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Cytotoxic Effect of Some Furanosesquiterpenes from Balsamodendron myrrha Nees. Oleo-gum-Resin on Brine Shrimp *(Artemia salina)* Larvae

Ayesha Asif1, M. Asif Saeed[2*]

1 Faculty of Pharmacy, University of Central Punjab, Lahore, Pakistan.
2 Department of Pharmacognosy, University of the Punjab, Lahore, Pakistan.

ABSTRACT

Furanosesquiterpenes from *Balsamodendron myrrha* oleo-gum-resin were assessed for their cytotoxic effect. Six Furanosesquiterpenes from hexane insoluble part of EtOH extract of *B. myrrha* oleoresin was separated, purified by column, preparative TLC and recrystallization. These were identified by comparative spectroscopic data. Cytotoxic effect of these compounds was carried out on brine shrimps' (*Artemia salina*) larvae (nauplii), evaluated by LD_{50} after compared with positive controlled colchicine. Six isolated furanosesquiterpenes demonstrated cytotoxic effect against brine shrimps' larvae compared with colchicine. Four sesquiterpenes, compound-1 (myrracadinol-A), compound-2 (myrracadinol-B), compound-5 (furanodiene-6-one) and compound-6 (2-methoxy-furanodiene) was the most active compound compared with compound-3 (furanodiene) and compound-4 (2-acetoxy-furanodiene).From the hexane insoluble part of ethanol extract of *B. myrrha* oleo-gum-resin, four furanosesquiterpenes were noxious while other two were mild harmful to the brine shrimps' larvae.

Keywords: Cytotoxic effect, furanosesquiterpenes, oleo-gum-resin, *Balsamodendron myrrha*, LD_{50}.

INTRODUCTION

Members of the family Burseraceae including *Balsamodendron myrrha* Nees., exude resins in peculiar and definite cavities or passages with a complex chemical nature[1-3]. Some investigators trusted that these resins were aerobiotic prod-

*Corresponding author: Mustafa Nadhim Owaid, e-mail: moasif1605@yahoo.com
Ayesha Asif ORCID Number: 0000-0002-1387-735X
M. Asif Saeed ORCID Number: 0000-0002-6003-7871
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ucts of essential oils[3,4]. Resin occurs solitary or in combination with volatile oils or gums[1,3]. When they are in the form of a consistent mixture with volatile oil, they were often described as oleoresins. Oleoresins also occur in admixture with gums which were then called oleo-gum-resins[1,3-5]. It has been investigated that the members of the family Burseraceae yielded resins as oleo-gum-resins in resin canals, present in their phloem which were established schizogenously or as schizolysigenous cavities [1,3,4]. An appreciable amount of oleo-gum-resin was accumulated in such cavities. The internal secretory system of the resin canals was also extended over the bark, pericycle, secondary cortex and medullary rays of the trees[1,3,4].

Many species of this family are economically valuable on account of their resins which have been exploited medicinally, but many species are used mainly in incense and perfumes[1-5]. The most important were frankincense or olibanum (from *Boswellia* species) and myrrha (from *Balsamodendron* or *Commiphora* species). These oleo-gum-resins were procured by tapping or by cutting their barks. These were further processed and coalesced with various seed's or root's oils to make versatile incense[6]. Both these species were native to Northeast Africa (Somalia and Ethiopia) and Arabia (Oman and Yemen), but their dispersion and utilization have been extended over to Indo-Pak region and China[6-8]. The resins from these trees were exploited in Indian Ayurvedic medicines and in Chinese herbal medicines to regale various complaints. In Indian and in Chinese oriental medicines small doses of these resins were appreciated for encouraging blood flow and for crusading the 'qi', i.e., the 'life force' or 'spiritual energy[7,8]. *B. myrrha* plant is a spiny glabrous small trees frequently attain a height of about four to ten meters. Branches of the tree are often with thorny tips and knotted. Bark of the tree is usually lustrous silvery white or agleam bluish grey in color on its outer surface while with greenish underbark. The trees transude scarcely odorous, glutinous matter that acquired a hard-translucent yellow gum-resin[4-7].

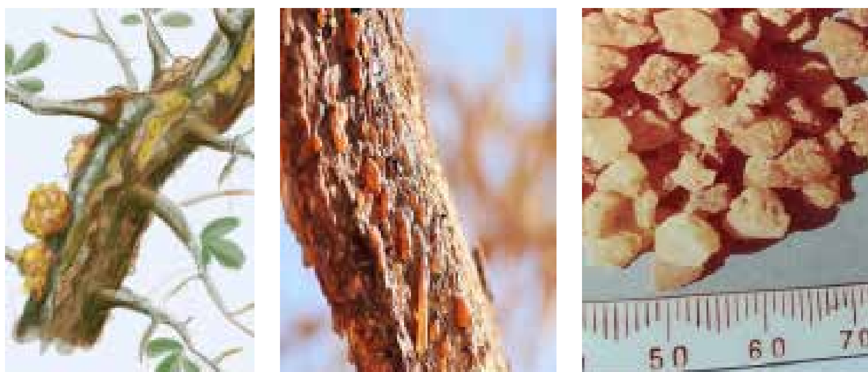

Figure 1. *Balsamodendron myrrha* Nees. tree with resin   Oleo-Gum-resin Tears

Although this plant was connatural to Peninsula, Somalia, Kenya, Ethiopia, Sudan, North East Africa, Abyssinia and Southern Arabia[8], yet it had also been established in a dried hilly tract of Mansehra and Abbottabad (Hilly areas north of Pakistan), at an altitude of about 200 to 1000m with lime-stone containing soil and 200-250mm of mean annual rainfall. The plant was also cultivated at some moist areas of Lahore regions (The capital of Punjab Province and central part of Pakistan)[9].

