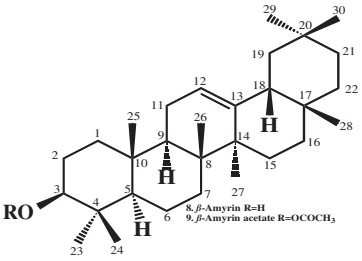
4.24-nor-12-ursene



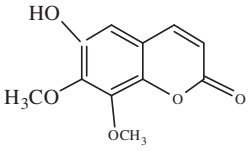
5.24-hydroxy-4-octacosanone



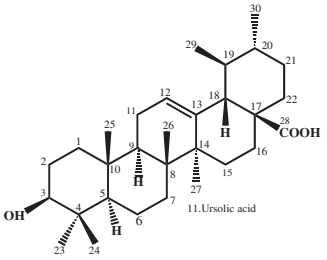
6.Lupeol R =OH
7.Lupeol acetate R=OCOCH$_3$



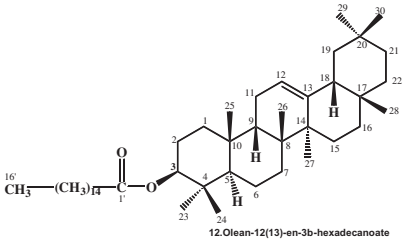
8. β-Amyrin R=H
9. β-Amyrin acetate R=OCOCH$_3$



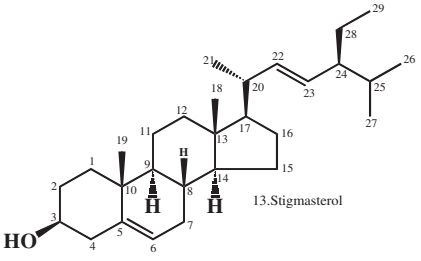
10. 7,8 dimthoxy -6- hydroxy cumarine



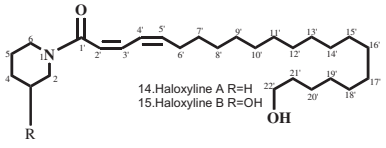
11.Ursolic acid



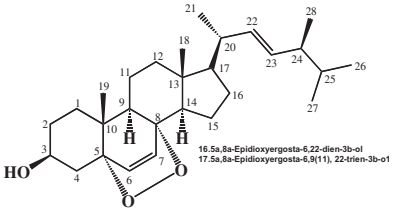
12.Olean-12(13)-en-3b-hexadecanoate



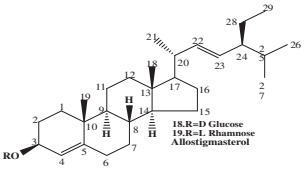
13.Stigmasterol



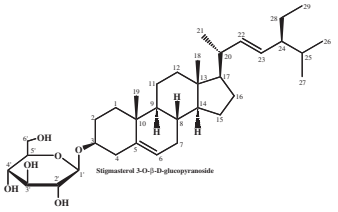
14.Haloxyline A R=H
15.Haloxyline B R=OH



16.5a,8a-Epidioxyergosta-6,22-dien-3b-ol
17.5a,8a-Epidioxyergosta-6,9(11), 22-trien-3b-o1



18.R=D Glucose
19.R=L Rhamnose
Allostigmasterol



Stigmasterol 3-O-β-D-glucopyranoside

Figure 1. Structures of the compounds.

and water. The aqueous fraction was successively partitioned with $CHCl_3$, EtOAc and *n*-butanol. The $CHCl_3$ soluble fraction (200 g) was subjected to column chromatography over silica gel eluting with *n*-hexane–$CHCl_3$, $CHCl_3$, $CHCl_3$–MeOH and MeOH in increasing order of polarity. Here, were a total 20 fractions which were selected for the study. The fraction obtained from *n*-hexane–$CHCl_3$ (7.0:3.0) gave four spots on TLC which on preparative TLC in the same solvent system gave 1′-hydroxytetracosanyl cyclohexane (1), 1′-hydroxydocasanyl cyclohexane (2), 24-ethyl cholesta-3,5-diene (3) and 24-nor-12-ursene (4), respectively. The fraction obtained from *n*-hexane–$CHCl_3$ (5.0:5.0) gave three spots on TLC which on preparative TLC in the same solvent system gave 24-hydroxy-4-octacosanone (5), Lupeol (6) and Lupeol acetate (7). The fraction obtained from *n*-hexane–$CHCl_3$ (4.0:6.0) gave three spots on TLC which on preparative TLC in the same solvent system gave β-amyrin (8), β-amyrin acetate (9), stigmasterol (13), respectively. The fractions obtained from *n*-hexane–$CHCl_3$ (3.0:7.0) were combined and rechromatographed over silica gel using solvent system *n*-hexane–$CHCl_3$ to provide ursolic acid (11) and olean-12(13) en-3β-hexadecanoate (12) from the top and tail fractions, respectively. The fractions obtained from *n*-hexane–$CHCl_3$ (2.0:8.0) gave two spots on TLC which on preparative TLC using solvent system *n*-hexane–$CHCl_3$ (1.5:8.5), respectively, provided 7,8-dimethyl-6-hydroxy cumarine (10), and haloxyline A (14). The fraction obtained from *n*-hexane–$CHCl_3$ (2.5:7.5) was a mixture of two components which were separated by silica gel columns chromatography using solvent system *n*-hexane–$CHCl_3$ (2.8:1.2), collecting 10 ml fractions in each case, to obtain haloxyline B (15) from fractions 20–30 and 5α-epidioxyergosta-6,9(11), 22-triene, (16) from fractions 40–60 and α,8α-epidioxyergosta-6,22-dien-3β-ol (17) from fractions 70–90. The fractions which were obtained from $CHCl_3$–MeOH (9.0:1.0) were combined and rechromatographed over silica gel eluting with $CHCl_3$–MeOH in increasing order of polarity.

