Evaluation of *In-vitro* ovarian anticancer activity (Ovarian Cancer Cells-PA1) and zebrafish embryo toxicity of *Parmeliaperlata* ethanolic extract

TITLE SUGGESTED

EVALUATION OF IN VITRO ANTICANCER ACTIVITY (OVARIAN CANCER CELLS -PA1)AND ZEBRAFISH EMBRYO TOXICITY OF *PARMELIA PERLATA* ETHANOLIC EXTRACT

Abstract

The Phytochemical composition and bioactivity of an ethanolic extract of *Parmeliaperlata* were analyzed in this (investigation) studyusing established procedures. The extract revealed a rich array of phytochemicals, showcasing its diverse composition, and notably exhibited strong antioxidant activity according to the 1, 1-diphenyl-2- picrylhydrazyl (DPPH) test. Furthermore, the research explored the extract's potential in preventing Bovine Serum Albumin (BSA) denaturation, comparing it with the well-known medication Diclofenac. The concentration-dependent inhibition of BSA denaturation by *P.perlata* ethanolic extract was comparable to the effects observed with diclofenac on BSA. The ethanolic extract demonstrated concentration-dependent antibacterial activity against gram-negative and gram-positive bacterial isolates. While gentamicin served as a positive control with larger zones of inhibition, *P.perlata* extract displayed significant antibacterial efficacy against all six tested organisms. Moreover, *in vitro* testing against human ovarian (PA-1) cell lines demonstrated the extract's anticancer potential. A concentration-dependent reduction in cell viability, culminating in the lowest percentage at 1000 µg/ml, was observed. The IC50 value of 31.2 µg/mL showed that the *P.perlata* extract strongly inhibited the PA-1 cell line. This was confirmed by the observed changes in the cellular morphology when exposed to higher concentrations of the extract. Further, the ethanolic extract of *P. perlata* was subjected to various concentrations to evaluate its embryotoxicity on zebrafish embryos. The concentration of extract was determined to have a safety limit of less than 50 µg/L. In summary, *P.perlata* ethanolic extract has emerged as a promising natural resource, exhibiting a spectrum of bioactivities, including potent antioxidant, anti-inflammatory, antibacterial, anticancer properties, and embryonic toxicology study. These

findings suggest that its potential utility in pharmaceutical applications is warranted, and further exploration and development are being suggested.

Keywords: *Parmeliaperlata*, antioxidant, anti-inflammatory, antibacterial, anticancer, ovarian cancer cell.

1. Introduction

Cancer is presently the foremost obstacle (to) inattaining a desirable lifespan in most countries; it is the leading cause of death worldwide [1]. Following uterine and cervical cancers, ovarian cancer ranks as the third most prevalent gynecologic malignancy [2]. The prognosis (was) isthe worst, and the death rate (was) isthe highest. In contrast to the prevalence of breast cancer, ovarian cancer is considerably rarer but three times more lethal. The death rate associated with this disease is projected to increase considerably by 2040. Inadequate screening, the tumor's furtive and silent growth, and the delayed onset of symptoms all contribute to the high mortality rate associated with ovarian cancer [3]. The inadequate diagnostic effectiveness of contemporary cancer screening methods adds to this distress. A comprehensive gynecological examination, including transvaginal ultrasound and blood testing, and Other major early detection methods, including the cancer antigen-125 (CA-125) assay, have failed to substantially reduce the morbidity and mortality associated with this particular cancer [4]. After surgical debulking, traditional medicine prescribes chemotherapy. Chemotherapy [5] is a frequently employed therapeutic approach targeting malignancy. However, the detrimental consequences of chemotherapy medications are highly toxic and abhorrent. There is an ongoing investigation into several novel approaches aimed at the treatment and administration of cancer in human subjects. As cancer treatments, herbal remedies are becoming increasingly popular [6-7] because of their reduced risk and lower cost. This (investigation)research work isaimed to evaluate the anticancer activity of an ethanolic extract from *Parmeliaperlata* using a PA-1 ovarian cancer cell line.

INCORPORATE AIMS AND OBJECTIVES HERE

2. Materials and Methods

2.1 Chemicals: Ethanol, 1, 1-diphenyl-2- picrylhydrazyl (DPPH), Ascorbic acid, Methanol, Nutrient broth, Muller Hinton agar, Dimethyl sulfoxide (DMSO), Diclofenac sodium, Potassium Chloride (KCL), Sodium Chloride (NaCl), sodium hydrogen phosphate (Na_2HPO_4), Phosphate Buffer Saline (6.4), potassium dihydrogen phosphate (KH_2PO_4), Bovine Serum Albumin (BSA), fluconazole and MTT(3- (4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2H-tetrazolium bromide) were of analytical grade.