Myrrh had been a significant natural plant product which was utilized in various applications including pharmacology, food, flavor, liqueur, beverage, cosmetics, perfumery and many others[1-5,10]. Myrrh had numerous indigenous assiduities in medicinal, hygienic and insecticidal domains[1-5,10]. In canonical medicines it had been utilized for revitalizing of numerous complaints from embalming to cancer, leprosy, bronchitis, diarrhoea, dysentery, typhoid fever, inflammatory complaints, hepatitis, contagious diseases, for wound healing, coughs, tumor and many others[10]. A myrrh resin has a mild disinfectant and local stimulant action on mucous membranes. Its tincture has also been applied in gargles and mouthwashes for mouth and pharynx ulcers[1-4,6-8]. It has also been employed to demolish a cacophonous odor, to promote granulations and to diminish the amount of released matter during nauseous ulcers[1-4,6-8]. Many research workers had investigated that both the myrrh oil and resin exhibited strong anti-inflammatory, analgesic[11], anti-microbial[12-14], antibacterial[15] and antifungal effects[16]. It has also been implemented as dentifrice for cleaning and polishing the teeth[2,10]. Internally myrrh was a carminative and flatus-relieving agent while in small doses it commonly encouraged appetite, produced warmth in stomach and provoked the flow of gastric juice[2,10]. Myrrh acted as a local anaesthetic on nerve cells[14] and as an antiparasitic agent[17]. Myrrh had stimulating effects on female uterus and was utilized as an emmenagogue[1-3,8]. *In vitro* anti diabetic and antiobesity activities of myrrh was also assessed[18]. It had also been probed that myrrh tinctures were effectively improved the glucose tolerance in both normal and diabetic rats[18-20]. Toxicity of solvent extracts of myrrh on goats[22] and cytotoxic effects on human gynecologic cancer cells had been reported[23]. Myrrh had been employed from the distant ages as a component of incense and perfume, in the holy oil of Jew and the 'kyphi' of Egyptian for embalming and fumigations. Skin sensitizing potential of myrrh had also been reported[24,25].

40 to 60% of partly water-soluble or a partly-ether soluble portion of myrrh oleo-gum-resin was designated as gum, about 40% of an alcohol soluble portion was consisted of volatile oil and resins mixture[28]. Among the alcohol soluble portions, about 25 to 40% was belonging to a resin[28]. The volatile oils isolated from oleo-gum-resin of *Balsamodendron* species exhibited diverging percentages of mono, sesqui- and diterpenes in addition to some long chain hydro-

carbons[1-4,7,8]. Chemistry of myrrh oil was first investigated in 1906 by Lewinsohn[27] and in 1907 by Friedrichs[28]. These research workers found α-pinene, dipentene, limonene, ±limonene. Later on these workers abstracted a number of compounds from *B. myrrha* oleo-resin, like –cumin aldehyde, cinnamic aldehyde, eugenol, m-cresol, isolinalyl acetate, 3-epilupenyl acetate, lupeone, 3-epi-α-amirin, α-amirone, acetyl-β-eudesmol, cadinene, a sesquiterpene lactone, a bicyclic sesquiterpene (with $C_{15}H_{24}$), heerabolene (a tricyclic sesquiterpene), formic acid, acetic acid, myrrholic acid (with $C_{16}H_{21}O_3$.COOH) and palmitic acid[27,28]. Other groups of research workers depicted following phytochemical compounds in myrrh essential oil. –curzerene[30-32], furanoeudesma-1,3-diene, 10(15)-furano-dien-6-one[33,34], lindestrene [35-37], curzerenone[38,39], furanodien-6-one, dihydropyro-curzerenone, 3-methoxy-10(15)-dihydrofurano-dien-6-one, 3-meth-oxyfuranoguaia-9-en-8-one, 2-methoxy-4,5-dihydrofuranodien-6-one, 3-methoxy-10-methylene-furanogerma-cra-1-en-6-one[38,39] and different aromatic sesqui-terpenes[40,41]. Ahmad etal (2006)[42] recognized six compounds named as myrracadinol-A (cadina-3-en-15-ol); myrracalamene-A (7,8-seco-2,5-dihydr-oxy-12-acetoxycalam-8-ene); myrracala-mene-B (7,8-seco-2,3,5-hydroxy-12-acetoxy- calame-8-ene); myrracadinol-B (7,8-seco-cadin-3,8-diene-2b,12-diol); myrracalamene-C (7,8-seco-12-hydroxy-calam-8-ene) and myrracadinol-C (7,8-secocadin-3,7-dien-5a,10a-diol). On the other hands, Shuaib etal. (2014)[43] isolated three tetracyclic triterpenoids named myrrhalanostenyl acetate (lanost-5,9(11),20(21), (*Z*)-23,25-pentaene-3β-yl acetate); myrrhalanostenol (lanost-5,(*Z*)-22,25(27)tri-en-3β-ol-21,24 aα-olide) and myrrhalanostenoic acid (lanost-5-en-3α-ol-21,24α-olide-26-oic acid), along with n-heptadecanyl capriate; n-dodecanyl myristate and n-henetriacosanyl laurate aliphatic esters[43]. γ-elemene and *Z*-γ-bisabolene were also separated as major constituents from *B. myrrha* oleo-gum-resins[44]. Hosseinkhani etal[45] found that although six unlike samples of myrrh oleoresin had different yields of their essential oils, yet all the samples revealed furanoeudesma-1,3-diene, curzerene and lindestrene as their main constituents. Alcohol insoluble matter of myrrh gum mostly contained protein and carbohydrates. D-galactose, L-arabinose, D-glucuronic acid and 4-methyl-D-glucuronic acid as main carbohydrates were detected[46]. Branched polysaccharide of myrrh gum on hydrolysis furnished high yield of a mixture of sugars and acidic oligosaccharides. Acidic oligosaccharides were a admixture of two aldobiuronic acids, identified as 6-O-(4-O-methyl-β-D-glucuro-nosyl)-D-galactose and 4-O-(4-O-methyl-α-D-glucuronosyl)-D-galac-tose[46,47]. Among the protein portion of myrrh gum after hydrolysis, 15 amino acids were detected[47] along with an oxidase enzyme whose activities were destroyed at 100° C[47].

