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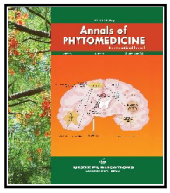

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Phytochemical profile, antioxidant and cytotoxic activities of aquatic weed *Landoltia punctata* (G. Mey.) Les & D.J. Crawford

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Abstract

This study investigates the phytochemical, antioxidant and cytotoxic activity of *Landoltia punctata* (G. Mey.) Les & D.J. Crawford, a floating aquatic plant. The total flavonoid content (TFC), total phenolic content (TPC), tannin content, and the antioxidant activities (NO and DPPH free radical scavenging assay) were studied. Outcomes showed that the hydroalcoholic fraction had the highest TPC (47.77 ± 1.27 mg GAE/g), subsequently followed by the n-butanol fraction (33.23 ± 0.93 mg GAE/g). The highest TFC (13.03 ± 1.43 mg QE/g) was exhibited by the n-butanol fraction, subsequently followed by the hydroalcoholic fraction (23.73 ± 1.44 mg QE/g). The tannin content in each fraction was articulated as milligrams of tannic acid equivalents per gram of dry weight (mg TAE/g), with the hydroalcoholic fraction having the highest amount at 47.77 ± 1.27 mg. The n-butanol fraction had the highest antioxidant activity in the NO and DPPH free radical scavenging assay indicating its potential therapeutic applications. The study evaluated the cytotoxic activity of different fractions of *L. punctata* and doxorubicin on four cell lines (HepG2, HeLa, A549, and MCF-7) and on human umbilical vein endothelial cells (HUVECs). The findings showed that the most potent cytotoxic effect on the tested cell lines was exhibited by the n-butanol fraction, with the lowest IC_{50} values for MCF-7, A549, and HeLa cells. The ethyl acetate fraction had intermediate cytotoxicity, while the hydroalcoholic fraction had the least of the three fractions. However, the n-butanol fraction had a more substantial cytotoxic effect on A549 cells than doxorubicin. The study also found that all fractions had higher IC_{50} values for HUVECs, indicating less cytotoxicity to healthy cells, which is desirable for potential therapeutic applications.

1. Introduction

An imbalance in the production and degradation of or reactive nitrogen species or reactive oxygen species (ROS) is known as oxidative stress. ROS, which includes singlet oxygen, hydroxyl radicals, hydrogen peroxide, and superoxide radicals, can damage essential cellular structures such as lipids, proteins, and nucleic acids. Free radicals produced by oxidative stress are mainly responsible for diseases like cancer, diabetes, metabolic disorders, and cardiovascular diseases (Dokuparthi and Reddy, 2021).

Free radicals contain one or more unpaired electrons and are highly reactive, unstable and can accept or donate electrons from molecules. Hence, they behave as either reductants or oxidants. Mitochondria primarily produce reactive oxygen species, and their production depends on enzymatic and non-enzymatic reactions (Sudheer *et al*., 2021). In several diseases, the oxygen-containing free radicals that occur most frequently include the peroxy nitrite radical, nitric oxide radical, hypochlorite, singlet oxygen, hydrogen peroxide, superoxide anion radical, and hydroxyl radical. These extremely reactive species have the potential to harm crucial biological molecules include lipids, carbohydrates, proteins, and DNA found in the cell membranes and nucleus. They mainly focus on macromolecules, causing harm to cells (Mahalakshmi *et al.,* 2021; Sujatha *et al*., 2022).

Antioxidants inhibit oxidation and neutralize excess free radicals to prevent cellular damage. Antioxidants act as radical scavengers, which contain both non-enzymatic and enzymatic antioxidants (Karunakar *et al*., 2021). Low molecular weight antioxidants can engage with free radicals and halt the chain reaction before it leads to harm (Kyrou *et al*., 2007). The body produces antioxidants molecules during metabolism that can counteract the free radicals and maintain the homeostasis (Yaribeygi *et al*., 2017). The second biggest cause of mortality globally, according to the World Health Organization, is cancer. The uncontrolled proliferation of tumor-forming cells, which can be malignant or benign, is what defines cancer (Venugopal *et al*., 2019). Malignant tumours metastasize, or spread to different areas of the body. The leading causes of cancer are mutations resulting from environmental and lifestyle factors (Tracey *et al*., 2000-2013; Swati *et al*., 2020).