2.2 Collection of Lichen: The sample was collected from Tirupattur district, Tamil Nadu, India, in sterile polythene bags. The collected lichen material was identified as *P.perlata*using standard manuals (Fig. 1).Following complete rinsing with tap water and drying in the shade, the lichen was ground into a fine powder and stored at 4°C in an airtight bottle.

Figure 1. Collected *Parmeliaperlata*

2.3 Preparation of Ethanolic *P. perlata*extract:A 10g sample of *P.perlata* lichen powder that had been stored was extracted using the Soxhlet extraction method with 100 mL of aqueous and ethanol-based solvents, which is shown in Fig. 2.The lichen extract was then collected and stored for further analysis (Fig. 3).

Figure 2: Soxhlet Extraction

Figure 3: Ethanolic extract

2.4 Phytochemical Qualitative Analysis:

Using the standard procedure [8-10], the plant extract was analyzed for the presence of various phytochemicals in the ethanolic extract of *P.perlata*.

2.5 In Vitro Antioxidant Activity:

2.5.1 Antioxidant activity using the DPPH (1, 1-diphenyl-2- picrylhydrazyl) free radical scavenging assay:

The radical scavenging activity of *P. perlata*was assessed using the DPPH assay [8-12]. A 1 mL solution of DPPH (0.1 mM) was combined with 3 mL of *P. perlata*extract at various concentrations (10–50µg/ml). Subsequently, the mixture was placed in the dark at room temperature for 30 min. The measurement of absorbance was noted at a wavelength of 517 nm

using a UV–visible spectrophotometer (Systron-ics-119). The DPPH radical scavenging activity can be determined based ona previous study [13].

2.6 Anti-inflammatory properties of the *Permaliaperlata* ethanolic extract:

The Mendili et al. (2022) [14] method was used to determine the inhibition of BSA denaturation.

BSA (500 mg) was dissolved in 100 mL of water containing Phosphate Buffer Saline. The pH level is 6.3. Dissolved 8 g of NaCl, 0.2 g of KCl, 1.44 g of NaHPO□, and 0.24 g of KH□PO□ in 800 mL of purified water. The pH was modified to 6.3 by adding HCI, and the volume was adjusted to 1000 mL with distilled water (H_2O). The test solution (0.5mL) comprises 0.45 mL of BSA (0.5%W/V aqueous solution) and 0.05mL of the test solution with different concentrations.

- The control solution was composed of 0.45mL of bovine serum albumin (a 0.5%W/V aqueous solution) and 0.05mL of H_2O.
- The product control (0.5mL) was composed of 0.45mL of H_2O and 0.05 mL of the test solution.
- The standard solution (0.5mL) comprised 0.45mL of BSA (0.5%w/v aqueous solution) and 0.05mL of Diclofenac sodium at different doses.

2.6.1 Procedure:

A volume of 0.05 mL of test drugs at concentrations ranging from 100 to 500 µg/mL, as well as a volume of 0.05 mL of diclofenac sodium at concentrations ranging from 100 to 500 µg/mL, were taken separately. These volumes were then combined with 0.45 mL of a solution containing 0.5% w/v BSA. The samples were incubated at 37°C for 20 min, after which the temperature was elevated to maintain the specimens at 57°C for 3 min. After the solutions have cooled, add 2.5 mL of phosphate buffer. The measurement of absorbance was conducted using a UV-visible spectrophotometer at a wavelength of 255 nm. The control demonstrates complete protein denaturation. The results were compared with diclofenac sodium. The following calculation is based on a previous study [15-16].

2.7 Antibacterial activity of the *P. perlata*ethanolic extract:

The ethanolic extract of *P. perlata*was tested against pathogenic bacterial strains such as*Escherichia coli, Staphylococcus aureus, Enterococcus sp.,Bacillus subtilis, Klebsiella*

*pneumoniae,*and *Pseudomonas sp.*Antibacterial activity was assessed using the standard agar well diffusion method [8-9]. Different concentrations of the ethanolic *P. perlata*extract (50, 100, and 150 µg/ml) were prepared using 2% DMSO (dimethyl sulfoxide). The 24h test cultures were spread onto respective Mueller-Hinton agar media using the spread plate method. Gentamycin was used as the positive control. The plates were placed in an incubator at 37°C for 24 h. The antibacterial activity was evaluated by measuring the diameter of the inhibition zone [11-13, 17].