B. myrrha Nees. plant was ascertained in dried hilly tract of Mansehra and Abbottabad (Hilly north part of Pakistan), at an altitude of about 200 to 1000m with limestone soil and 200-250mm mean annual rainfall. A toxic reaction on hands of indigenous inhabitants was perceived during the assemblage of oleo-gum-resin from this plant. In spite of its copious usage in folk medicine and with its untoward reactions, no endeavor had been made to appraise its jejune/hostile effects. In the present report, we delineated a comparative cytotoxic effect of some furanosesquiterpenes from this oleoresin on mature larvae (nauplii) of brine shrimps (*Artemia salina*), followed by fractionation to isolate and characterize its active compound/s, whose cytotoxic effect was further assessed by calculating their LD_{50}.

METHODOLOGY

General Experimental Procedures

Unless otherwise stated, all the chemicals used were of analytical grade. Concentrations were executed under reduced pressure at bath temperatures not exceeding 50°C. Melting points were determinated on Perfit apparatus with the help of open capillary tubing and were unrebuked. UV spectra were deliberated on Hitachi-270-30 spectrophotometer in MeOH. IR spectra were secured as KBr disc or as thin film on NaCl discs on a Pye-Unicam SP-8-400 spectrophotometer. ^{1}HNMR spectra were procured in DMSO-d_6 at 270 MHz using TMS as an internal standard. ^{13}C NMR spectra were conducted on Bruker AM-300 NMR, spectrometers with 75MH_Z, at 28°C and with 0.2-0.5 mM/ml concentrations of the samples, using 10mm tubes and TMS as an internal reference. EI and FD mass spectra were commemorated on a Varian MAT-312 double focusing mass spectrometer using the direct inlet method. FAB (positive), in glycerin, was acquitted on JEOL JMS-110 spectrometer. Column chromatography was executed on silica gel 60 (70-230 mesh ASTM No. 7734 of E. Merck, Damstadt, Germany), monitored its fractions by TLC. Both the analytical and preparatory TLC were performed with silica gel $PF^{254+366}$ (from E. Merck Damstadt Germany) on 10×20 or 20×20cm glass plates. Analytical TLC with a depth of 0.25 mm thicknesses and preparatory TLC with 0.75 mm thick was utilized. The samples were applied as thin sports on analytical TLC and as narrow bands on preparatory TLC. Spots on chromatographs were visualized by a combination of UV fluorescence, exposing to 254/365 nm UV light, or with I_2 vapors, or with vanillin/H_2SO_4 reagent or with anisaldehyde/ H_2SO_4 reagent[48,49]. The separated bands on preparatory TLC were abraded off and eluted with methanol.

Plant materials

Clean myrrh oleo-gum-resin tears (about 3kg) from *B. myrrha* Nees. were accumulated in August/September 2018 from the local people living around a hilly tract of Mansehra and Abbottabad at an altitude of nearly 900 meters. These tears were authenticated by Prof. Dr. Zaheer-ud-Khan, in charge herbarium, Department of Botany, Government College University, Lahore, Pakistan. A voucher specimen of the sample (No. **P-cog.0157**) was deposited in the Herbarium of Pharmacognosy Section, Faculty of Pharmacy, University of Central Punjab, Lahore for further reference. The myrrh tears were placed overnight in air at laboratory temperature and stored as such in amber colored glass bottles.

Extraction and isolation

2.10kg of the drug was dried in an oven at about 40°C for three days. It was fine-granulated and extracted extensively with 96% ethanol in a Soxhlet extractor. Ethanol extract was concentrated under reduced pressure. The resulting resinous material was extracted repeatedly with hexane. The hexane extract was dried under reduced pressure to secure an oily viscous liquid (about 6.21g, yield = 0.3%). A dark brown, viscous mass (380g, yields = 18.90%) from hexane insoluble portion after drying was procured. 250g of this extract was incorporated with a minimum amount of silica gel using methanol and after drying, it was pulverized into a fine powder. It was then adsorbed over silica gel column and chromatographed in light petroleum ether (40–60°C). The column was eluted with petroleum ether, chloroform and methanol in order of increasing polarities. The eluents were accumulated after monitoring with TLC and fractions with similar compounds were pooled.