Herbal medicines have been used for many years, with 70% of people using herbal drugs as primary healthcare due to their fewer side effects. The use of herbal medicines is increasing day-by-day (Bakhodir *et al*., 2020). Various parts of plants are used as herbal drugs, such as seeds, leaves, stems, bark, roots, and flowers (Bushra *et al.*, 2020). Plants contain secondary metabolites which show various activities such as antimicrobial and anti-infective agents. Drugs obtained from nature include plants, microbes, marine organisms, and animals (Mehrotra *et al*., 2021).

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Araceae family member *L. punctata* is a free-floating water plant that grows rapidly. Phosphorus is a vital source for plant growth and reproduction. It is mostly used for cleaning water pollutants (Mirinda *et al*., 2020). *L. punctata* mainly consists of flavonoids, mostly c-glycosides, accumulating much starch. *L. punctata* grows rapidly, producing biomass and bioethanol while removing nitrogen and phosphorus from wastewater. It is reported to have antioxidant and anticancer activity. (Jacono *et al*., 2023).

2. Materials and Methods

2.1 Extraction and preliminary phytochemical screening

L. punctata is collected from Hyderabad, Telangana. The whole plant is collected, washed, and dried under shade. The leaves were dried, ground into a powder, subjected to a hydroalcoholic extraction with the ratio of 8:2 (water and ethanol) and fractionated with ethyl acetate and n-Butanol. Later it is subjected to drying using a rotary evaporator (Pratap *et al*., 2021). According to the established procedures, the preliminary phytochemical assessment was carried out (Harborne *et al*., 1973).

2.2 Total phenolic content

The extract's total phenolic content was assessed utilizing the Folin-Ciocalteu reagent technique. In a nutshell, 2.5 ml of 10% sodium carbonate solution and 0.5 ml of the Folin-Ciocalteu reagent were added to 0.1 ml of the extract. The blend was tested for absorbance using UV-Vis spectrophotometry at a wavelength of 725 nm after an hour of incubation at room temperature. The findings have been presented as mg of gallic acid equivalents (GAE) per gram of the dry weight of the extract employing gallic acid as the reference (Abbagoni *et al*., 2021).

2.3 Total flavonoid content

The overall flavonoid content of the extract was assessed utilizing the Dowd (aluminium chloride colorimetric) technique. 0.5 ml of the extract, 2.8 ml of distilled water and 0.1 ml of 1 M potassium acetate, 1.5 ml of 95% ethanol, 0.1 ml of 10% aluminum chloride were used in the procedure. A UV-Vis spectrophotometer was used to test the blend's absorbance at 415 nm after it had rested for 30 min at room temperature. The outcomes have been represented as mg of quercetin equivalents (QE) per gram of the extract's dry weight employing quercetin as the reference (Sushant *et al*., 2019).

2.4 Total tannin content

The total tannin concentration of the extract was calculated using the Broadhurst technique. In a nutshell, 0.4 ml sample solution was mixed with a 4% vanillin solution in MeOH (3 ml) and 1.5 ml of concentrated HCl, and left to incubate for 15 min while absorbance values at 500 nm were recorded at regular intervals. The findings were reported as mg of tannic acid equivalents (TAE) per gram of dry weight of the extract utilizing tannic acid as the reference (Broadhurst *et al*., 1978).

2.5 Antioxidant activity by DPPH assay

An antioxidant causes DPPH, a stable free radical, to change colour to purple. A 0.1 mM solution of DPPH in methanol is produced for the test, and 900 µl of the DPPH solution is combined with 100 µl of the sample solution. In the dark and at room temperature, the mixture is permitted to stand for 30 min. A UV-Vis spectrophotometer is employed to determine a solution's absorbance at 517 nm (Alam *et al*., 2013).

$$\text{DPPH scavenging activity (\%)} = [(A_{control} - A_{sample})/A_{control}] \times 100$$

2.6 No free radical scavenging assay

The Griess reagent method is employed. The samples are weighed and dissolved in methanol to obtain the desired concentration. In phosphate-buffered saline (PBS) a 1 mM solution of sodium nitroprusside (SNP) is prepared for the experiment. The mixture is then incubated at 25°C for 2 h after being combined with 900 µl of SNP solution in a volume of 100 µl. After the incubation period, the mixture is added to 100 µl of the Griess reagent, which contains 0.1% N-(1-naphthyl) ethylenediamine dihydrochloride and 1% sulfanilamide in 2.5% H_3PO_4. Finally, a UV-Vis spectrophotometer is employed for determining the mixture's absorbance at 540 nm (Marcocci *et al*., 1994). The following equation is used to determine the sample's NO scavenging capacity.

$$\text{NO scavenging activity (\%)} = [(A_{control} - A_{sample})/A_{control}] \times 100$$