2.8 Anticancer Activity of *P. perlata*ethanolic extract against the ovarian cancer cell line:

2.8.1 Cell line and culture:

The PA-1 ovarian cancer cell line was acquired from the National Center for Cell Sciences (NCCS), Pune. The cells were cultured in Dulbecco's Modified Eagle's medium (DMEM) with 10% fetal bovine serum (FBS), penicillin (100 U/mL), and streptomycin (100 µg/mL) at 37°C in a humidified environment containing 5% carbon dioxide (CO_2).

2.8.2*In Vitro* assay for anticancer activity by the MTT assay:

Cells were plated at a density of 1×10^5 cells per well in 24-well plates and incubated at 37°C in a 5% CO_2 atmosphere. Once the cell achieved its maximum density, different concentrations of the samples were introduced and incubated for 24 h. Following incubation, the sample was extracted from the well and rinsed with phosphate-buffered saline (pH 7.4) or DMEM lacking serum. Each well was supplemented with 100µL of a 5mg/mL solution of 0.5% MTT and incubated for 4 h. Following incubation, 1 mL of DMSO was added to each well. A spectrophotometer was used to measure the absorbance at 570nm, with DMSO serving as the blank [16, 18]. Measurements were conducted, and the concentration needed to achieve 50% inhibition (IC50) was established using graphical methods. The percentage of cell viability was determined using the previous study formula [19].

2.9 Zebrafish embryonic (toxicology)toxicityevaluation of *P. perlata* ethanolic extract

Based on the Rajesh Kumar et al. 2022 procedure, zebrafish (*Danio rerio*) embryos were maintained and exposed to *P. perlata* ethanolic extract.The progression of *D. rerio* embryos' growth was seen using a stereo microscope. The embryos were exposed to different concentrations of *P. perlata* ethanolic extract (5, 10, 20, 40, and 80 µg/mL) for a duration of 24 to 72 h after fertilization (hpf). The evaluation of embryonic death and hatching rates was

conducted at 24 h intervals. The study assessed the mortality of embryos and hatchlings, the rate of hatching, and the identification and documentation of any abnormalities in the embryos and larvae of both the control and treatment groups. Malformed embryos were observed using a COSLAB- Model HL-10A light microscope, and the number of abnormal embryos was noted every 24 h. [20]

(Results)Resultand Discussion(RESULT AND DISCUSSION SHOULD BE SEPARATED)

3.1 Phytochemical Screening of *P.perlata* extract:

The Indian Siddha system is a renowned traditional form of treatment that has been employed since ancient times to heal a wide range of illnesses. Ayurvedic Indian medicine uses several lichen extracts as remedies for various ailments [21]. The phytochemical screening of the *P. perlata* extract was evaluated using a conventional approach described by Behera et al. (2024) and Mursaliyeva et al. (2023). Table 1 provides a comprehensive list of phytochemical components found in *P. perlata* extracts.

The ethanolic extract contained all phytochemicals such as glycosides, carbohydrates, tannins, saponins, terpenoids, alkaloids, flavonoids, quinone, phenols, and steroids.Similarly, Momoh and Adikwu 2008[22]study, that the lichen *P. perlata* ethanolic extract contains saponins, flavonoids, tannins, glycosides, steroidal aglycone, and carbohydrates.

S. No	Test	Ethanol Extract
1.	Carbohydrate	+
2.	Tannins	+
3.	Saponins	+
4.	Alkaloid	+
5.	Flavonoids	+
6.	Glycosides	+
7.	Quinone	+
8.	Phenols	+

9.	Terpenoid	+
10.	Steroid	+

Table 1. Phytochemical Screening of *P. perlata* Ethanolic extract.

Key: (+) Present and(-) Absent

3.2 Antioxidant activity of *P.perlata*ethanolic extract:

Antioxidant activity was assessed using the DPPH assay. It was demonstrated that*P.perlata* ethanolic extract exhibits a higher degree of free radical scavenging activity, indicating a stronger potential for antioxidants. By increasing absorbance with concentration, the DPPH assay indicated a lower power potential. Strong reducing power was shown by the ethanolic extract of *P.perlata* employed in the study; the reported inhibition percentage was 70%shown in Fig: 4. Kello et al. (2023) assessed the antioxidant properties of acetone extracts from several lichen species, comparing them to ascorbic acid, which showed scavenging activities. According to Us D et al. (2023), DPPH radical scavenging activities of lichen methanolic extracts increase (proportionally)proportionatelywith concentration. Absorbance values of these extracts and standard antioxidants were assessed at 30 µg/mL [11-12].

