IN VITRO LEISHMANICIDAL, ANTIBACTERIAL, ANTIFUNGAL, ANTICANCER (MCF-7, 3T3 AND HELA CELL LINES) ACTIVITIES OF EXTRACT AND FRACTIONS OF *PEROTIS HORDEIFORMIS* AND GC-MS ANALYSIS OF *PEROTIS HORDEIFORMIS* WHOLE PLANT BUTANOL FRACTION (PHWBF)

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Abstract. In this research study, leishmanicidal, antibacterial, antifungal, anticancer (MCF-7, 3T3 and HeLa cell lines) activities and GC-MS studies of *Perotis hordeiformis* extract and fractions were examined. Leishmanicidal bioassay, 96 Well Plate Method, Agar tube dilution method and MTT assay were the methods used for leishmanicidal, antibacterial, antifungal and anticancer activities. *Perotis hordeiformis* whole plant butanol fraction (PHWBF) exhibited leishmanicidal activity with IC_{50} 53.31 ± 0.59. *Perotis hordeiformis* whole plant hexane fraction (PHWHF) showed activity against bacteria such as *Staphylococcus aureus* having an inhibition percentage of 58.5%. *Perotis hordeiformis* whole plant methanol extract (PHWME) showed activity against fungi such as *Microsporum canis and Fusarium lini* having an inhibition percentage of 55% and 50%, respectively. *Perotis hordeiformis* whole plant hexane fraction (PHWHF) showed activity against *Aspergillus niger* having an inhibition percentage of 40% while *Perotis hordeiformis* whole plant aqueous fraction (PHWAF) showed activity against *Microsporum canis and Fusarium lini* having an inhibition percentage of 100% and 40%, respectively. *Perotis hordeiformis* whole plant butanol fraction (PHWBF) showed activity against cancer cell lines such as HeLa cell line and MCF-7 cell line with an inhibition percentage of 55% and 48%, respectively. Other extract and fractions were less active against cancer cell lines. GC-MS analysis showed 8 compounds in *Perotis hordeiformis* whole plant butanol fraction (PHWBF) which exhibited leishmanicidal and anticancer activities.

Keywords: *leishmaniasis, bacterial strains, fungal strains, cell lines, PHWME, PHWHF, PHWAF, PHWBF*

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Introduction

Traditional plants are the main sources of phytochemicals, used for the preservation of human health and alleviating infectious diseases of mankind since prehistoric times. At present, the entire world has interest in green medicines and demands medicines originating from traditional plants rather than from a synthetic source. This is due to the fact that traditional drugs are safer than synthetic medicines which have toxicity and side effects. This stimulates the researchers to develop new medicines against microbes (Cordell et al., 2000; Nair et al., 2007). The drugs which are synthesized, are expensive, have side effects and the diseases are not properly treated. Hence, new antimicrobial agents are needed to be developed from traditional plant sources (Sieradzki et al., 1999; Dabur et al., 2007). According to the WHO, 80% of the population of the world use traditional medicines to cure infectious diseases (WHO, 1993). The compounds which are extracted from plant sources are more than 50% of current drugs (Baker et al., 1995). The medicinal plant, *Perotis hordeiformis* is a short lived perennial or annual and belongs to the Poaceae family. Sandy places are the main locations for the presence of this ethnomedicinal plant. This traditional plant is mainly distributed in Nepal, Thailand, Indonesia, India, Pakistan, Sri Lanka and Myanmer. This plant has significant antileishmanial, cytotoxic and antioxidant activities. *Perotis hordeiformis* has close resemblance with *Perotis indica* (Baloch et al., 2013). In this study, *Perotis hordeiformis* whole plant extract and fractions are used against leishmania major, six bacteria, five fungi and three cancer cell lines.

Materials and Methods

Plant material

Perotis hordeiformis whole plant was the plant material in this analysis.

Extraction

The medicinal plant *Perotis hordeiformis* was collected from Soorab, Balochistan, Pakistan and was authenticated by Prof. Dr. Rasool Bakhsh Tareen, Department of Botany, UoB, Quetta, Pakistan. *Perotis hordeiformis* was kept for one month under the shade and then powdered in a grinder. 1.5 kg powdered *Perotis hordeiformis* was macerated in 14 L of methanol for the period of seven days and then the mixture was filtered, and vaporized in a rotary evaporator. The crude extract of *Perotis hordeiformis* whole plant methanol extract (PHWME) was 24.52 g.

