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In Vitro Leishmanicidal Effect and Gas Chromatography Mass Spectrometry analysis of Chloroform fraction(CF) of *Allium Cepa*

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Abstract:

The Gas Chromatography and Mass Spectrometrystudyshown that chloroform fraction (CF) ofAlliumcepacomprises of 26 compounds which shown effect against Leishmania major (DESO). Inhibitory concentrations observed for chloroform fraction (CF)of Allium cepa(IC50=73.3 ±2.2ug/ml). In the course of this assay, Amphotericin B (IC50= 0.29 ± 0.05 ug/ml) as well aspentamidine (IC50=5.09 ±0.09 ug/ml) have been used as positive control drugs to compare the parasite inhibition with that by the chloroform fraction (CF) of Allium cepa. During this bioassay, Incubation temperature and

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Incubation period were 22C°and 72hours. Methanol extract (ME), Hexane fraction (HF), Ether fraction (EF), and Diethyl ether fraction (DEF) of Allium ceparevealed noleishmanicidal effect.

Key words: chloroform fraction (CF); Gas Chromatography and Mass Spectrometry; Leishmania major; Amphotericin B; pentamidine

INTRODUCTION

Leishmaniasis forms a variety of multifaceted diseases. Because of defence system developed by the host and different varieties of parasite, it can lead to syndromes which can range from slight self-limiting cutaneous lesion to lethal visceral diseases. The diseases take place in three forms that is: Visceral Leishmaniasis (VL), Mucocutaneous Leishmaniasis (MCL), and Cutaneous Leishmaniasis (CL) [1]. Cutaneous Leishmaniasis has several native alternative expressions for instance, Tropical sore, Oriental sore, Aleppo sore, or Baghdad sore. Cutaneous Leishmaniasis is noticeable through the presence of irritated sores, lesions (ulcers) and enlargement of the lymph nodes on arm, nose, legs or face and lower limbs [2].Mucocutaneous Leishmaniasis (Espundia) creates far-reaching defacing, devastation of mucosa and cartilage of the mouth, nose, ear, and pharynx which leads towards austere disfigurement of the face. Latter on secondary bacterial or fungal infection arises when this disease become worse [3, 4]. Visceral Leishmaniasis "Kala-azar" is a universal infection in which the parasites are capable to occupy the spleen and liver which causes a persistent splenomegaly.[1] [5, 6].Chemotherapy of Leishmaniasis is targeted at reducing illness and death related with the disease. Drugs used for the treatments of Leishmaniasisare pentavalentantimonials; meglumineantimoniate (Glucantime), pentamidine (aromatic diamidine) and sodium stibogluconate (SbV) (Pentostam),

liposomal amphotericin B, (AmBisome), Paromomycin (Humatin) and miltefosine (Miltex) [7, 8, 9, 10,].Pentavalent antimonial, Pentamidine, Amphotericin B, Paromomycin and Miltefosine are the first line of medications which have been approved for the cure of leishmaniasis[11,12,13,14,15]however, Azoles, Allopurinol, Sitamaquine, and Imiquimod are the medications in experimental trial for the cure of leishmaniasis[16,17,18,19].*Allium cepa* is one of the important specie of the Liliacec[20]. .*Allium* comprises around five hundred and fifty types [21]. Alliumcepa is significant fornutritional as well as for medicationsin "folk medicine", [22]. In this research study we evaluate the leishmaniacidal effect of chloroform fraction *(*CF)of *Allium cepa.*

MATERIAL AND METHODS

PLANT MATERIAL

Garden-fresh*Allium cepa* were collected from local market of Quetta City, Balochistan, Pakistan. **EXTRACTION OF *ALLIUM CEPA***

This research of extraction was completed in the Prof. Dr. M. Anwar Panezai lab, Institute of Biochemistry University of Balochistan Quetta. Five kg of onion also known as *Allium cepa*were soaked in methanol[23] and was kept for six days. During this period, the soaked Allium cepa was shaken daily. After the completion of six days, the soaked Allium cepa in methanol was filtered and the filterate containing methanol was vaporized in the rotary evaporator. Finally, the crude methanolic extract (ME) was collected and measured 165.42 gm[24].