Isolation of Compound-1

Compound-1 was eluated from silica gel column with light petroleum ether with the initial 35 fractions (50ml each) of 100% pure petroleum ether and by preparatory TLC after utilization of petroleum ether/$CHCl_3$ (95:5). It prevailed a colorless amorphous powder which on recrystallization with hot Me_2CO/MeOH (75:25) acquired 321mg (with yield = 0.14%) that appeared on TLC as a single spot (with pet. ether/$CHCl_3$ 97:3) at hRf = 28. It had mp. = 152.5–153°C. $[\alpha]D^{28}$+80° (C 0.06 $CHCl_3$). FABMS, m/z (rel. intens. %): 222 [M^+] ($C_{15}H_{26}O$) (3.6), 154 (100). UV λ_{max} (MeOH): 204 nm (log e = 3.4). IR bands: 3442 (strong OH), 2960 (C-H), 2848, 1664 (strong ketonic C=O), 1468 (C=C), 1344,1120cm^{-1}. ^{1}HNMR ($CDCl_3$), δ: 5.70 (1H, m, H-3), 3.35 (1H, d, *J* = 7.3 Hz, H2-15a), 3.31 (1H d, *J* = 7.5 Hz, H-15b), 2.36 (2H, broad s, H2-2), 2.27 (6H, broad s, H2-5, H2-8, H2-9), 2.25 (2H, broad s, H-1, H-6), 2.15 (2H, broad s, H-7, H-10), 1.70

(3H, broad s, Me-11), 1.16 (2H, m, H-12), 1.06 (3H, d, *J* = 6.5 Hz, Me-13), 1.08 (3H, d, *J* = 6.7 Hz, Me-14). ^{13}CNMR ($CDCl_3$), δ: 51.17 (C-1), 29.09 (C-2), 121.96 (C-3), 127.24 (C-4), 26.73 (C-5), 44.08 (C-6), 35.48 (C-7), 20.65 (C-8), 19.23 (C-9), 37.31 (C-10), 7.63 (C-11), 34.26 (C-12), 14.72 (C-13), 17.24 (C-14), 65.17 (C-15). The compound-1 was identified by comparing its spectral data with the reported data as myrracadinol-A[42] (Figure 2.).

Isolation of Compound-2

Compound-2 was eluted from the column with 100% light petroleum ether (with further 80 to 120 fractions of 50 ml each) and by preparatory TLC after employing petroleum ether/$CHCl_3$ 90:10 as a solvent system. It came out as pale yellow precipitates which after re-crystallization from hot Me_2CO/MeOH (70:30) mixture rendered 385 mg of pale-yellow amorphous powder (with 1.013% yield). It appeared on TLC at hRf = 71 (with pet. ether/$CHCl_3$ 80:20) as a single spot and have mp. = 142.5–144°C. $[\alpha]_D^{28}$+89° (C 0.05 $CHCl_3$). FABMS m/z (rel. inten.%): 238 [M^+] ($C_{15}H_{26}O_2$) (6.8), 222 (12.2), 204 (23.8), 189 (19.9), 185 (16.5), 168 (22.9), 152 (21.6), 130 (24.8), 115 (37.3). UV λ_{max} (MeOH): 206 nm (log e = 4.3). IR (KBr) bands: 3460 (OH), 1656 (Ketonic C=O), 1525, 1475 (C=C), 1398, 1120 cm^{-1}. ^{1}HNMR ($CDCl_3$) δ: 5.89 (1H, m, H-3), 5.52 (1H, m, H-9), 4.96 (1H, m, H2-8a), 4.71 (1H, d, *J* = 6.3Hz, H2-8b), 3.12 (1H, broad m, *J* = 8.3Hz, H-2a), 2.26 (2H, m, H2-5), 1.91 (1H, m, H-10), 1.80 (1H, m, H-1), 1.73 (3H, broad s, Me-11), 1.53 (1H, m, H-6), 1.52 (2H, broad s, H2-7), 1.28 (6H, broad s, Me-13, Me-14), 0.82 (3H, d, *J* = 6.2 Hz, Me-15). ^{13}C NMR ($CDCl_3$) δ: 47.55(C-1), 70.46 (C-2), 127.26 (C-3), 141.38 (C-4), 26.26 (C-5), 40.23 (C-6), 16.80 (C-7), 111.26 (C-8), 115.40 (C-9), 35.26 (C-10), 7.46 (C-11), 69.38 (C-12), 20.30 (C-13), 15.34 (C-14), 28.72 (C-15). Compound-2 was identified by comparing its spectral data with the reported data as myrracadinol-B[42] (Figure 2).

Isolation of Compound-3

Compound-3 was eluted from the column with light petrolum ether/$CHCl_3$ (90:10) from further 121 to 160 (each 50 ml) fractions and by preparatory TLC after using petroleum ether/$CHCl_3$ (85:15) as a solvent system. It came out as oily grumous matter. It was kept at 6°C for overnight and re-crystallized from hot MeOH. It furnished about 3.25g (1.3% yield) of achromatic crystalline material. It appeared on TLC at hRf = 16 (with pet. ether/$CHCl_3$ 70:30) as a single spot, quenching under UV, gave a yellowish orange spot with anisaldehyde/H_2SO_4 and have mp. = 65.5–67°C. EIMS (rel. intens. %): m/z 216 [M^+] ($C_{15}H_{20}O$) (2.5), 215 (30), 149 (9), 136 (14), 109 (10), 107 (12), 105 (12), 95 (12), 93 (16), 91 (16), 85 (71), 79 (12), 47 (30), 45 (22), 43 (100), 41 (20). UV λ_{max} (MeOH): 256 nm. IR (KBr) bands: 3466 (strong OH), 1660 (strong ketonic C=O), 1530, 1480