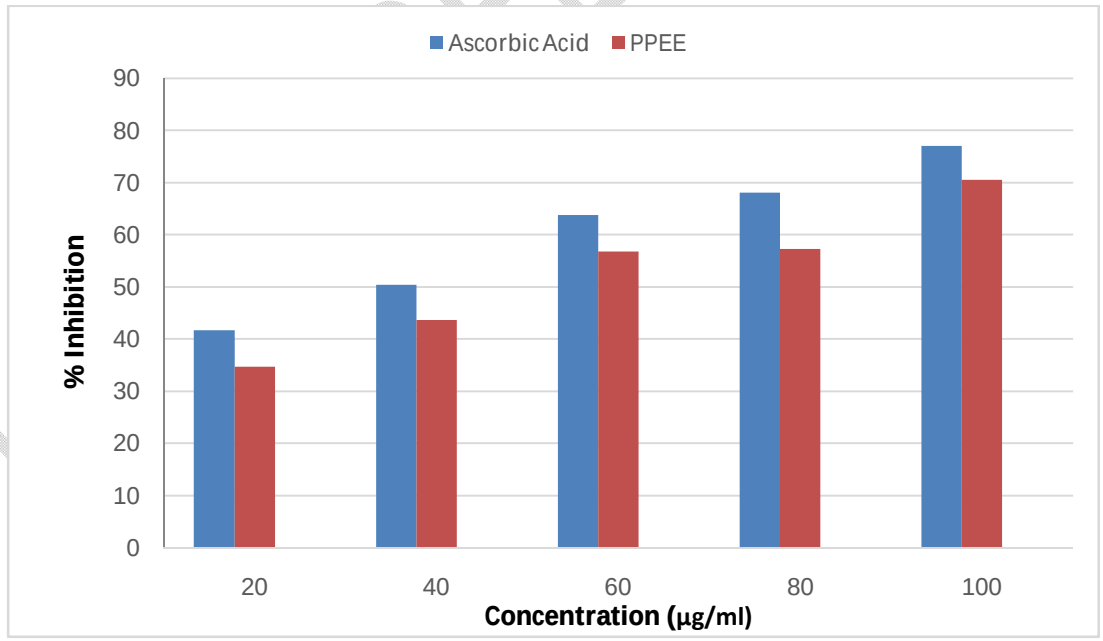


Fig 4. Antioxidant activity of *Parmeliaperlata* Ethanolic extract.

3.3 Anti-inflammatory properties of the *Permaliaperlata* ethanolic extract:

Using spectrophotometry at 660 nm, the effects of the common medication Diclofenac and *P. perlata* ethanolic extract ranging from 100 to 500 (µg/mL)on the prevention of BSA denaturation were assessed. The lowest dose of 100 µg of *P. perlata* ethanolic extract effectively inhibited the denaturation of the albumin protein (Fig: 5). As the concentration of *P. perlata* ethanolic extract increased, the proportion of denaturation inhibition grew steadily. In *P. perlata*, 500µg of albumin-like denaturation inhibition resulted in a maximum denaturation inhibition of 51.72% shown in Table: 2. Diclofenac was demonstrated to have a denaturation-inhibiting effect on BSA at dosages of 100–500µg/mL, whereas*P. perlata* ethanolic extract had the same effect.The lichen extract that was examined had a dose-dependent protective effect against the denaturation of BSA caused by heat. According to Tartouga et al. (2022), the extract demonstrated a 38.39% inhibition of BSA denaturation at a concentration of 1000 µg/mL [23].

Tatipamula et al. (2018) reported that extracts from lichen had significant anti-inflammatory properties in vitro. At a concentration of 1 mg/mL, the extracts acetone and ethyl acetate showed inhibition levels that were comparable to indomethacin, with acetone inhibiting inflammation by 89.85% and ethyl acetate by 82.36% [24].