Fractionation of crude extract

The methanolic crude extract was fractionated with n-hexane and aqueous solvents in a separatory funnel and vaporized in a rotary evaporator to form *Perotis hordeiformis* whole plant hexane fraction (PHWHF) 8.1 g and *Perotis hordeiformis* whole plant aqueous fraction (PHWAF) 17.3 g. *Perotis hordeiformis* whole plant aqueous fraction (PHWAF) was fractionated with butanol to form *Perotis hordeiformis* whole plant butanol fraction (PHWBF) 4 g (Bakht et al., 2013; Achakzai et al., 2016, 2019).

Leishmanicidal bioassay

At 3000 rpm for 10 min, the leishmanial parasite such as promastigotes was sedimented. This parasite was counted by Neubaur chamber, diluted to a concentration

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of $1x10^6$ with fresh medium. In 96 well plate, 180 uL of parasite culture was added. The sample with the concentration of 20 uL was added and then serially diluted till final concentration of sample 1 ug/mL. $1x10^6$ cells/mL of parasite density was kept for negative control while for positive control, it varied. The 96 well plate including parasites, sample, positive and negative control was kept in an incubator between 21 to 22 ºC for the duration of 72 h. With the help of Neubaur chamber, parasites with IC_{50} values were counted (Atta-ur Rahman et al., 2001).

Antibacterial assay

96 well plate method

For the growth of bacterial strains, Muller Hinton medium was used. McFarland turbidity index with 0.5 was used for the adjustment of inoculums. In DMSO, extracts/fractions were added and from this stock solution were formed. In wells, media and samples were added while control wells were without extracts and fractions. Up to 200 uL, the wells were filled. $5x10^6$ cells were added in both control and test wells, which were then sealed with parafilm and kept in an incubator for 18-20 h. Alamar Blue Dye was added to all wells, which were then shaken for 2-3 h at 80 RPM. Blue to pink color change of dye indicated the growth of bacteria. Absorbance was recorded at 570 nm with ELISA reader (Pettit et al., 2005).

Antifungal assay

Agar tube dilution method

In this assay, in 1 mL of DMSO, 24 mg of sample was dissolved. SDA with the concentration of 32.5 g was dissolved in 0.5 L of Distilled water. With the help of steam, this growth medium was completely dissolved. This medium with 4 mL was poured in tubes with screw cap, autoclaved for 15 min at 121 ºC, cooled till 50 ºC. 66.6 uL of sample was loaded into non-solidified SDA. At room temperature, in a slanting position, tubes were solidified. Fungus was inoculated with 4 mm diameter into tubes. In other media, reference antifungal drug and DMSO were used as positive and negative control. Tubes were kept in an incubator for one week at 27-29 ºC, and examined twice in a week (Choudhary et al., 1995).

Calculating Inhibition % of fungal growth

$$Inhibition\% = 100 - \frac{linear\ growth\ in\ test\ (mm)}{linear\ growth\ in\ control\ (mm)} \times 100 \qquad \text{(Eq.1)}$$

MTT assay

In this study, cancer cell lines were cultured in Dulbecco's Eagle modified medium with 10% FBS, 2% antibiotics were used and then kept in 5% CO_2 in incubator at 37 °C. After the development of confluency, cell lines were harvested. In a 96 well flat, 5×10^4 cells/well were added and then after one day, sample with the concentration of 50 ug/mL was added, and kept for 48 hours in an incubator. The sample was removed after incubation. MTT with the concentration of 0.5 mg/mL was added, kept at 37 °C for hours in an incubator. Formazan crystals were formed when MTT was reduced. With the help of 100 ul DMSO, Formazan crystals were dissolved. Micro-plate reader was used for recording absorbance at 570 nm (Spectra Max plus, Molecular Devices,