(C=C), 1378, 1129 cm^{-1}. ^{1}HNMR ($CDCl_3$) δ: 7.07 (1H s, H-12), 4.93 (1H, dd, *J* = 6.5, 10.6 Hz, H-1), 4.65 (1H broad t, *J* = 7.1 H-5), 3.56 (1H d, *J* = 14.2, H-9α), 3.45 (1H d, *J* = 14.1, H-9β), 3.21 (2H, broad d, *J* = 6.7, H-2α, H-2β), 2.78 (1H td, *J* = 11.5 3.4, H-6β), 2.10-2.61 (2-H, m, H-3α, H-3β), 1.89 (3H s, H-13), 1.80 (1H dt, *J* = 7.1 11.6, H-6α), 1.62 (3H s, H-14), 1.29 (3H s, H-15). ^{13}C NMR ($CDCl_3$) δ: 130.0 (C-1), 24.35 (C-2), 26.79 (C-3), 128.71 (C-4), 127.4 (C-5), 39.42 (C-6), 118.82 (C-7), 149.63 (C-8), 40.81 (C-9), 134.31 (C-10), 121.80 (C-11), 135.9 (C-12), 8.89 (C-13), 16.39 (C-14), 16.20 (C-15). Compound-3 was identified by comparing its spectral data with the reported data and with CAS ID = 19912-61-9 as a germacrane sesquiterpenoid named as furanodiene [51,54] (Figure 2).

Isolation of Compound-4

Compound-4 was eluted from the column with petroleum ether/$CHCl_3$ (70:30) from further 161 to 190 (each 50 ml) fractions and by preparatory TLC after utilizing petroleum ether/$CHCl_3$ (80:15) as the band eluting solvent. This compound appeared to be light yellowish viscous liquid with 102 mg quantity (0.041% yield). It was homogenous on analytical TLC, as a single spot with hRf = 65 (with pet. ether/$CHCl_3$/ MeOH 80:3:1). It gave light blue colored spots with vanillin/H_2SO_4. HRMS: m/z 274.1561 [M]+ (calcd. for $C_{17}H_{22}O_3$, 274.1568). EIMS (rel. intens. %): m/z 274 [M^+] ($C_{17}H_{22}O_3$) (23), 232 (20), 214 (35), 199 (24), 177 (14), 159 (12), 149 (21), 146 (42), 135 (15), 123 (17), 109 (13), 108 (100), 107 (16), 105 (14), 95 (13), 93 (19), 91 (25), 85 (79), 79 (13), 47 (32), 45 (23), 43 (45), 41 (22). UV λ_{max} (MeOH): 252 nm. IR (thin film) bands: 2930 (strong OH), 2870, 1730 (strong ketonic C=O), 1600, 1530 (strong), 1450 (C=C), 1435, 1375, 1240, 1100, 1020, 960, 850 and 760cm^{-1}. ^{1}HNMR ($CDCl_3$) δ: 7.05 (1H, s H-12), 5.43 (1H, broad m, H-9), 5.05 (1H t), 4.98 (1H, d, H-2), 3.49 (2H s), 3.39 (3H s, H-2), 3.02 (2H, d, *J* = 8Hz, H-5), 2.06 (3H s), 1.92 (3H, d, *J* = 4 Hz, H-10), 1.66 (3H s), 1.40 (3H s). Compound-4 was identified by comparing its spectral data with those reported in the literature as 2-acetoxyfuranodiene[54,55,57] (Figure 2).

Isolation of Compound-5

Compound-5 was eluted from the column with petroleum ether/$CHCl_3$ (50:50) from further 191 to 240 fractions (50 ml each) and by preparatory TLC after using pet. ether/$CHCl_3$ (70:30) as a band eluting solvent. This compound appeared to be colorless viscous liquid and was homogenous on analytical TLC as a single spot with hRf = 0.75 (in pet. ether/$CHCl_3$/MeOH 90:10:5). It afforded an intense violet colored spot with vanillin/H_2SO_4. HRMS: m/z 232.1542 [M]+ (calcd. for $C_{15}H_{20}O_2$, 232.1562). EIMS (rel. intens. %): m/z 232 [M^+] ($C_{15}H_{20}O_2$) (59), 204 (37), 189 (25), 176 (30), 175 (100), 162 (26), 161 (74), 150 (19), 149 (26), 147 (15), 137 (22), 133 (15), 123 (16), 122 (25), 121 (50), 119 (28), 109 (30),

107 (31), 105 (42), 95 (41), 94 (17), 93 (44), 91 (33), 80 (48), 79 (30), 77 (19), 71 (32), 67 (30), 65 (9), 56 (41), 53 (20), 41 (50). UV λ_{max} (MeOH): 234, 280 nm. IR (thin film) bands: 2938 (strong OH), 2870, 1700 (strong ketonic C=O), 1670, 1520, 1450 (strong C=C), 1370, 1230, 1104, 1090, 1030, 1018, 900, 895, 760 and 737cm^{-1}. ^{1}HNMR ($CDCl_3$) δ: 7.09 (broad d, H-11), 6.91 (1H, broad s, H-12), 5.75 (broad s, H-5), 5.34 (1H, broad t, *J* = 9Hz, H-9), 5.12 (1H broad dd, 6.5, *J* = 4.0 Hz H-1), 3.67 (broad s, H-9), 3.30 (2H m), 3.01 (d, *J* = 1.0Hz, H-13), 2.80 (2H, m, a multiplet centered around δ 2.31), 2.46 (td, 11.2, *J* = 3.7 Hz), 2.30 (2H m), 2.17 (dt, 11.6, *J* = 3.4Hz, H-2), 2.05(broad s, H-14), 1.92 (3H d, *J* = 4Mz), 1.87 (dt 10.8, *J* = 4.0, H-3), 1.62 (3H broad s, H-2), 1.40 (broad s, H-15), 0.97 (3H, d, *J* = 7Hz, H-5). ^{13}C NMR ($CDCl_3$) δ: 129.7 (C-1), 25.6 (C-2), 41.5 (C-3), 145.3 (C-4), 131.6 (C-5), 187.2 (C-6), 122.3 (C-7), 157.2 (C-8), 40.0 (C-9), 136.1 (C-10), 125.2 (C-11), 139.4 (C-12), 10.2 (C-13), 17.8 (C-14), 17.3 (C-15). Compound-5 was identified by comparing its spectral data with the reported data as furanodiene-6-one[58] (Figure 2).