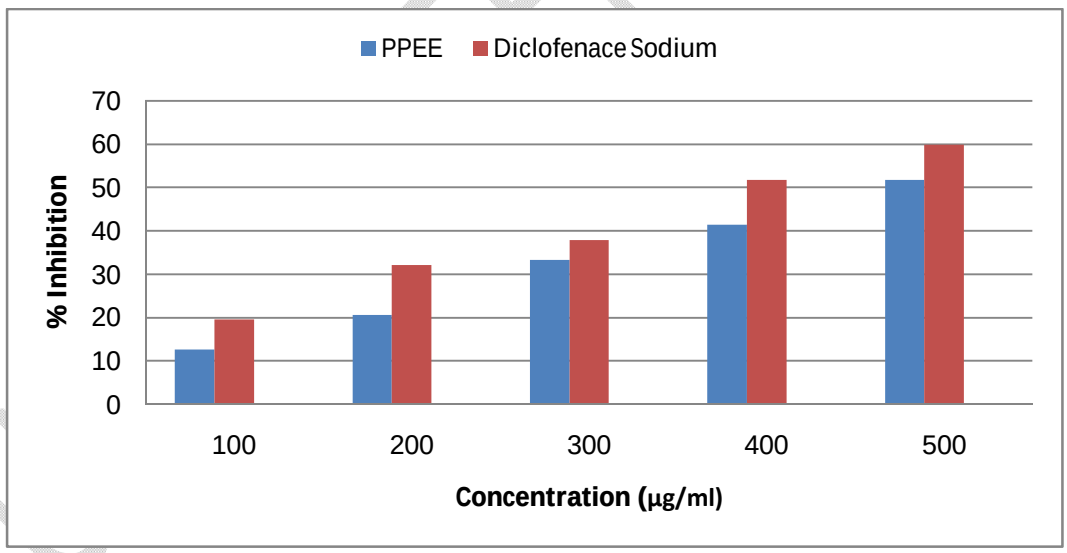


Fig. 5.Anti-inflammatory activity of *ParmeliaPerlata* ethanolic Extract.

Concentration in µg/mL	**Percentage of inhibition (%)**	
	Diclofenac Sodium	***Parmeliaperlata*eth**

		anolic extract
100	19.54	12.64
200	32.18	20.68
300	37.93	33.33
400	51.72	41.37
500	59.77	51.72

Table 2: Anti-inflammatory activity of *ParmeliaPerlata* Ethanolic extract.

3.4Antibacterial activity of ethanolic *Parmeliaperlata* extract:

The antibacterial properties of an ethanolic extract of *P. perlata*were evaluated against six bacterial strains, consisting of three gram-positive and three gram-negative strains, using the agar well diffusion method. The bacteria included *S. aureus, B. subtilis, Enterococcussp.,E. coli, K. pneumoniae,*and *Pseudomonas sp.*(Fig: 6 (a), (b), (c), (d), (e), and (f)). Six harmful microorganisms were evaluated using ethanolic extract of *P. perlata* at several concentrations (50, 100, and 150 µg/mL). The increasing concentration gradients indicate a high zone of inhibition (ZOI) in the *P. perlata* ethanolic extract. In all six organisms, the ZOI for the positive control gentamicin is larger than the ZOI for the other three concentrations (50, 100, and 150µg/mL) of *P. perlata* ethanolic extract. Nonetheless, there is a noticeable ZOI in the ethanolic *P. perlata* extract, which is appropriate to illustrate its antibacterial activity.

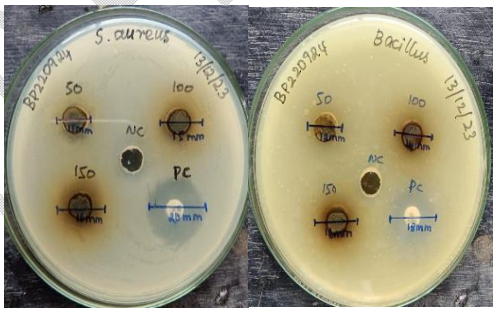
The results indicate that the acetone extract of lichen had superior antibacterial activity compared to the methanolic extract, particularly against Gram-positive bacteria. Nevertheless, it had little efficacy against Gram-negative bacteria such as *E. coli, S. enterica, S. typhimurium,* and *K. pneumoniae* when assessed using MIC and MBC techniques as per Kocovic et al. study in 2022 [8].

Kello et al. (2023) evaluated by extracting seven lichen extracts. These extracts were then tested against both Gram-positive bacteria (*S. aureus*) and Gram-negative bacteria (*E. coli*) at a

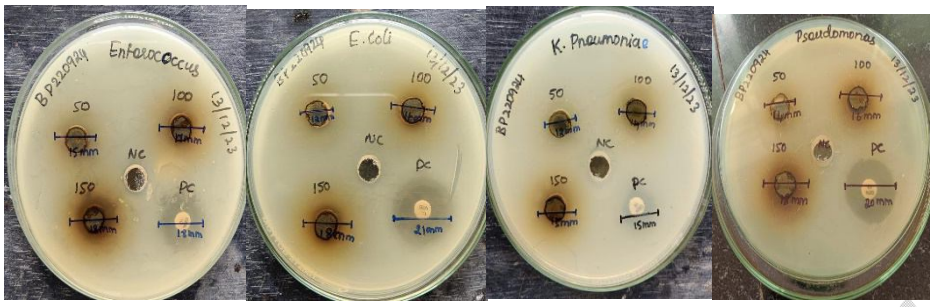
concentration of 1 mg/mL. The treatment showed no efficacy against *E. coli*, but showed efficacy against *S. aureus*, with lichens exhibiting the most pronounced inhibitory impact [11].

