**In silico and in vitro cytotoxicity profile of hydroalcoholic extract/fraction(s) of Pachygone ovata**

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**ABSTRACT**

Medicinal plants have been used in the past for the treatment of diseases and continue to be an important reservoir for the development of new drugs. With the increasing burden of cancer globally, there is a need to find newer anticancer agents. The process of identification and evaluation of cytotoxic molecules from plants can be achieved conveniently by using simple yet reliable screening models and combining with *in silico* techniques. *Pachygone ovata*, least explored plant from Menispermaceae family, is known to be rich in alkaloids. This study aimed to identify the cytotoxic constituents from *Pachygone ovata* through bioactivity-guided fractionation using Brine shrimp lethality bioassay as a screening model. The active fraction in this assay was evaluated for its *in vitro* cytotoxic activity on human tumor cell lines. Some reported alkaloids were studied for their binding affinities with topoisomerase II by molecular docking. The study revealed the cytotoxic constituents from *P. ovata*. The study also revealed alkaloids with higher binding affinity with topoisomerase II, and the scope for further use leads to the development of new drugs.

**INTRODUCTION**

In the past, medicinal herbs were used to treat even the most complex conditions related to cancer such as inflammation, swellings, growths, and warts. Over 2,000 plant species of medicinal value have been recorded and are used for therapeutic purpose in different forms (Biljana, 2012). Cancer begins as a result of altered cell function, due to genetic and epigenetic changes within the cell leading to genetic instability (Anna, 2019). At present, cancer is the second largest cause of death worldwide. According to the WHO, cancer accounted for an estimated 9.6 million deaths in 2018 and continues to grow globally (WHO, 2020). The complication in treatment arises due to the uncontrolled proliferation of cells and invasion into other tissues, and thus, ineffective treatment and toxic side effects associated with chemotherapy necessitate the discovery of alternative treatment options (Chidambaram et al., 2011).

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Medicinal plants still prove to be an important resource in the development of new drugs. Currently, a number of drugs derived from plants have been approved for clinical use, including cancer therapy (Cragg and Newman, 2005). Extensive research findings suggest that phytochemicals and their derived analogs are reported to inhibit the progression of cancerous cells through various mechanisms and have the most promising alternative for the treatment of cancer (Ana et al., 2018). The Indian system of medicine offers a number of plants that possess cytotoxic activities (Petrovska, 2012). *Pachygone ovata* (Poir.) Miers ex Hook. f. Et Thoms belonging to the family Menispermaceae is a deciduous woody shrub that can climb up to 15 m or more. The dried fruits of *P. ovata* have been used traditionally to repel insects and as fish poison (Shirin et al., 2014). It is one of the least explored plants, which is rich in active phytoconstituents, especially alkaloids. Different benzyl-isoquinoline-derived alkaloids have been reported from *P. ovata* stems, leaves, and roots (El-Kawi et al., 1984). Alkaloids have been well known for their inhibitory action on a wide range of tumors through different mechanisms (Lu et al., 2012).
The brine shrimp lethality (BSL) assay is reported to be an effective screening model for potential cytotoxic constituents and has led to the identification of many cytotoxic compounds through bioactivity-guided approach (Meyer et al., 2005). Computational tools, in recent years, are being employed to study the modes of interaction, bioavailability, and toxicities of the possible lead compound with the target protein (Rosales-Hernandez et al., 2009). The inhibition of topoisomerase enzymes is one of the many modes, through which drugs may exert their effects on cancer cells. Important cellular functions such as replication, recombination, transcription, and DNA repair are governed by the activities of topoisomerase I and II (Kumar et al., 2013). The inhibition of such enzymes has been the target in anticancer drug research since recent years (Sivakumar et al., 2010). The use of simple and reliable screening models combined with modern in silico techniques can make the process of identification of bioactive constituents from plants effective and convenient (Khanal et al. 2019a, 2019b). Thus, this study was carried out to identify the potential cytotoxic constituents from *P. ovata* through the bioactivity-guided approach and molecular docking, which could be promising in the development of new drugs. The phytoconstituents used for docking i.e., coreximine, isoboldine, liriodenine, norjuziphine, pachygonine, reticuline, nortrilobine, and trilobine including standard etoposide are shown in Figure 1.