FRACTIONATION OF ALLIUM CEPA

One gm from crude methanolic extract(ME) used for leishmanicidal effect and GC-MS analysis while the remaining extract was fractionated with the help of different solvents such

as n-Hexane, water, ether, chloroform, diethyl ether. In a separatory funnel, with methanolic crude extract, n-Hexane and water have been added. After shaking, n-Hexane layer has been separated from aquous layer. Both n-Hexane and aquous layer were separately vaporized in the rotary evaporator to get n-Hexanefraction (HF), andaquous fraction (AF).

FRACTIONATION OF n-HEXANE FRACTION (HF)

One gm from n-Hexane fraction (HF) used for leishmanicidal effect and GC-MS analysis while the remaining fraction was fractionated with the help of different solvents such as ether, chloroform, diethyl ether.

FORMATION OF ETHER FRACTION (EF)

In a separatory funnel, with n-Hexane fraction (HF), n-Hexane and ether have been added. After shaking, n-Hexane layer has been separated from ether layer. Both n-Hexane and ether layer were separately vaporized in the rotary evaporator to get n-Hexane fraction (HF) and etherfractions (EF).Ether fraction (EF) was used forleishmanicidal effect.

FORMATION OF CHLOROFORMFRACTION (CF)

In a separatory funnel, with n-Hexane fraction (HF), n-Hexane and chloroform have been added. After shaking, n-Hexane layer has been separated from chloroform layer. Both n-Hexane and chloroform layer were separately vaporized in the rotary evaporator to get n-Hexane fraction (HF) and chloroform fraction (CF).Chloroform fraction (CF) was used forleishmanicidal effect.

FORMATION OF DIETHYL ETHER FRACTION

In a separatory funnel, with n-Hexane fraction (HF), n-Hexane and diethyl ether have been added. After shaking, n-Hexane layer has been separated from diethylether layer. Both n-Hexane and diethylether layer were separately vaporized in the

rotary evaporator to get n-Hexane fraction (HF) and diethyletherfraction (DEF). DiethylEther fraction (DEF) was used forleishmanicidal effect.

Finally, methanolicextract (ME), n-Hexane fraction (HF), ether fraction (EF), chloroform fraction (CF) anddiethylether fraction (DEF) were prepared for leishmanicidal effect.

ANTILEISHMANIAL ACTIVITY OF *ALLIUM CEPA* FRACTION

The test was performed according to Atta-urRahman[25]. Concisely, L. major promastigotes in stationary phase were seeded at 40,000 parasites/400ul/well in 24-"well plate i]n RPMI-FCS". The extracts were dissolved in DMSO and added further 400ul/well to give final concentrations of 1mg per ml and serial two-fold dilution thereof. Promastigotes were incubated over a period of 6 days at 25 C^{o} and the amount of parasites in each well determined on days 2, 4 and 6 of experiment using neubar chamber under a microscope. Amphotericin B (0.5 mg/ml (was used as positive control, culture media was used as negative control and DMSO was used as solvent control.

GAS CHROMATOGRAPHY MASS SPECTROMETRY (GC-MS) ANALYSIS TRIPLE QUADRUPOLE ACQUISITION METHOD-MS PARAMETERS

For identification and quantification of *Allium cepa*compounds: 2 ul of *Allium cepa* extract or fraction was directly injected into the gas chromatograph mod.6890N Network GC System (Agilent Technologies "Palo Alto, CA") together in the presence of mass spectrometer mod. "5973 Network Mass Selective Detector" (Agilent Technologies "Palo Alto, CA") and furnished in the presence of "a column HP-5MS (30 m length, 0.25 mm interior diameter, 0.25 um film width" Agilent Technologies, "Palo Alto, CA"). Helium gas was off. Injection was made into a

split-splitless injector (split ratio 30:1) at 250 Cº. The oven program was the following: "70 Cº for 3 min then 6 Cº /min to 180 for 5 min, then 6 Cº /min to 280 Cº for 10 min, then 8 Cº /min to 290 Cº for 20 min". The MSD transfer line was set at a temperature of 250 Cº ; MSD temperature quadrupole was of 150 Cº and ionization temperature was 230 Cº, Mass specra were seventy electrovolts and scan achievement was accomplished in the series between thirty five and 300m/z. The identification of the components of the *Allium cepa* extract or fraction was assigned by matching their "mass spectra with those available in the libraries NIST 02 and WILEY"[26].