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CA, USA). In this assay, doxorubicin was used as a standard drug. The decrease in viable cells or percent inhibition was calculated with the help of the following formula:

$$\%\ Inhibition = 100 - \frac{mean\ of\ O.D.\ of\ test\ compound - mean\ of\ O.D.\ of\ negative\ control}{mean\ of\ O.D.\ of\ positive\ control - mean\ of\ O.D.\ of\ negative\ control} \times 100 \quad \text{(Eq.2)}$$

For the calculation of IC_{50} 20 mM stock solution of extracts/fractions were diluted into working solution with 50 uM and then in order to get less than 50 percent inhibition, working solution was further diluted in serial dilutions. With the help of EZ-fit5 software, IC50 was calculated (Scudiere et al., 1988).

Gas chromatography mass spectrometry (GC-MS) analysis triple quadrupole acquisition method MS parameters

For identification and quantification of *Perotis hordeiformis* compounds: 2 ul of *Perotis hordeiformis* 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 ºC. The oven program was the following: 70 ºC for 3 min then 6 ºC /min to 180 for 5 min, then 6 ºC /min to 280 ºC for 10 min, then 8 ºC /min to 290 ºC for 20 min. The MSD transfer line was set at a temperature of 250 ºC; MSD temperature quadrupole was of 150 ºC and ionization temperature was 230 ºC, Mass spectra were seventy electrovolts and scan achievement was accomplished in the series between thirty-five and 300 m/z. The identification of the components of the *Perotis hordeiformis* extract or fraction was assigned by matching their mass spectra with those available in the libraries NIST 02 and WILEY (El-Wakil et al., 2015).

Results and Discussion

Perotis hordeiformis whole plant butanol fraction (PHWBF) exhibited leishmanicidal activity with IC_{50} 53.31 ± 0.59. None of other *Perotis hordeiformis* extract and fractions exhibited leishmanicidal activity. Leishmanicidal activities of extract and fractions of *Perotis hordeiformis* are shown in *Table 1*.

Table 1. *Leishmanicidal analysis of extract/fractions of whole plant of Perotis hordeiformis*

Extract/Fractions	**IC_{50} (ug/mL) ± S.D.**
PHWME	>100
PHWHF	>100
PHWAF	>100
PHWBF	53.31 ± 0.59 moderate activity
Standard (Pentamidine)	4.08 ± 0.8
Standard (Amphotericin B)	0.30 ± 0.4

Perotis hordeiformis whole plant hexane fraction (PHWHF) showed activity against bacteria such as *Staphylococcus aureus* having an inhibition percentage of 58.5%.

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Perotis hordeiformis whole plant methanol extract (PHWME), *Perotis hordeiformis* whole plant aqueous fraction (PHWAF) and *Perotis hordeiformis* whole plant butanol fraction (PHWBF) showed no antibacterial activity. The antibacterial activities of *Perotis hordeiformis* whole plant extract and fractions are shown in *Table 2*.

Table 2. *Antibacterial activities of Perotis hordeiformis whole plant extract and fractions*

	Escherichia coli **(%) Inhibition**	Bacillus subtilis **(%) Inhibition**	Shigella flexenari **(%) Inhibition**	Staphylococcus aureus **(%) Inhibition**	Pseudomonas aeruginosa **(%) Inhibition**	Salmonella typhi **(%) Inhibition**
PHWME	-	19%	-	7.2%	-	-
PHWHF	-	11.5%	-	58.5%	-	-
PHWAF	-	-	-	-	-	-
PHWBF	-	-	-	-	-	-
Standard (ofloxacin)	87.6%	95.6%	-	93.7	95.5%	96.2%

Perotis hordeiformis whole plant methanol extract (PHWME) showed activity against fungi such as *Microsporum canis and Fusarium lini* having an inhibition percentage of 55% and 50%. *Perotis hordeiformis* whole plant hexane fraction (PHWHF) showed activity against *Aspergillus niger* having an inhibition percentage of 40% while *Perotis hordeiformis* whole plant aqueous fraction (PHWAF) showed activity against *Microsporum canis and Fusarium lini* having an inhibition percentage of 100% and 40%. *Perotis hordeiformis* whole plant butanol fraction (PHWBF) showed no antifungal activity. The antifungal activities of *Perotis hordeiformis* whole plant extract and fractions are shown in *Table 3*.