The fractions which were obtained from $CHCl_3$–MeOH (9.0:1.0) gave three spots on TLC which on preparative TLC by using solvent system $CHCl_3$–MeOH (8.8:1.2), afforded 24β (24*S*)-ethyl-cholesta-4-22 *E* diene 3-*O*-β-D-glucoside (18), 24β (24*S*)-ethyl-cholesta-4-22*E* diene 3-*O*-α-L-rhamnosid (19) and stigmasterol 3-*O*-β-D-glucopyranoside (20).

The chemical constituents of *H. salicornicum* including four fractions and 20 pure compounds were dissolved in DMSO to prepare stock solutions (1 mg/ml). Isoniazid (INH) (control drug) was dissolved in D/W (0.1 mg/ml). Stock solutions of reagents XTT ((2,3-bis(2-methoxy-4-nitro-5-sulphonyl)-5[(phenylamino)carbonyl]-2*H*-tetrazolium hydroxide)) and Phenazine methosulfate (PMS) (Sigma) were prepared in PBS at concentration of 1 mg/ml.

Log phase culture was prepared by growing *M. tuberculosis* H37Rv (ATCC 27294) for 10 days in Middlebrook 7H9 broth (DIFCO) (supplemented with 10% ADC, glycerol and 0.05% Tween). The culture ($\sim 10^8$ CFU/ml) was diluted 1:50 in Middlebrook 7H9 complete medium. Old stationary phase culture was prepared by growing *M. tuberculosis* H37Rv in Middlebrook 7H9 complete medium (supplemented with 10% ADC, glycerol and 0.05% Tween) up to 9 weeks without shaking. Cells were centrifuged, washed and resuspended in minimal 7H9 broth (without ADC and glycerol, pH 5.8) to match turbidity to McFarland standard tube no. 1.

Screening of compounds against the logarithmic phase culture: Compounds were screened against the logarithmic phase culture of *M. tuberculosis* H37Rv by rapid micro-colorimetric method using XTT dye as growth indicator.[15] Briefly, in a 96-well flat bottom microtitre plate, compounds were added in Middlebrook 7H9 complete medium (at pH 6.8) to the concentrations of 200 and 100 μg/ml and inoculated with 100 μl culture suspension at the concentration of 10^5 cells/ml in 200 μl final volume. Isoniazid (final concn 0.1 μg/ml) was tested as control drugs. Following incubation for about 7 days, 50 μl of a solution prepared by mixing 100 μl of PMS (Phenazine methosulfate) and 900 μl of XTT, was added to the control wells and incubated for another 3–4 h or longer (overnight). Color change (from pale yellow to red) indicated growth in control wells, the dye was added to the remaining wells. Compounds that exhibited activity were further tested for their MICs by micro broth dilution method.[16] Series of concentrations from 100 to 0.8 μg/ml were tested and the MICs were recorded as the lowest concentration that inhibited the visible growth after 2 weeks of incubation.

Screening of compounds against stationary phase culture: The anti-TB activities of the compounds against the stationary phase culture of *M. Tuberculosis* H37Rv were determined by drug exposure method.[17] Aliquots of the undiluted old culture were exposed to compounds at concentrations of 50, 100 and 200 μg/ml in acidic minimal 7H9 broth (pH 5.8) for 3 days. Pyrazinamide (50 and 100 μg/ml) was used as control drug. Following exposure to the compounds; cells were washed to remove the drug, appropriate dilutions were plated for CFU count and the plates were incubated at 37 °C for 4 weeks to determine the MBCs.