**MATERIALS AND METHODS**

**Collection, authentication, extraction, and fractionation of plant**

The wild-grown whole plant of *P. ovata* was collected in the month of July–September, from areas of Tirupati, Eastern Ghats, Andhra Pradesh, authenticated at Sri Venkateswara University, Tirupati, Andhra Pradesh, and the herbarium of the same was deposited with voucher number 0827 for future reference. The dried plant material was extracted by cold maceration using 70% v/v ethanol for 24 hours. After filtration, the marc was dried and further extracted by Soxhlet extraction. The macerate and percolate were then combined and concentrated using a rotary evaporator (IKA-RV Digital) to obtain the final extract. The fractionation of extract was carried as explained by Cos et al. (2006) to obtain methanol, petroleum ether, dichloromethane, and aqueous fractions.

**Cell culture**

MCF-7 (human, breast cancer), HT-29 (human, colon cancer), A-549 (human, small-cell lung carcinoma), HepG-2 (human, hepatic cancer), and L-6 (rat, normal skeletal muscle) cell lines were purchased from National Centre for Cell Science, Pune, India. The cells were cultured in 25 cm² culture flasks with Dulbecco’s modified eagle medium (DMEM) supplemented with 10% inactivated fetal bovine serum (FBS), penicillin (100 IU/ml), streptomycin (100 mg/ml), and amphotericin B (5 mg/ml) in a humidified atmosphere of 5% CO₂ at 37°C until confluent. The cells were dissociated with trypsin phosphate versene glucose solution 0.2% trypsin, 0.02% Ethylenediaminetetraacetic acid (EDTA) and 0.05% glucose in Phosphate-buffered saline (PBS)).

**Evaluation of cytotoxicity**

**BSL bioassay**

The brine shrimp (*Artemia salina* Leach.) eggs were purchased from a local vendor. The procedure was carried out according to the procedure reported (Mc Laughlin and Roggers,

![Figure 1. Structure of (a) coreximine, (b) isoboldine, (c) liriodenine, (d) norjuziphine, (e) nortrilobine, (f) pachygonine, (g) reticuline, (h) trilobine and (i) etoposide.](image-url)
with modifications. Briefly, the stock solution of extracts/fractions was prepared in 1% DMSO and serially diluted using sea water to obtain solutions of 10, 50, 100, 500, and 1,000 µg/ml. Ten nauplii were added to 5 ml of each test solution. Control tubes contained equal volumes of distilled water. The assay was carried out in triplicate for each concentration. The tubes were kept under illumination, and the number of survivors was counted after 24 hours and the percentage of mortality was calculated. LC$_{50}$ values were calculated by Probit analysis using SPSS-10.0.5 software (Armonk, NY).

The active fractions from BSL bioassay were screened for cytotoxicity by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay as previously described (Lombardi et al., 2017). Cells were collected by trypsinization, and the cell count was adjusted to 1.0 × 10$^5$ cells/ml per well using DMEM medium containing 10% FBS. After 24 hours of incubation at 37°C in 5% CO$_2$ atmosphere, 100 µl of the various concentrations of the sample were added to the wells and incubated. After 48 hours, 20 µl of MTT was added to each well and incubated for 4 hours. The supernatant was discarded, and DMSO was added to each well. The absorbance was measured using a microplate reader at a wavelength of 540 nm. The percentage growth inhibition and IC$_{50}$ values were calculated.

Percentage growth inhibition (%) = \[ \frac{\text{Absorbance of control} - \text{Absorbance of sample}}{\text{Absorbance of control}} \times 100 \]

where A (control)= Absorbance of control, A (sample) = Absorbance of sample

### Molecular docking

The selected ligand molecules, i.e., coreximine, isoboldine, norjuziphine, nortrilobine, trilobine, liriodenine, pachygonine, and reticuline were retrieved from the PubChem database (.sdf format), converted into .pdb using Discovery Studio 2017 and minimized using mmff 94 force field and conjugate gradients as an optimization algorithm. After minimization, the pose with the least energy was chosen for docking. Topoisomerase II (PDB: 4GHF) was retrieved from the RCSB database and used as a template for query sequence for Accession number: P11388.3 for the homology modeling by adding the missing amino acid using Modeller 9.10. The protein molecule was made free from heteromolecules using Discovery Studio 2017 to avoid docking interference and saved in .pdb format, and the docking was carried by using AutoDock 4.0 under Lamarckian GA 4.2. The protein was viewed in Ramachandran plot to assess the distributed amino acid residues using Procheck (https://services.mbi.ucla.edu/PROCHECK/). After docking, the pose scoring minimum binding energy was chosen to visualize the ligand–protein interaction using Discovery Studio 2017. All the docking results were compared with the known topoisomerase II inhibitor, i.e., etoposide.