2.7 Cytotoxicity assay by MTT assay

The MTT assay was employed to assess the test sample's cytotoxicity. The test sample was added to the cultured cells, *viz*., A549 (lung cancer), HepG2 (liver cancer), PC-3 (prostate cancer), HCT-116 (colorectal cancer), and MRC-5 (Human fibroblast) and was incubated for 24 h. MTT solution was added after the cells had been cultured for 24 h, and the cells were then given another 4 h of incubation. The formazan crystals were subsequently dispersed in DMSO, and their absorbance was recorded with a microplate reader at 570 nm. In order to compare the test sample's IC_{50} value to the standard Cisplatin, the IC_{50} value was computed as the concentration that may impede 50% of cell growth (Pravalika *et al*., 2021).

3. Results

3.1 Preliminary phytochemical analysis

The extracts listed in Table 1 included various types of plant metabolites, according to preliminary phytochemical examinations.

Table 1: Preliminary phytochemical analysis

Metabolites	Ethyl acetate fraction	n-Butanol fraction	Hydroalcoholic fraction
Amino acids	-	+	+
Carbohydrates	-	-	+
Proteins	-	-	-
Tannins	-	-	+
steroids	+	-	-
Terpenoids	+	+	-
Flavonoids	-	+	+
Glycosides	-	+	+
Alkaloids	-	-	-

3.2 Total phenolic content

It is possible to directly correlate phenols' ability to protect the organism from cellular stress. Using the Folin-Ciocalteu technique

and gallic acid as the reference, the total phenolic content of a methanolic extract of *L. punctata* was calculated. The absorbance measurements were plotted against various gallic acid concentrations to create a calibration curve (y = 0.0065 x + 0.0602, R^2 = 0.9904). The calibration curve's regression equation was then used to compute the extract's total phenolic content, which was then reported as mg of gallic acid equivalents (GAE) per gram of the sample's dry weight (mg/g).

The findings indicate that the hydroalcoholic fraction, with a value of 47.77 1.27 mg GAE/g, has the highest TPC. The ethyl acetate fraction has the lowest TPC, 21.13 ± 1.56 mg GAE/g, whereas the n-butanol fraction has an intermediate TPC of 33.23 ± 0.93 mg GAE/g. These findings imply that the hydroalcoholic fraction, followed by the n-butanol fraction, is the main source of phenolic chemicals in *L. punctata*. The relatively low TPC in the ethyl acetate fraction could be because this fraction selectively extracts fewer phenolic compounds listed in Table 2.

Table 2: Total phenolic content of various fractions of *L. punctata*

Ethyl acetate fraction	n-butanol fraction	Hydroalcoholic fraction
21.13 ± 1.56 mg GAE/g	33.23 ± 0.93 mg GAE/g	47.77 ± 1.27 mg GAE/g

Values are uttered as Mean ± SD, n = 3

3.3 Total flavonoid content

Flavonoids are polyphenolic compounds crucial as adaptogenic agents in helping the body cope with harsh environments. These substances also include protective qualities that can enhance human health by treating chronic illnesses. A colorimetric approach based on the Dowd technique was used to measure the flavonoid content of *L. punctata*, and the result was a value of 54 ± 0.67 of gram equivalence of quercetin at 415 nm. Using linear regression, the calibration curve was produced, and the findings were presented in three copies. The calibration curve's regression equation was then used to determine the extract's total phenolic content, which was then computed and represented as mg of quercetin equivalents per gram of the sample's dry weight (mg QE/g).

The n-butanol fraction exhibited the highest TFC, with a value of 13.03 ± 1.43 mg QE/g, followed by the hydroalcoholic fraction with a TFC of 23.73 ± 1.44 mg QE/g, while the ethyl acetate fraction produced the lowest TFC, with a value of 20.07 ± 1.66 mg QE/g, as shown in Table 3.

Table 3: Total flavonoid content of various fractions of *L. punctata*

Ethyl acetate fraction	n-butanol fraction	Hydroalcoholic fraction
20.07 ± 1.66 mg QE/g	23.73 ± 1.44 mg QE/g	13.03 ± 1.43 mg QE/g

Values are uttered as Mean ± SD, n = 3

3.4 Total tannin content

The total amount of tannin in the *L. punctata* was calculated employing the Broadhurst technique. Different tannic acid concentrations were used to create a calibration curve (y=0.0079 x - 0.0508, R^2 = 0.9931) to determine the tannin content. Following that, the amount of tannin in each fraction was calculated as mgs of tannic acid equivalents (mg TAE/g) of the sample's dry weight. The results showed that the extract fractions had varying levels of tannin content, with the ethyl acetate fraction having 21.13 ± 1.56 mg of gram equivalence of tannic acid, the n-butanol fraction having 33.23 ± 0.93 mg, and the hydroalcoholic fraction having the highest amount at 47.77 ± 1.27 mg. These findings provide valuable insights into the distribution of condensed tannins in different fractions of *L. punctata*. The hydroalcoholic fraction was found to have the highest concentration of tannins, followed by the ethyl acetate fraction and the n-butanol fraction listed in Table 4.