RESULTS

ANTILEISHMANIAL ACTIVITY OF *ALLIUM CEPA*FRACTIONS

Antileishmanial activity of *Allium cepa* extract and fractions was performed against Leishmania major (DESO). Effective antileishmanial activity was observed for chloroform fraction CF) while other extract and fractions such as Methanol extract (ME), Hexane fraction (HF), Ether fraction (EF), and Diethyl ether fraction (DEF) exhibited no antileishmanial activities. Inhibitory concentration of chloroform fraction (CF) observed (IC50=73.3 ±2.2ug/ml). In this bioassay, Amphotericin B (IC50= 0.29 ± 0.05 ug/ml) and pentamidine (IC50=5.09 ±0.09 ug/ml) were used as positive control drugs to compare the parasite inhibition with that by the *Allium cepa* extract and fractions. This activity was perceived under Incubation period 72 h and Incubation Temperature 22 Cº.

Table 1: Antileishmanial activities of extracts and fractions of *Allium cepa*

S.NO	Name of extracts	IC50 ug/ml
1	ME	-
2	HF	-
3	EF	-
4	CF	73.3 ±2.2ug/ml
5	DEF	-
6	Amphotericin B	0.29 ± 0.05 ug/ml
7	pentamidine	5.09 0.09 ug/ml

CHEMICAL COMPOSITION OF CHLOROFORM FRACTION (CF)

The chloroform fraction *(*CF) which showed antileihmanial activity has been analyzed by Gas Chromatography Mass Spectrometry and results are represented in tables. Chloroform fraction **(CF)**consists of 26 compounds. 26 compounds were evaluated by RT matching with library and MS spectra.

Table 2: Molecular formula, Molecular mass, Structure, m/z and RT of 1-7 compounds in chloroform fraction (CF)

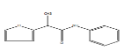
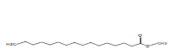
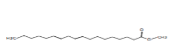
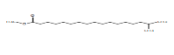
Compd	Molecular formula	Molecular mass	Structure	m/z	RT
1	C13H13NO2	215		93	11.72
2	C15H30O2	242		74	22.351
3	C17H34O2	270		74	28.038
4	C19H34O2	294		67.1	32.066
5	C19H36O2	296		55.1	32.243
6	C19H36O2	296		55.1	32.331
7	C19H38O2	298		74	32.726

Table 3: Molecular formula, Molecular mass, Structure, m/z and RT of 8-14compounds in chloroform fraction (CF)

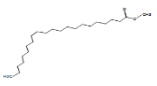
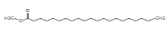
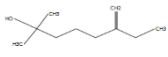
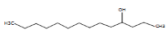
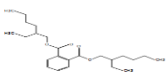
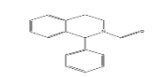
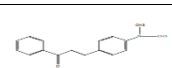
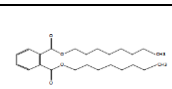
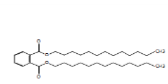
Compd	Molecular formula	Molecular mass	Structure	m/z	RT
8	C23H44O2	352		55.1	35.834
9	C22H44O2	340		74	36.253
10	C10H20O	156		59.1	36.769
11	C26H54O	382		55.1	37.261
12	C14H30O	214		73	38.54
13	C24H38O4	390		149	39.5
14	C16H15NO	237		237	40.1

Table 4: Molecular formula, Molecular mass, Structure, m/z and RT of 15-21 compounds in chloroform fraction (CF)