Despite the availability of a powerful treatment course (DOTS; 6 months therapy with multiple drug regimen), tuberculosis control is a big challenge due to the development of multiple drug resistance tuberculosis (MDR-TB) resistant to the main firstline anti-TB drug that is, isoniazid and now the extensive drug resistance XDR-TB (MDR strain that is resistant to any fluoroquinolone and at least one of the injectable secondline drugs).[18–22] Moreover, the current treatment regimens for MDR-TB are quite expensive and more complex with only 50% survival rate.[23,24] Therefore, the search for new, more effective and less toxic antituberculosis agents has become increasingly important for the treatment of MDR-TB cases. *H. salicornicum* is a wild plant widely distributed in Central and South East Asia including Pakistan and is traditionally reported for its toxicity and applied externally on insect stings.[25] The ash of the plant is used for internal ulcers.[12] In this study, 24 chemical constituents (four fractions and 20 pure compounds) isolated from *H. salicornicum* were screened for their antituberculosis activities by colorimetric XXT method and MICs of the compounds were determined by Broth Microdilution method. Results are presented in (Tables 1 and 2). The MICs were found to be 50 and 100 μg/ml. Among four fractions tested, the chloroform and ethylacetate fraction showed significant activity at 50 μg/ml (Table 1) whereas similar activity was observed in three of the pure compounds namely Haloxyline B, 24β (24*S*)-ethyl cholesta-4-22 *E* diene 3-*O*-α-L-rhamnoside, and stigmasterol 3-*O*-β-D-glucopyranoside (Table 2). The compounds were also tested against the stationary phase culture of *M. tuberculosis* H37Rv by drug exposure method where no activity was observed after the culture was treated with compounds up to 200 μg/ml for 3 day in minimal 7H9 broth medium at pH 5.8. In an earlier study, Haloxyline A and B have also been shown to exhibit antifungal activities.[26] Wahab et al. recently reported that the methanolic extract of *Haloxylon recurvum* and its butanol fraction have been found to possess antibacterial and antifungal activities.[27]A number of studies are available reporting moderate to high antituberculosis activities of plant derived alkaloids, terpenes, saponins and sterols.[8,28] Jin reviewed the diverse nature of alkaloid containing natural products exhibiting biological activities such as antibacterial, antifungal

Table 1
Anti-TB activities of the fractions isolated from *H. salicornicum* by micro broth dilution method

S. No	Fractions	MIC (μg/ml)
1	Hexane	100
2	Chloroform	50
3	Ethylacetate	50
4	Butanol	100

Table 2
Anti-TB activities of the pure compounds isolated from *H. salicornicum* by micro broth dilution method

S. No	Pure compounds	MIC (μg/ml)
1	1′-Hydroxytetracosanyl cyclohexane	100
2	1′-Hydroxydocasanyl cyclohexane	100
3	24-Ethyl cholesta-3,5-diene	100
4	24-Nor-12-ursene	100
5	24-Hydroxy-4-octacosanone	100
6	Lupeol	100
7	Lupeol acetate	100
8	β-Amyrin	100
9	β-Amyrin acetate	100
10	7,8-Dimthoxy-6-hydroxy cumarine	100
11	Ursolic acid	100
12	Olean-12(13) en-3β-hexadecanoate	100
13	Stigmasterol	100
14	Haloxyline A	100
15	Haloxyline B	50
16	5α-Epidioxyergosta-6,9(11), 22-triene	100
17	α,8α-Epidioxyergosta-6,22-dien-3β-ol	100
18	24β (24*S*)-Ethyl-cholesta-4-22 *E* diene 3-*O*-β-D-glucoside	100
19	24β (24*S*)-Ethyl cholesta-4-22 *E* diene 3-*O*-α-L-rhamnoside	50
20	Stigmasterol 3-*O*-β-D-glucopyranoside	50
21	Isoniazid	0.02

and antiviral activities.[29] The promising antituberculosis activities exhibited by the chemical constituents of *H. salicornicum* reported in this study together with the antifungal activities of its alkaloids justify the ethnopharmacological use of *H. salicornicum* in different diseased conditions and provide an evidence of their potential to be used as broad spectrum antituberculosis agents.

H. salicornicum constituents did not exhibit antituberculosis activity against the non replicating stationary phase culture of *M. tuberculosis* (up to 200 μg/ml) when tested in acidic medium representing the intracellular microenvironment during established infection. These observations indicate that like many other existing anti-infective agents, *H. Salicornicum* constituents also exert their antimicrobial activity on actively multiplying cells during the macromolecular synthesis. *Mycobacterium tuberculosis* is hard to treat organism being equipped with lipid and waxed cell wall rendering most of the antibiotics unable to penetrate.[30] The alkaloids, terpenes and saponins because of the lipophilic nature can easily penetrate into mycobacterial cell wall to inhibit their growth. Berberine and harmane for example are the two highly aromatic planar quaternary alkaloids that have the ability to intercalate with DNA.[31] Whereas, the detrimental effect of saponins may largely lie on their ability to cause membrane damage and leakage of cellular materials leading to cell death.[32] But this has to be confirmed by mechanistic studies. Further studies are required to explore the in vivo efficacy of the bioactive compounds isolated from *H. salicornicum* to use them for the treatment of tuberculosis, along with other anti tuberculosis drugs.

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Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bmcl.2010.05.061.

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