Table 4: Different fractions of *L. punctata* total tannin content

Hydroalcoholic fraction	n-butanol fraction	Ethyl acetate fraction
7.17 ± 0.79 mg TAE/g	13.05 ± 0.79 mg TAE/g	11.04 ± 0.91 mg TAE/g

Values are uttered as Mean ± SD, n = 3

Table 5: DPPH scavenging property of various fractions of *L. punctata*

Concentration (µg/ml)	Ascorbic acid	Ethyl acetate fraction	n-butanol fraction	Hydroalcoholic fraction
100	81.55	57.83	97.06	68.65
75	76.07	52.03	90.54	64.16
50	64.66	45.21	76.96	55.80
25	39.11	25.25	46.55	29.60
15	20.31	14.85	24.17	17.24
10	16.97	11.50	20.20	13.15
IC_{50}	**48.19**	**74.61**	**38.48**	**59.01**

3.5 DPPH assay

This test evaluated the test samples' capacity to neutralize the free radical DPPH (2,2-diphenyl-1-picrylhydrazyl). The IC_{50}values for the fractions were 74.61 µg/ml for the ethyl acetate fraction, 38.48 ìg/ml for the n-butanol fraction, and 59.01 µg/ml for the hydroalcoholic fraction. The IC_{50}values suggest that the n-butanol fraction of the test sample demonstrated the significant antioxidant activity among the fractions, with an IC_{50}value of 38.48 µg/ml, indicating that this fraction required the lowest concentration to scavenge 50% of the DPPH free radical. This finding implies that the n-butanol fraction may have compounds with strong antioxidant properties, which could have potential therapeutic applications listed in Table 5.

3.6 NO free radical scavenging assay

A prevalent method for assessing the potential antioxidant efficacy of natural products is the NO (nitric oxide) free radical scavenging test. The test samples' capacity to scavenge the NO free radical is assessed in this experiment. The test sample concentration needed to scavenge 50% of the NO free radical is known as the IC_{50} value. The IC_{50}values were 88.67 µg/ml for ascorbic acid, 135.03 µg/ml for the ethyl acetate fraction, 79.89 µg/ml for the n-butanol fraction, and 120.24 µg/ml for the hydroalcoholic fraction listed in Table 6. The highest antioxidant efficacy was exhibited the n-butanol fraction, while the ascorbic acid standard exhibited the highest activity overall in Table 6.

Table 6: NO free radical scavenging property of various fractions of *L. punctata*

Concentration (µg/ml)	Ascorbic acid	Ethyl acetate fraction	n-butanol fraction	Hydroalcoholic fraction
100	48.93	34.00	53.57	37.07
75	45.64	30.59	49.98	34.65
50	38.80	26.58	42.48	30.13
25	23.47	14.85	25.70	15.98
15	12.18	8.73	13.34	9.31
10	10.18	6.76	11.15	7.10
IC_{50}	**88.67**	**135.03**	**79.89**	**120.24**

3.7 MTT assay

The results show that the IC_{50}values for the different fractions of *L. punctata* and doxorubicin vary for the cell lines tested. For the MCF-7 cell line, the n-butanol fraction (IC_{50}= 52.38 ± 1.62 µg/ml) showed the highest cytotoxicity among the fractions tested. In contrast, the ethyl acetate fraction (IC_{50}= 84.36 ± 2.33 µg/ml) showed lower cytotoxicity than the other fractions. For the A549 cell line, the n-butanol fraction (IC_{50}= 18.34 ± 1.55 µg/ml) again showed the highest cytotoxicity and hydroalcoholic fraction (IC_{50}= 98.54 ± 1.22 µg/ml) lower cytotoxicity compared to the other fractions. For the HeLa cell line, the n-butanol fraction (IC_{50}= 20.34 ± 1.67 µg/ml) showed the highest cytotoxicity, and the hydroalcoholic fraction (IC_{50}= 135.24 ± 1.88 µg/ml) showed lower cytotoxicity compared to the other fractions. For the HepG2 cell line, the ethyl acetate fraction (IC_{50}= 85.3 ± 1.34 µg/ml) showed the highest cytotoxicity, and the hydroalcoholic fraction (IC_{50}= 173.96 ± 1.46 µg/ml) showed lower cytotoxicity. For HUVECs, all the fractions showed higher IC_{50}values compared to the cancer cell lines tested. This indicates that the fractions are less cytotoxic to the healthy cells, which is desirable for potential therapeutic applications.