### Statistical analysis

Data were expressed as mean ± SD using GraphPad Prism version 5.0. The IC50 was calculated using a linear regression model.

### RESULTS

#### BSL bioassay

The hydroalcoholic extract of *P. ovata* displayed high toxicity toward shrimp nauplii with LC$_{50}$: 58.411 ± 1.33 µg/ml. The fractions showed toxicity toward shrimp nauplii in a concentration-dependent manner and are shown in Figure 2. Fraction 3 showed the highest percentage mortality of nauplii and LC$_{50}$: 3.47 ± 1.66 µg/ml. Fraction 4 showed the least percentage mortality in this assay. The LC$_{50}$ of hydroalcoholic extract/fraction is shown in Table 1.

#### MTT assay

Alkaloid-rich Fraction 3 of *P. ovata* displayed a notable cytotoxicity in the cell lines. The cytotoxicity increased with increasing concentration and is shown in Figure 3. The

![Figure 2. Brine shrimp mortality by various fractions of *P. ovata*.](image)

![Figure 3. Cell growth inhibition of Fraction 3 of *P. ovata* in different cell lines.](image)

| Test agent                       | LC$_{50}$ (µg/ml) |
|----------------------------------|-------------------|
| *P. ovata* alcoholic extract     | 58.411 ± 3.33     |
| Fraction 1                       | 261.57 ± 2.14     |
| Fraction 2                       | 163.92 ± 3.73     |
| Fraction 3                       | 30.47 ± 1.66      |
| Fraction 4                       | 429.94 ± 3.27     |
most cytotoxicity was observed towards the A-549 cell line compared to the others displaying IC$_{50}$ = 84.76 ± 1.47µg/ml. The least cytotoxicity was noted in the MCF-7 cell line, whereas no cytotoxicity was observed in the normal cell line L6. Table 2 shows the IC$_{50}$ of a fraction rich in alkaloids in multiple cell lines.

| Test agent                  | A-549  | MCF-7  | HT-29  | HepG2  | L-6   |
|-----------------------------|--------|--------|--------|--------|-------|
| Fraction rich in alkaloids  | 84.76±1.47 | 333.7±4.23 | 91.12±2.17 | 625.10±2.73 | >1000 |
| Paclitaxel                  | 7.43±0.33 | 10.52± | 12.67±1.29 | 15.81±2.26 | 323±2.73 |

**Molecular docking**

In the homology modeled protein, the residues in most favored, allowed, and generously allowed regions were 81.5%, 14.2%, and 2.7%, respectively (Figure 4). The docking study predicted trilobine to possess the highest binding affinity (−11.2

**Figure 4.** Ramachandran plot of homology modeled topoisomerase II.
kcal/mol) compared to others. However, no hydrogen bond interactions were found with the amino acids of topoisomerase II. Although reticuline was predicted to have binding energy −7.2 kcal/mol, it was found to be interactive with two amino acids, i.e., GLU1494 and MET1500. Table 3 shows the binding affinity of each ligand molecule with topoisomerase II. Figure 5 shows the interaction of liriodenine, pachygonine, norjuziphine, trilobine, nortrilobin, coreximine, isoboldine, reticuline, and etoposide with topoisomerase II.

DISCUSSION

The BSL bioassay, a general test for screening bioactive compounds, was used in this study to identify the potential cytotoxic constituents in *P. ovata*. According to several reports, the BSL test predicts cytotoxicity and has been successfully utilized in the bioassay-guided fractionation of active cytotoxic and antitumor agents (Arullappan et al., 2015; Zhang et al., 2015). The reports suggest a significant correlation between the brine shrimp assay and *in vitro* inhibition of human tumor cell lines (Anderson et al., 1991).