Compd	Molecular formula	Molecular mass	Structure	m/z	RT
15	C17H17NO	251		251.1	40.61
16	C24H38O4	390		149	41.13
17	C34H58O4	530		149	41.92
18	C22H43NO4	385		116	43.1

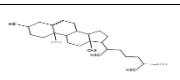
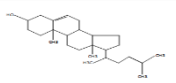
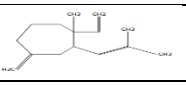
19	C27H46O	386		43.1	47.47
20	C26H42O	370		55.1	49.02
21	C15H24	204		69.1	51.434

Table 5: Molecular formula, Molecular mass, Structure, m/z and RT of 22-26 compounds in chloroform fraction (CF)

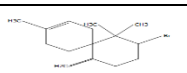
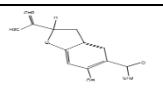
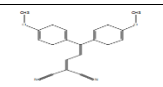
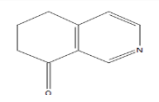
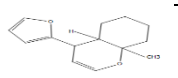
Compd	Molecular formula	Molecular mass	Structure	m/z	RT
22	C15H23Br	283		69.1	53.967
23	C13H14O3	218		218.1	55.452
24	C20H16N2O2	316		316.2	60.324
25	C9H9NO	147		147	60.365
26	C14H18O2	218		95	66.119

Table 6: Mass spectra of 1-7 compounds in chloroform fraction (CF)

Compd	**m/z (% Relative abundance)**
1	215(M^+),95(14440), 93(46197), 85 (2360.8), 83.1 (3233.4), 77 (2272.6), 76 (4344.3), 57.1 (19890), 49 (3199), 48(2804), 43.1 (15559)
2	242(M^+),143.1(4129.5), 87 (22208),75.1(5600.6), 74 (34315), 69.1 (4488.3), 59.1 (4065), 57.1 (5013.8),55.1(8950.7), 43.1 (10207), 41.1 (5431.1)
3	270(M^+),143.1(191427.9),87 (982781.6),75.1 (265683.6), 74 (1473550), 69.1(201160.1), 59.1(142191.7), 57.1 (242415.4), 55.1 (416671.7), 43.1 (396394.8), 41.1(195992.4)
4	294(M^+),96(67181.3), 95 (107656.9), 82 (87786.3), 81 (162562.4) , 79(79615.2), 69.1(64992.5) , 68.1(87309.7), 67.1 (206363.5), 55.1 (149260.7), 54.1(86297.8)
5	296(M^+),97(281998.9), 96(258945.3), 87 (272790.8), 84 (264819.1), 83.1 (343315.6), 74 (387162.9), 69.1(450111.5), 67.1 (262389.1), 55.1 (743192), 43.1 (274578.7)
6	296(M^+),97(21334), 96(19655), 87 (20334), 83.1 (26118), 74 (29895) , 69.1(33821),67.1(21657), 55.1 (60695), 43.1 (25131)
7	298(M^+), 143.1(40205.2), 87 (195141.5),83.1(27565.6),75.1(62133.7), 74

	(290450.7),69.1(46621.6), 57.1 (60433.3), 55.1(89768.1)

Table 7: Mass spectra of 8-14 compounds in chloroform fraction (CF)