The IC_{50} values indicate that the n-butanol fraction generally had the strongest cytotoxic effect on the cell lines tested, with the lowest IC_{50} values for MCF-7, A549, and HeLa cells in contrast to the other fractions. The intermediate cytotoxicity was showed by the ethyl acetate fraction, with IC_{50}values generally falling between the n-butanol and hydroalcoholic fractions. The hydroalcoholic fraction had the highest IC_{50}values for all cell lines, indicating the most negligible cytotoxicity of the three fractions. All fractions generally had higher IC_{50} values than the positive control doxorubicin, indicating lower cytotoxicity. However, the n-butanol fraction had a lower IC_{50}value for A549 cells than doxorubicin, indicating a more substantial cytotoxic effect on that cell line listed in Table 7.

Table 7: Cytotoxic potential of various fractions of *L. punctata*

Cell Type	Ethyl acetate fraction	n-butanol fraction	Hydroalcoholic fraction
MCF-7	84.36 ± 2.33 µg/ml	52.38 ± 1.62 µg/ml	120 ± 1.65 µg/ml
A549	28.34 ± 2.66 µg/ml	18.34 ± 1.55 µg/ml	98.54 ± 1.22 µg/ml
HeLa	43.52 ± 2.37 µg/ml	20.34 ± 1.67 µg/ml	135.24 ± 1.88 µg/ml
HepG2	85.3 ± 1.34 µg/ml	76.17 ± 0.97 µg/ml	173.96 ± 1.46 µg/ml
HUVECs	184.34 ± 1.28 µg/ml	157.36 ± 1.82 µg/ml	171.55 ± 1.87 µg/ml

4. Discussion

L. punctata is an aquatic plant used in traditional medicine for treating various ailments. The plant contains several secondary metabolites, like flavonoids, phenols, and tannins, known to possess beneficial health effects. Utilizing established techniques, the phytochemical profile of the plant extract was assessed. The total tannin content (TTC) was assessed by Broadhurst approach, the total flavonoid

content (TFC) was assessed by Dowd technique, the total phenolic content (TFC) was assessed by Folin-Ciocalteu technique. Employing NO and the DPPH free radical scavenging assay determined the plant extract antioxidant potential. In the different fractions of *L. punctata* the existence of tannins, flavonoids, and phenols were determined by phytochemical examination. The ethyl acetate fraction had the lowest TPC of 21.13 ± 1.56 mg GAE/g and with a TPC of 447.77 ± 1.27 mg GAE/g, the hydroalcoholic fraction exhibited the highest TPC, subsequently followed by the n-butanol fraction, which had a TPC of 33.23 ± 0.93 mg GAE/g. The ethyl acetate fraction displayed the lowest TFC of 20.07 ± 1.66 mg QE/g, while the n-butanol fraction showed the highest TFC value, *i.e.*, 13.03 ± 1.43 mg QE/g, subsequently followed by the hydroalcoholic fraction with a TFC of 23.73 ± 1.44 mg QE/g. The hydroalcoholic fraction displayed the lowest TTC of 11.04 ± 0.91 mg TAE/g, while the n-butanol fraction showed the highest TTC of 47.77 ± 1.27 mg TAE/g, subsequently followed by the ethyl acetate fraction with a TTC of 33.23 ± 0.93mg TAE/g. With IC_{50} values of 79.89 µg/ml and 38.48 ig/ml, respectively, the n-butanol fraction demonstrated the strongest antioxidant activity in the NO and DPPH free radical scavenging tests.

5. Conclusion

The findings of this investigation imply that *L. punctata* may be a source of natural antioxidants. The different fractions of the plant extract showed varying levels of phenolic, flavonoid, and tannin content and antioxidant activity. In both assays, the highest antioxidant activity was exhibited by the n-butanol fraction, indicating that this fraction may contain compounds with potent antioxidant properties, which could have potential therapeutic applications. The high levels of phenolic, flavonoid and tannin content in the hydroalcoholic fraction suggest that this fraction may also have potential health benefits. To determine and isolate the precise substances that are responsible for the antioxidant activity observed in this study, as well as to assess their potential therapeutic uses, more research is required.

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Conflict of interest

The authors declare no conflicts of interest relevant to this article.

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