Isolation of Compound-6

Compound-6 was eluted from the column with $CHCl_3$: MeOH (90:10) from further 241 to 352 (each 50 ml) fractions and by preparatory TLC after eleuting the bands with pet. ether/$CHCl_3$ (65:35). This compound came out as white amorphous powder, re-crystallized from hot Me_2CO/MeOH (80:20) which afforded 380 mg amount (about 0.152% yield) and was homogenous on analytical TLC. It came out as a single spot with hRf = 65 (in pet. ether/$CHCl_3$/MeOH 90:20:7). It had a strong quenching under UV, gave a pink spot with vanillin/H_2SO_4 and with mp. 59–61°C. EIMS (rel. intens. %) m/z: 246 [M^+] ($C_{16}H_{22}O_2$) (25), 215(15), 176(20), 166(20), 159(30), 149(15), 147(27), 138(15), 123(30), 119(15), 107(18), 91 (42), 85(43), 77(40), 55(42), 45(100), 40(15), 39(20). UV λ_{max} (MeOH): 210nm. IR (thin film) bands: 2970(strong OH), 2930, 2910, 2860, 2840, 1670(strong ketonic C=O), 1600, 1560, 1460(C=C), 1420, 1380, 1180, 1160, 1120, 1090, 960, 810 and 760cm^{-1}. ^{1}HNMR ($CDCl_3$) δ: 7.04 (1H, broad s, H-12), 5.03 (2H, m, H-9), 4.11 (1H m), 3.62 (2H broad s), 3.45 (3H s), 3.10 (2H, d, *J* = 7.5Hz), 2.50 (2H m), 1.91 (3H d, *J* = 3Hz, H-2), 1.61 (3H, broad s, H-5), 1.0 (3H broad s). The compound 6 was identified by comparing its spectral data with the reported data as 2-methoxyfuranodiene of furanogermacrane[59] (Figure 2).

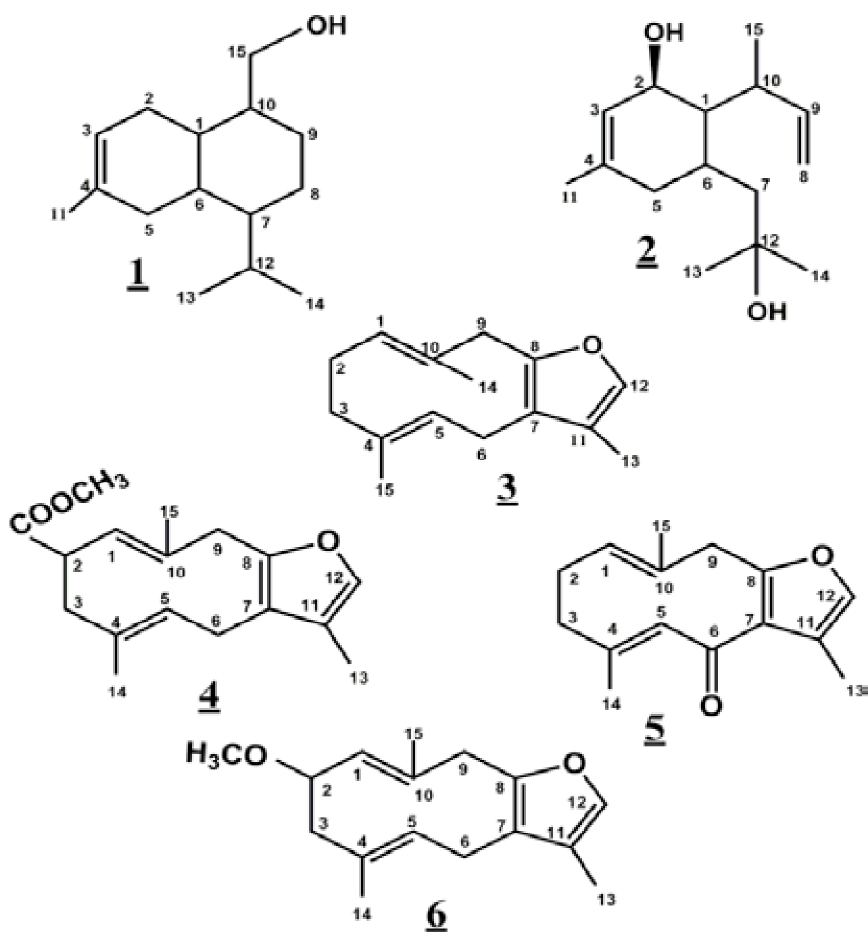

Figure 2. Furanosesquiterpenes isolated from *Balsamodendron myrrha* oleo-gum-resin