The methanol extracts of five lichen species exhibited antibacterial properties against 11 bacteria and 1 fungus. *C. calcarea*, *N. chlorophylla*, and *B. capillaris*showed significant effectiveness against *B. cereus*, *B. subtilis*, *C. perfringens*, and *E. coli*, resulting in ZOI ranging from 9 to 15 mm. In a study conducted by Us et al in 2023, it was shown that *C. calcarea* exhibited the most activity, resulting in a 15 mm inhibition zone against both *E. coli* and *B. cereus* [12].

The study assessed the antibacterial efficacy of solvent extracts derived from 12 lichen species against pathogenic bacteria (*P. aeruginosa*, *E. coli*, *S. aureus*,and *K. sp.*) and the fungus *Aspergillus sp.*, employing the disc diffusion method. The acetone extract of lichen species exhibited the most potent antibacterial action, namely against *E. coli*, *P. aeruginosa*, and *S. aureus*. The fungal strain examined by Khan et al 2023 showed the greatest inhibitory zones in *P. sulcata*, *Evernia mesomorpha*, and *Stictalambata*[17]. In (our)the present study, the antibacterial activity of *P. perlata* ethanolic extract was mostly reflected in a higher ZOI in *Enterococcus sp.*, *E. coli*, and *Pseudomonas sp.*, which is shown in Table: 3. The remaining cultures have less ZOI in measurement. Among the bacteria, *P. perlata* extract showed the best effect against *E. coli* in 150 µg/mL concentration.



(a) *Staphylococcus aureus*(b) *Bacillus subtilis*



(c) *Enterococcus* sp. (d) *E. coli* (e)*K. pneumoniae* (f) *Pseudomonas* sp.

Figure 6. Antibacterial activity of ethanolic *ParmeliaPerlata* extract.

S. No	Organism	DMSO	Gentamycin	Zone of inhibition		
				50 µg/mL	100 µg/mL	150 µg/mL
1.	*Staphylococcus aureus*	-	20mm	11mm	15mm	16mm
2.	*Bacillus subtilis*	-	18mm	12mm	14mm	16mm
3.	*Enterococcus*sp.	-	18mm	15mm	17mm	18mm
4.	*Escherichia coli*	-	21mm	12mm	16mm	19mm
5.	*Klebsiellapneumoniae*	-	15mm	12mm	14mm	15mm
6.	*Pseudomonas*sp.	-	20mm	14mm	16mm	18mm

Table 3. Antibacterial activity of ethanolic *Parmeliaperlata* extract.

3.5*Invitro* anticancer activity of ethanolic extract of *Parmeliaperlata* against ovarian cancer cell line:

In this study, the anticancer effects of *P. perlata* were evaluated in vitro on the viability of the human ovarian (PA-1) cell line. Various concentrations of *P. perlata* ranging from 7.8 to 1000 µg/mL were used for testing. The outcome found that as the concentration of the test substance *P. perlata* increases, the percentage of cell viability of the PA-1 cell line decreases. The lowest percentage of cell viability was found in 1000 µg/ml.It was discovered that the matching IC_{50} value was 31.2 µg/mL (Table: 4). Comparing the Morphology of Human Ovarian (PA-1) Cell

Lines Treated with Different Doses of the Test Drug *P. perlata*, the standard Control and concentrations are shown in Fig: 7.

Similarly, Fankam et al. (2023) examined the potential role of apoptosis in the cell death of ovarian cancer cells induced byguttiferone(GBL)isolatedfrom *Allanblackiagabonensis.* The study of the cell cycle revealed a significant prevent with 8.5% of cells in the sub-G0 phase at a low dosage and 30.7% at the IC50 dosage, in contrast to 5.4% in cells that were not treated [25].

According to Nikhitha et al. (2021), *Adhatodavasica* extract was used to treat PA1 teratocarcinoma cells and showed significant anticancer effects. Observations were conducted for a duration of 48 h in order to evaluate the suppression of metastatic characteristics. The mRNA obtained from both treated and untreated cells was transformed into complementary DNA (cDNA) and then amplified in order to assess the gene expressions of p53, p21, and GAPDH. The findings demonstrated the efficacy of A. vasica extract in combating ovarian cancer[26].

In (our)the present study, *P. perlata*ethanolic extract was used for the *in vitro* anticancer activity, which shows significant activity against human ovarian (PA-1) cell line.