compd	m/z (% Relative abundance)
8	352(M^+), 97(3049.2), 83.1 (3774),74 (2757.8), 73 (6972.1), 72 (5157), 71.1 (3724.9),69.1(4327.3),57.1(5377.4),55.1(8499.4), 43.1 (6041.3)
9	340(M^+),143.1(3957.7), 87(18461), 83.1 (3842.2), 75.1 (6727.5),74(26340), 69.1 (4914.2), 57.1 (6218.3),55.1(8879.5),43.1(9331.1), 41.1 (3710)
10	156(M^+),83.1(2373.8), 81 (2309), 72 (8354.3), 69.1(2100.3), 67.1(1883.9), 59.1(14445), 55.1(5201.7), 44(1845.5), 43.1(3342.9), 41.1 (2694.7)
11	382(M^+),251.1(1781.3), 97 (2681), 83.1 (3142.6), 71.1(2417.2), 70.1 (1522.6), 69.1(2266.2), 57.1(2941.1), 56.1(1688.4), 55.1 (3509.4), 43.1(2495)
12	214(M^+),98(3277.6), 97 (3544.1), 83.1 (4845.1), 73(14963), 69.1 (4772.7), 57.1(3416.7), 55.1 (10820), 45 (2867.6), 43.1(4189.3), 41.1 (2779.4)
13	390(M^+), 167(119897.5), 150 (43304.8), 149(392165.7), 113(40650.1), 71.1(106907), 70.1 (90466.5), 57.1(165805.4), 55.1(64162.4), 43.1(83894.1), 41.1(45352.9)
14	237(M^+),237(3687.9), 236.1 (1644.6), 160(2608.8), 132 (1752.3), 117(1725.8), 111.1(1398.2), 107 (2675.6), 97(2352.9), 83.1(2454),71.1(2370.6)

Table 8: Mass spectra of 15-21 compounds in chloroform fraction (CF)

compd	m/z (% Relative abundance)
15	251(M^+), 252.1(107316.9), 251.1(605137.9), 250.1 (257931.7), 174.1(360281.7), 146.1(178911.7),130(105656.7) , 121(186905.4), 105(111206.7), 77 (317788.8), 51.1 (111913.4)
16	390(M^+), 162(3423.3), 150 (3599.5), 149(13019), 132(2682.6), 113 (2326.2),104 (2705.8), 71.1 (5239.4), 57.1 (6806.1), 55.1(4014.1), 43.1(4480.4)
17	530(M^+), 149(18515), 127(10710), 109(4527.6), 108(5495.5), 83.1(6002.7), 71.1(7071.8),69.1(4518.6), 57.1(8306.6), 55.1(5357.2), 43.1(15671)
18	385(M^+),133.9(4714.6),116(4814.2), 97 (2257.3), 88(2995.1), 83.1 (2668.7), 69.1 (1754.5),67.1(1839), 56.1 (1674.7), 55.1 (3591.4), 43.1 (2405.4)
19	386(M^+),107(3719.9),105(4160.5),95(4661.8), 93 (3123.8),91(3200.9), 81 (4478.4), 79(3601.5), 57.1 (3755.7), 55.1(4180.7), 43.1 (5025.2)
20	370(M^+),399.4(24977), 119(13415), 107 (13336), 105(15780), 95 (22140), 83.1(14822), 81(14718), 69.1(19287), 57.1 (21514), 55.1 (26423)
21	204(M^+), 109(30049), 107(33329), 95 (48101), 93 (34056), 91 (29122), 81 (41142), 79(30006), 69.1(83050), 55.1(56910), 41.1 (28791)

Table 9: Mass spectra of 22-26 compounds in chloroform fraction (CF)

compd	m/z (% Relative abundance)
22	283(M^+), 135(715.5), 121(4761.9), 109(6506.1), 105(5549.9), 95(9577.8), 93 (6855.6), 81(7868.7), 79 (5861.8), 69.1(15446), 67.1(4831.5)
23	218(M^+), 281(1176), 218.1 (3086.2), 203 (2036.6), 119(1251.5), 109 (1241.5), 107 (1119.4), 105 (1482.2), 69.1 (1229.4), 55.1 (1171.1), 43.1(1560.7)
24	316(M^+), 316.9(1564.9), 316.2(4130.3), 175.1(1172.9), 163.1 (823.2), 159(1297.1), 135 (2008.2), 129 (1169.6), 109(953), 107 (1233.5), 59.1(1001.6)
25	147(M^+), 207.9(785.3), 194.9 (615.8), 149(1166.7), 147(2605.2), 131(1554.5), 119(1907.9), 117(1463.9), 91 (2363.2), 58.1(1245.8), 53 (782.6)
26	(M^+), 192.9(622.7), 190.9 (728.7), 147(677.4), 133 (952.5), 119 (730.2), 117 (622.9), 105 (961.3), 96 (642.2), 95(1362.9), 59.1(755.6)

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