Table 3. *Antifungal activities of Perotis hordeiformis whole plant extract and fractions*

	Candida albicans **(%) Inhibition/MIC**	Trichphyton rubrum **(%) Inhibition/MIC**	Aspergillus niger **(%) Inhibition/MIC**	Microsporum canis **(%) Inhibition/MIC**	Fusarium lini **(%) Inhibition/MIC**
PHWME	0%	0%	0%	55%	50%
PHWHF	0%	0%	40%	0%	20%
PHWAF	12.5%	0%	30%	100%	40%
PHWBF	0%	0%	0%	0%	0%
Standard (Miconazole) Mic (ug/mol)	113.5	97.8	20.70	98.1	73.50

Perotis hordeiformis whole plant butanol fraction (PHWBF) showed anticancer activity against HeLa cell line and MCF-7 cell line with percent inhibition 55% and 48%. Other extract and fractions are less active against cancer cell lines. The anticancer activities of extract and fractions of whole plant of *Perotis hordeiformis* are shown in *Table 4*.

Molecular formula, molecular mass, structure, m/z and RT of compounds 1-8 of *Perotis hordeiformis* whole plant butanol fraction (PHWBF) are shown in *Tables 5 and 6* while mass spectra interpretation of compounds 1-8 of *Perotis hordeiformis* whole plant butanol fraction (PHWBF) are shown in *Tables 7 and 8*.

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Table 4. *Anticancer activities of extract and fractions of whole plant of Perotis hordeiformis*

	MCF-7 **(%) Inhibition**	3T3 **(%) Inhibition**	HeLa **(%) Inhibition**
PHWME	12%	25%	36%
PHWHF	8%	32%	32%
PHWAF	16%	25%	6%
PHWBF	48%	25%	55%
Standard Doxorubicin	87.6%	71%	70%

Table 5. *Molecular formula, molecular mass, structure, m/z and RT of compounds 1-4 of Perotis hordeiformis whole plant butanol fraction (PHWBF)*

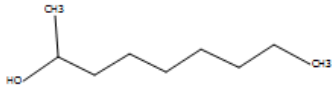
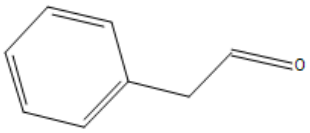
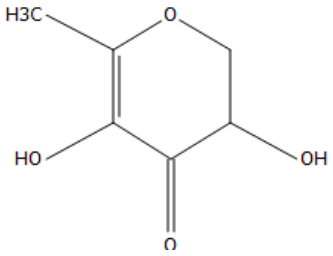
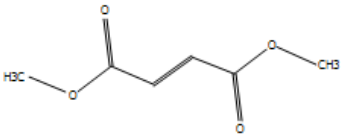
compd	Molecular Formula	Molecular Mass	Structure	m/z	RT
1	C9H20O	144		45.1	6.681
2	C8H8O	120		91	7.503
3	C6H8O4	144		43.1	9.983
4	C6H8O4	144		53.1	12.39

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Table 6. *Molecular formula, molecular mass, structure, m/z and RT of compounds 5-8 of Perotis hordeiformis whole plant butanol fraction (PHWBF)*

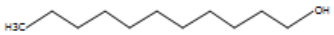
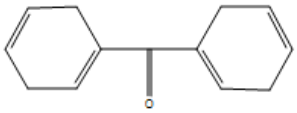
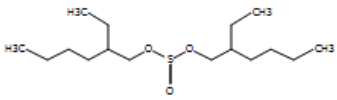
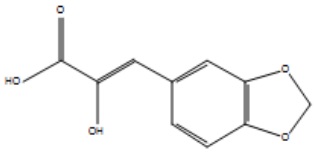
compd	Molecular Formula	Molecular Mass	Structure	m/z	RT
5	C11H24O	172		43.1	17.44
6	C13H10O	182		104.9	20.61
7	C16H34O3S	306		57.1	35.25
8	C10H8O5	208		135	68.13