Brine Shrimp (*Artemia salina* Leach) Lethality Bioassay

This assay was embraced from the method already portrayed in literature[61-65]. The eggs of brine shrimps (*Artemia salina* Leach) were redeemed from an indigenous fish shop. A brine shrimp container was filled with artificial sea water (about 3.7%)[63,65]. The seawater was merged with salts like $MgCl_2 \cdot 6H_2O$, Na_2SO_4, $CaCl_2 \cdot 2H_2O$ or $CaCl_2 \cdot 6H_2O$[62,63]. Sea salt and yeast suspension (in the ratio of 3mg dried yeast for each 5ml of sea water), were also purchased from the local fish store. Syringes of 5ml, 1ml, 500µl's, 100µl, 50µl and 10µl ability and 2-dram vials (9 per sample and 3+3 for each control) were also ransomed from the domestic market. Sea salt solution was produced by disbanding 38 g sea salts in 1000 ml of distilled water and filtered. This solution was taken in a small

fictile tubful that was divided by a uniformed muddle partition with consistent holes in it. The eggs were besprent on one side of the muddled partition which was then embraced with a carbon paper. Other half of the tubful was illuminated with an electric lamp to draw in the hatched shrimps. The solution in the tubful was incessantly provided with a regular air flow with an average pressure and proper light conditions which were necessary for hatching litigates[63,64]. After 48 hours, the shrimps hatched and matured as nauplii. The mature nauplii was then utilized further for the experiment. 20 mg of each of the compound was taken in small vial and dissolved in 2 ml of methanol to swear out as a stock solution. From the stock solution, 500 µl, 400 µl, 300 µl, 200 µl, 100 µl, 50 µl, 40 µl, 30 µl, 20 µl, 10 µl and 5 µl (corresponding to the 1000, 800, 600, 400, 200, 100, 80, 60, 40, 20 and 10 µg respectively) were channelized to the vials with three replicates of each engrossment of the isolated compound. The vials were then placed in an open area for 24 hours for a complete removal of methanol after vaporization. 2 ml of sea salt solution was then added in each vial. 10 brine shrimp's larvae were transferred to each vial (30 brine shrimps per dilution) with the help of a long-tipped dropper. Total volume of the liquid in each vial was adjusted to 5 ml with sea salt solution. After 24 hours, the facetious or nonresonant brine shrimps were counted for all the concentrations of the isolated compounds[61-64]. Colchicine[64,65] in the same concentrations was employed as a positive control. Total number of devastated brine shrimp's larvae per dilution of each compound was tabulated. LC_{50} (lethal concentration in 50% individuals) was computed by probit analysis[66], using a computer program[67]. The number of exterminated brine shrimp's larvae due to the upshot produced by the six isolated compounds from *B. myrrha* oleo-gum-resin and also by colchicine, their LC_{50}, has been outlined in Table-1.

RESULTS AND DISCUSSION

The indecorous outcomes due to *B. myrrha* oleo-gum-resin (myrrh oleo-gum-resin) were conglomerated by the indigenous inhabitants living in the hilly areas of Mansehra and Abbottabad, at an altitude of about 200 to 1000m. A toxic reaction on the hands was ascertained during the accumulation of myrrh. Skins of abaxial sides of hands and arms were often involved. It frequently developed erythematous inflammation, after prolong handling the fresh myrrh oleo-gum-resin. The apparent toxic reaction induced by the oily material from fresh *B. myrrha* oleo-gum-resin was resolved after five or six days. This type of atrocious salacious reaction of the myrrh oleo-gum-resin on human skin incited us to carry out an investigation about the chemical nature of their hostile active compounds.

Table 1. Cytotoxic effect of the compounds isolated from *B. myrrha* oleo-gum-resin on brine shrimps' larvae.

Dose levels (μg/ml)	Compounds						
	Comp. 1	Comp. 2	Comp. 3	Comp. 4	Comp. 5	Comp. 6	Colci.
400	27*/30†	22/30	12/30	10/30	22/30	21/30	28/30
200	25/30	20/30	11/30	8/30	21/30	20/30	27/30
100	21/30	19/30	9/30	6/30	20/30	18/30	25/30
80	20/30	18/30	8/30	5/30	19/30	17/30	21/30
60	18/30	16/30	7/30	2/30	18/30	15/30	20/30
40	17/30	13/30	5/30	1/30	17/30	14/30	18/30
20	16/30	12/30	3/30	0/30	15/30	12/30	17/30
10	14/30	11/30	2/30	0/30	14/30	10/30	15/30
5.0	12/30	10/30	0/30	—	9/30	8/30	13/30
2.5	10/30	8/30	—	—	7/30	5/30	11/30
1.25	8/30	4/30	—	—	5/30	3/30	4/30
LC_{50} (μg)	28.65	31.41	419.58	754.32	30.56	33.27	27.32
S.D.	0.85	0.84	0.81	0.79	0.84	0.83	0.78
x^2	1.78	2.35	1.52	0.48	1.56	1.67	1.58
d. f.	27	26	1.51	0.308	27	26	25

Comp. 1 = myrracadinol A; **Comp. 2** = myrracadinol B; **Comp. 3** = furanodiene; **Comp. 4** = 2-acetoxyfuranodiene;
Comp. 5 = furanodiene-6-one; **Comp. 6** = 2-methoxyfuranodiene; **Colci.** = Colchicine

*=Number of brine shrimps killed after 24 hours;
†=Total number of brine shrimps used;
LC50 = Lethal concentration where 50% brine shrimps larvae were killed;
S.D.= Standard deviation; $\chi 2$ = Chi square; d.f.= degree of freedom.