In this study, the fraction rich in alkaloids showed the highest mortality of the brine shrimp nauplii compared to the other three fractions, LC₅₀ = 30.47 ± 1.66 µg/ml, which is considered as cytotoxic as explained by Pimentel Montanher et al. (2002). Hence, the alkaloid fraction was further evaluated for its *in vitro* cytotoxicity using multiple human tumor cell lines using the MTT assay. Although there have not been notable reports regarding the cytotoxicity of *P. ovata*, there are, however, studies reporting the antioxidant (Amalarasi and Jothi, 2019) and anti-inflammatory (Marahel and Sharanaiah, 2016) activities of *P. ovata* extracts, both of which have a close association with the pathogenesis of cancer (Yoshikawa and Naito, 2002; Rayburn et al., 2009). In accordance, a noteworthy cytotoxic effect of the alkaloid rich fraction was observed in this study. The highest cytotoxicity (IC₅₀ = 84.76 ± 1.47 µg/ml) was noted against A-549 (human lung cancer) cell line. Moreover, worth noticing was that the fraction was relatively nontoxic toward the L-6 cell line indicating biocompatibility with the normal cell line. The preliminary screening of hydroalcoholic extract/fraction (s) in BSL bioassay was found to be effective in identifying potentially toxic fractions containing alkaloids, which further displayed significant cytotoxicity *in vitro*, and thus confirming its productive use in our study.

*P. ovata*, which is a member of the Menispermaceae family, is reported to constitute benzylisoquinoline alkaloids (El-Kawi et al., 1984). This study also identifies alkaloids from

| Ligand         | Binding affinity (kcal/mol) | Number of hydrogen bonds | Hydrogen bond residues     |
|----------------|----------------------------|--------------------------|---------------------------|
| Liriodenine    | −9.3                       | –                        | –                         |
| Norjuziphine   | −7.6                       | 1                        | MET1500                   |
| Pachygonine    | −8.4                       | 1                        | LYS512                    |
| Reticuline     | −7.2                       | 2                        | GLU1494, MET1500          |
| Trilobine      | −11.2                      | –                        | –                         |
| Coreximine     | −4.58                      | 2                        | GLY 474, LYS480           |
| Isoboldine     | −4.94                      | 1                        | TYR 481                   |
| Nortrilobine   | −4.42                      | –                        | –                         |
| Etoposide*     | −9.2                       | 3                        | HIS605, ASN508, GLN537    |

*Known molecule as topoisomerase inhibitor.*

![Figure 5. Interaction of (a) liriodenine, (b) pachygonine, (c) norjuziphine, (d) trilobine, (e) nortrilobin, (f) coreximine, (g) isoboldine, (h) reticuline, and (i) etoposide with topoisomerase II.](image-url)
*P. ovata* which could be a relatively promising group of natural products as a source of new anticancer agents. Hence, *in silico* docking studies were carried out on previously reported alkaloids (liriodenine, trilobine, pachygonine, coreximine, reticuline, isoboldine, norjuziphine, and nortrilobine) against Topoisomerase II. Topoisomerase II is an enzyme involved in DNA replication. Topoisomerase II is radically upregulated in cancer cells due to rapid cell division and growth (*Nainwal et al.*, 2014). Topo II is a potential target in the designing of newer anticancer agents (*Heck et al.*, 1986). In this study, alkaloids from *P. ovata* were found to interact with topoisomerase II, suggesting their involvement in cancer management.

It was reported that the stability of the ligand–protein complex depends on the binding energy as well as hydrogen bond interactions. In this study, although trilobine was found to have the highest binding affinity with topoisomerase II, the complex may not be stable since it could not form any hydrogen bond interactions with any amino acid of protein molecules. Although reticuline was predicted to have binding energy $-7.2 \text{ kcal/mol}$, it was found to be interactive with a protein molecule. Reticuline has previously shown *in vitro* cytotoxic activity in human tumor cell lines such as P-388, KB16, and A549 (*Chen et al.*, 1997; *Suresh et al.*, 2012), demonstrating its future scope in cancer research.

Further, cancer is a polygenic condition, in which multiple proteins are involved in its pathogenesis (*Bredberg*, 2011). The fraction rich in alkaloids could modulate the multiple proteins and pathways which can be accessed through the gene-set enrichment analysis (*Khanal and Patil*, 2019; *Khanal and Patil*, 2020) and network pharmacology (*Khanal et al.*, 2019c) which is also a future scope of the present study.

**CONCLUSION**

This study revealed potential cytotoxic alkaloids from *P. ovata*. Further, the docking study predicted the binding ability of alkaloids from *P. ovata* with topoisomerase II; however, further investigations need to be carried out to validate the findings and the use of alkaloids from *P. ovata* leads to cancer treatment.

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**CONFLICT OF INTEREST**

Authors declare that they do not have any competing interests.

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