Table 7. *Mass spectra of compounds 1-4 of Perotis hordeiformis whole plant butanol fraction (PHWBF)*

compd	m/z (% Relative abundance)
1	144(M^+), 126(2365.9), 69(2401), 57.1(3606.8), 56.1(2876.3), 55.1(1849.7), 54(2775.9), 53.1(3732.9), 45.1(8743.3), 44.1(4950.7), 41.1(2064.4)
2	119.9(M^+],13185), 91.9(21295), 91(83166), 88.9(4417.4), 64.9(18968), 62.9(7995.1), 62(3067.6), 51.1(6416.9), 50.1(3636.7), 39.1(2357.5)
3	144(M^+],58661.2), 101(56580), 73(39808.6), 72(47429.7), 58(7389.8), 55.1(43751.8), 45.1(39179.1), 44.1(108117.2), 43(145948), 42.1(7752.4)
4	144(M^+), 113.9(2272.7), 112.9(3354), 98(2209.6), 85(2140.8), 71(2930), 68(1637.4), 56.1(1855.7), 53.1(4503.1), 52(1566.3), 51.1(3247.2)

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Table 8. *Mass spectra of compounds 5-8 of Perotis hordeiformis whole plant butanol fraction (PHWBF)*

compd	m/z (% Relative abundance
5	172(M^+), 97(1101.3), 84(1288.9), 83(2228.8), 70(2031.5), 69(4248.8), 57.1(4541.2), 56.1(5445.9), 55.1(5725.6), 43.1(8861.9), 41.1(2510.4)
6	181.9(M^+],4016.4), 105.9(1127.1), 104.9(11055), 91(1654.5), 87(6040.8), 76.9(7954.4), 75.9(1546), 53.1(1837), 51.1(4023.6), 50.1(1050.9)
7	306(M^+), 135(6436.7), 112.9(13869), 111.9(6419.7), 88.9(16427), 71(27183), 70(11703), 57.1(33305), 55.1(9046.4), 43.1(13743), 41.1(6340.2)
8	208.9(M+1],672.6), 207.9(M^+],771.6), 196.9(816.7), 149(546.4), 135(1655.4), 104.9(543.5), 95.9(770.1), 76.9(541.6), 75(774.9), 44.1(829.8)

Conclusion

In this research study, *Perotis hordeiformis* whole plant butanol fraction (PHWBF) exhibited leishmanicidal activity with IC_{50} 53.31 ± 0.59. None of other extract and fractions of *Perotis hordeiformis* exhibited leishmanicidal activity. *Perotis hordeiformis* whole plant hexane fraction (PHWHF) showed activity against bacteria such as *Staphylococcus aureus* having an inhibition percentage of 58.5%. *Perotis hordeiformis* whole plant methanol extract (PHWME), *Perotis hordeiformis* whole plant aqueous fraction (PHWAF) and *Perotis hordeiformis* whole plant butanol fraction (PHWBF) showed no antibacterial activity. *Perotis hordeiformis* whole plant methanol extract (PHWME) showed activity against fungi such as *Microsporum canis and Fusarium lini* having an inhibition percentage of 55% and 50%, respectively. *Perotis hordeiformis* whole plant hexane fraction (PHWHF) showed antifungal activity against *Aspergillus niger* having an inhibition percentage of 40% while *Perotis hordeiformis* whole plant aqueous fraction (PHWAF) showed antifungal activity against *Microsporum canis and Fusarium lini* having an inhibition percentage of 100% and 40%, respectively. *Perotis hordeiformis* whole plant butanol fraction (PHWBF) exhibited no antifungal activity. *Perotis hordeiformis* whole plant butanol fraction (PHWBF) showed anticancer activity against HeLa cell line and MCF-7 cell line with an inhibition percentage 55% and 48%, respectively. Other extract and fractions are less active against cancer cell lines. GC-MS analysis showed 8 compounds in *Perotis hordeiformis* whole plant butanol fraction (PHWBF) which exhibited leishmanicidal and anticancer activities. In the near future, in the Institute of Biochemistry, University of Balochistan, Quetta, Pakistan, the compounds present in the *Perotis hordeiformis* whole plant butanol fraction (PHWBF) will be isolated and tested against cancer cell lines and leishmaniasis and will lead to drug development with least toxicity and side effects.

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