During the preliminary cytotoxic assay, it was detected that the ethanolic extract of *B. myrrha* oleo-gum-resin was fatal to the brine shrimps (*Artemia salina*) larvae (nauplii). The ethanolic extract was further fractionated into hexane soluble non-polar portion and hexane insoluble polar fraction which was chromatographed by column and preparative TLC to sequestrate their active cytotoxic constituent/s. Six cytotoxic compounds videlicet as compound-1 (myrracadinol-A); compound-2 (myrracadinol-B); compound-3 (furanodiene); compound-4 (2-acetoxy-furanodiene); compound-5 (furanodiene-6-one) and compound-6 (2-methoxy-furanodi-

ene) were abstracted from the hexane insoluble portion of ethanol extract by column chromatography and were purified by preparative TLC and recrystallization. These compounds were identified as furanosesquiterpenes by comparative physical and spectral data of previously reported similar compounds (Figure 2). Previously many research workers had isolated and characterized similar furanosesquiterpenes from myrrh oleo-gum-resin and from other oils and resins[41-45,50-60].

Many research workers had taken advantage of brine shrimp (the larvae i.e., nauplii of mature brine shrimps – *Artemia salina*), assay for assessing the cytotoxicity of crude solvent extracts, column fractions and phytochemical compounds isolated from various natural sources[61-65]. This assay was thus assisted to appraise the cytotoxic activity. It was therefore employed to expedite the bioactive directed column fractions and preparatory TLC bands that maneuver the bioactive phytochemical compounds from the natural products towards cytotoxicity. Bioactive furanosesquiterpenes from *B. myrrha* oleo-gum-resin (Figure 2) were manifested as lethality to the brine shrimp's larvae. It was believed that the difference between toxicity and efficacy of such compounds is only the dosage[61,62,65]. This assay often manouevre the column fractions from different solvent extracts of natural products towards worthful bioactive phytochemical compounds. Cytotoxic effects were frequently evinced by the research workers in ppm or in μg as LC_{50} values with 95% confident levels[61,62,65]. To compare the cytotoxic effect of furanosesquiterpenes the brine shrimp larvae's assay appeared to be an appropriate manner of quantifying the LD_{50} (Lethal dose in at least 50% individuals) at time, in which the destruction of such larvae was ascertained. Input data for a computer program consisted of the specific dose, total number of test animals' larvae used, and the number of test animals' larvae reacted (i.e., the number of dead larvae) for a particular dose. The computer program transmute the dose to the *log* dose and the proportion of test animals' larvae reacted to the *probit* of percentage response. It then reconciled with a probit regression line to the resulting points and computing the values of LD_{50} along with standard deviation and a value of χ^2 [66,67]. The output consisted of a listing of LD_{50}, standard deviation and a value of χ^2. The aim of the χ^2 test was to find out whether the assay, after transformation was sufficiently constituted by a probit regression line. If the χ^2 test pointed out, a deviation of transformed results from linear shape, these could not be assigned to a random biological variation (i.e. if χ^2 value is not significant at $p > 0.05$), then the results secured by probit analysis would not be legitimate[66]. The results pointed out that the six furanosesquiterpenes isolated from *B. myrrha* oleo-gum-resin demonstrated a cytotoxic effect against the brine shrimps' larvae when compared with the known cytotoxic compound colchicine[64,65], utilized in the same assiduity (Table 1).

The results further indicated that among the isolated compounds, the compound-**1** (myrracadinol A), compound-**2** (myrracadinol B), compound -**5** (furanodiene-6-one) and compound-**6** (2-methoxyfuranodiene) came out to be the most active compounds (with least LC_{50} = 28.65, 31.41, 30.56 and 33.27) when compared with other two like compound-**3** (furanodiene) and compound-**4** (2-acetoxy-furanodiene) (with LC_{50} = 419.58 and 754.32) and also with the positive controlled colchicine (with LC_{50} = 27.32) (Table 1). Out of the four most active compounds, compound-**1** (myrracadinol A) appeared to possess the highest cytotoxic activity with the least LC_{50}. Its cytotoxic potentiality seemed to be a terminus to the colchicine (Table 1). Furthermore, the results also pointed out that two of the furanosesquiterpenes, i.e., the compounds-**3** (furanodiene) and -**4** (2-acetoxyfuranodiene) exhibited lesser cytotoxic effect (Least LD_{50}) against the brine shrimps' larvae than other four furanosesquiterpenes and also lesser than colchicine (Table 1).

The toxic response on brine shrimps' larvae, caused by myrracadinol A, myrracadinol B, furanodiene-6-one and 2-methoxyfuranodiene from *B. myrrha* oleo-gum-resin was probably due to their rapid acculturation through the larvae's skin and quickly bio-available to the living tissues. These compounds perhaps barricaded the respiratory or nervous centers and caused speedy tissue deterioration in the larvae, leading to their death. The comparatively less toxic reactions of furanodiene and 2-acetoxyfuranodiene were perhaps due to their direct actions, at some of the receptor sites in the larvae.

We concluded that the hexane insoluble portion of EtOH extract of *B. myrrha* oleoresin restrained closely related cytotoxic furanosesquiterpenes which could be hostile, not only to the brine shrimps' larvae but also insalubrious to the bodies of higher animals and human beings.

Further work is the requisite to amplify this dimension through the preparation of these compound's derivatives which would possibly direct to the structure-activity relationship of such an important cytotoxic molecules, not only *in vivo* in the bodies of the lower and higher animals as well as in human being's torso but also *in vitro*. These cytotoxic molecules and their derivatives might also be an important against carcinogenic tissues both in animal and human's bodies, which could further be ascertained in conformity with the criterion procedures of WHO[68]. Further work is also contrived to find some cytotoxic inhibitor/s from our natural sources which could subjugate the hostile activeness of such phytochemical compounds from *B. myrrha* oleoresin and related species of the family Burseraceae.

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