S. No.	Concentration (µg/ml)	OD value	Cell viability (%)
1	1000	0.089	14.75
2	500	0.132	21.89
3	250	0.174	28.85
4	125	0.217	35.98
5	62.5	0.261	43.28
6	**31.2**	**0.304**	**50.41**
7	15.6	0.349	57.87
8	7.8	0.392	65.00
9	Cell control	0.603	100

Table 4. Anticancer effect of *P. perlata* on PA-1 cell line.

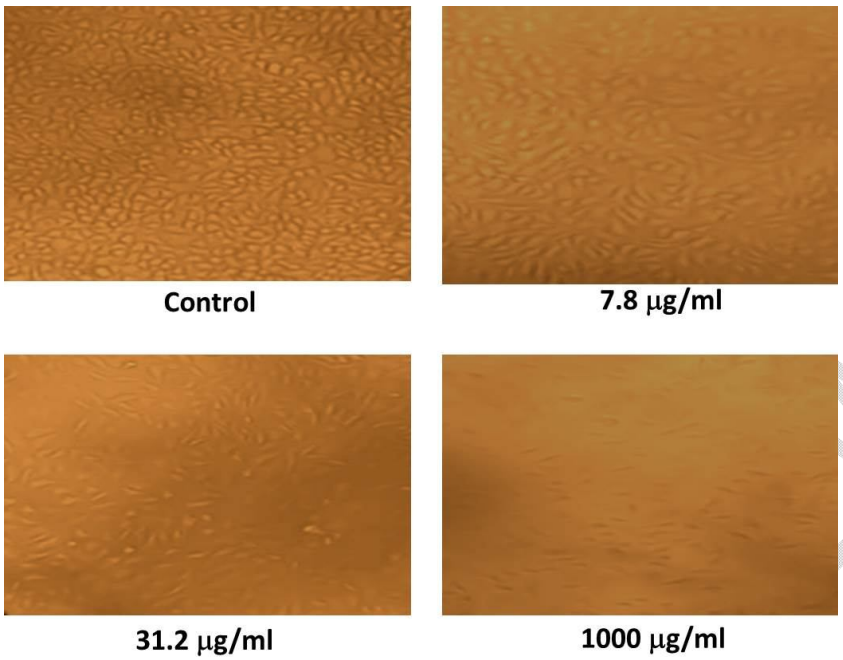


Figure 7: Morphological comparison of PA-1 cells treated with *Parmeliaperlata* at different concentrations and control.

3.6 Embryonic cytotoxicity study of *Parmeliaperlata*extract using Zebrafish:

The impact of *P. perlata* extract on zebrafish embryos was investigated by examining its effects on various developmental parameters such as hatching rates, mortality rates, deformities, and morphological abnormalities. The treated embryos were observed at 24, 48, and 72 h post-fertilization (hpf).

Shenbagam et al. (2021) treated zebrafish with *P. tinctorum* extract for 7 days. The behavior study revealed no observable physiological alterations or notable behavioral consequences, such as immobility, over the whole duration of the exposure [27].

Shenbagam et al. (2021) investigated the harmful effects of *P. tinctorum* on zebrafish embryos by exposing them to doses ranging from 50 to 250 µL for a duration of 72 h after fertilization. There were no notable variations in growth, survival, and heart rate across the different concentrations. The embryos exhibited good health and did not have any morphological defects. The results indicate that the safety is maintained at concentrations of up to 200 µg/mL [21].

The findings of (our)the presentstudy indicate that exposure to *P. perlata* extract at a concentration of 150 µg/mL has a significant impact on the hatching rates and viability rates, as shown by Figures 8 and 9. The embryos successfully hatched at a high rate. In addition, there were no significant differences in morphology and heart rate between the group exposed to *P. perlata* extract and the normal control group (Figures 8 and 9).

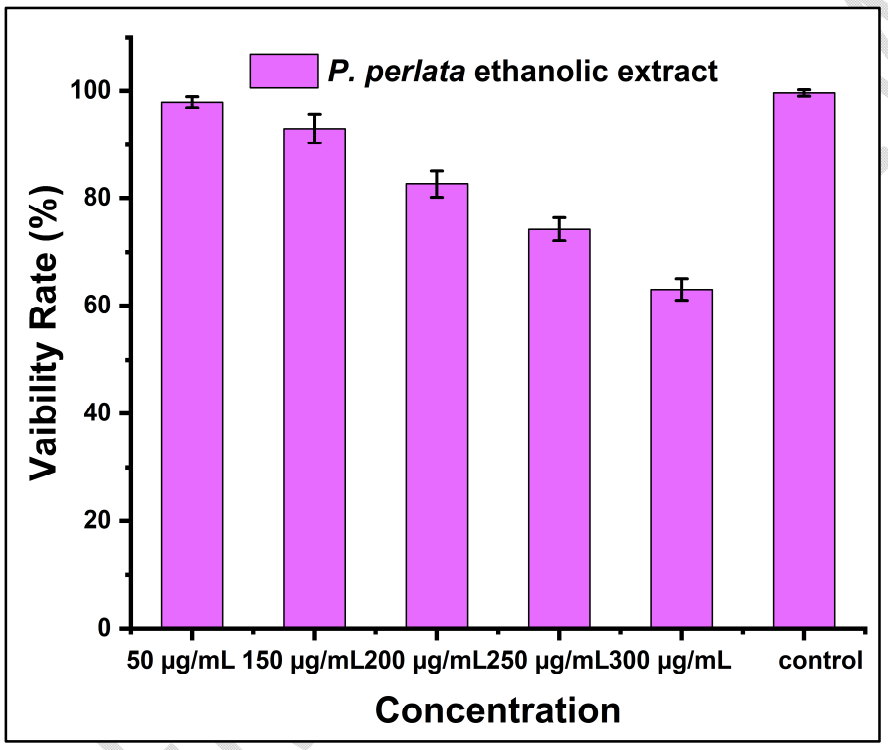


Figure 8: Viability Rate of Zebrafish treated with *Parmeliaperlata*extract

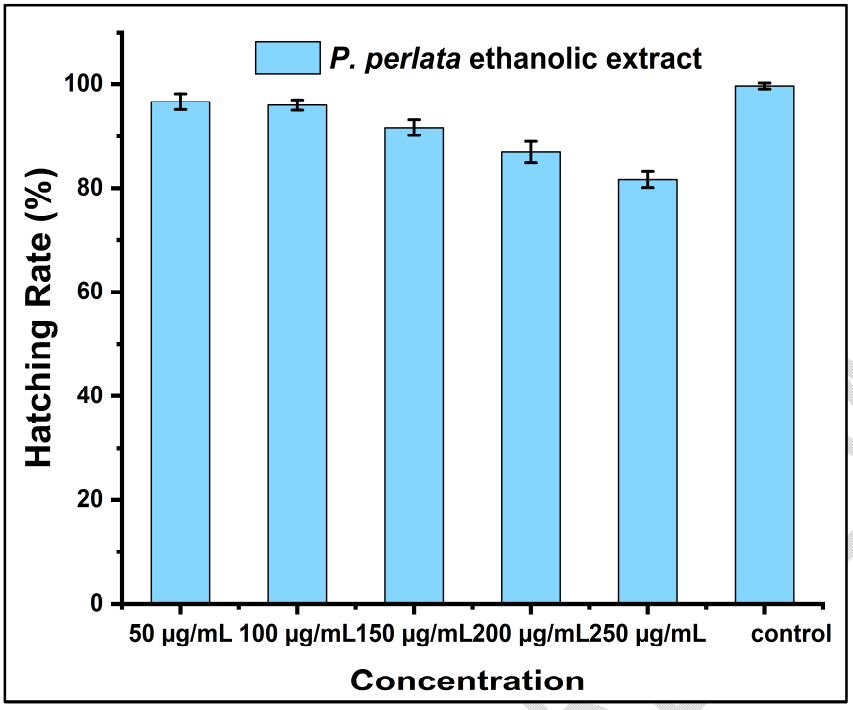


Figure 9: Hatching Rate of Zebrafish treated with *Parmeliaperlata*extract

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4. Conclusion:

The ethanolic extract of *P. perlata* was used to identify phytochemicals and several biochemical activities, including antioxidant, anti-inflammatory, and antibacterial properties by in vitro methods. The MTT test was employed to determine how various *P. perlata* concentrations affected the survival of PA-1 cell lines. This *in vitro* model is extensively utilized to assess the cytotoxicity of plant extracts against various types of cancer cells. *P. perlata* extract identified its toxic level by observing the morphological characteristics such as mortality, malformation rate, and heartbeat of the zebrafish model. The extract induces toxicity in embryonic development in a dose-dependent manner. This toxicity adversely impacts the development of the embryos, resulting in deformities, delayed development, and potentially even mortality. Numerous herbs found in the test medication contain phytochemicals that function as antioxidants, antibacterial and anticancer agents. Antioxidants are effective in preventing and treating cancer and other ailments because of their ability to protect cells from the harmful effects of highly reactive oxygen molecules, commonly referred to as "free radicals."

Declarations

Ethics approval: In this study, animal experiment is not applicable.

Consent to participate: In this study, animals and human trails is not applicable.

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