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Research

Exploring the phytochemicals and in-vitro anticancer activities of *Torenia crustacea*

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Abstract
Recent advances in herbal medicine have given it a unique place within society. This is perhaps due to its less harmful effects and plant-based products' use. The plants that we now call herbal remedies were used for centuries by many communities who relied on their traditional healers. The current study examines the phytochemicals and in-vitro anticancer activities properties of the *Torenia crustacea* after it have been extracted using various solvents. The plant was collected and authenticated, followed by drying, grinding and extraction using liquids with increasing polarity. The extracts obtained were subjected to phytochemical screening and TLC. The results were interpreted and used as a basis for the in vitro antioxidant and anticancer activities. The presence of phytoconstituents was further proved by TLC analysis, during which it was seen that the acetone extract exhibited two spots on the TLC plate, and the chloroform extract exhibited seven spots on the TLC plate. Various identification tests showed the presence of flavonoids, cardiac glycosides and anthraquinone glycosides. This was used as a basis for the in vitro studies, including antioxidant and anticancer studies. The results suggest that the plant under research exhibits promising in vitro antioxidant activity compared to the standard ascorbic acid with a low IC_{50} value. The in vitro anticancer experiments also yielded good findings, indicating that this plant has the potential to be used as a strong anticancer agent.

Keywords Herbal medicine · *Torenia crustacean* · Phytochemicals · Antioxidant and anticancer activities

Abbreviations

TLC	Thin-layer chromatography
ROS	Reactive oxygen species
NO	Nitric dioxide
HPLC	High performance liquid chromatography
MTT	3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide

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1 Introduction

Phytochemicals are becoming increasingly important in pharmacological studies [1]. Different phytochemicals have been shown to suppress a variety of human cancers in vitro and intravenously by inducing cell death and autophagy, arresting the progression of cell cycle, smothering cell movement and invasion, and activating a resistance response. The term oxidative stress describes an imbalance in the production of reactive metabolites and free radicals, also known as reactive oxygen species (ROS) or oxidants [2]. This results from a lack between their formation and removal through defensive mechanisms known as antioxidants. The mitochondrial respiratory system is responsible for most of the ROS produced in the cells. In the mitochondrial membranes, electron transport chains allow electron transfer from the respiratory chain to molecular O_2. The mitochondrial respiratory system can produce nitric dioxide (NO) under hypoxic conditions, which may lead to additional reactive nitrogen species [3]. Altering proteins and lipids can increase the chance of mutation. Over time, ROS is produced by prolonged environmental stress. This causes severe cell damage and dysfunction, leading to somatic mutations or neoplastic changes. In fact, oxidative stresses have been linked to the onset of cancer and its development. This is because they cause DNA damage or mutations, cell proliferation, and genomic instability. The first phase of inflammation occurs when the immune system is activated. Cancer development is influenced by chronic inflammation, which has been linked to many processes, such as cellular transformations, proliferation, invasion, and angiogenesis [4]. Cancer has at least three phases: Promotion, Progression, and Start. Oxidative stress influences all three stages of the cancer process. ROS can cause DNA damage at the initial stage of the cancer process by inducing gene mutations or structural changes. Finally, oxidative stresses may also contribute to cancer development by inducing further DNA mutations in the cell population. Flavonoids also have a dual function on ROS homeostasis—they serve as antioxidants under normal conditions and as powerful pro-oxidants in cancer cells, activating apoptotic pathways while suppressing pro-inflammatory signaling pathways *Torenia crustacea (L.) Cham. and Schltdl.* is a common medicinal herb in India, Indonesia, and Malaysia [5]. Even though India's Northeastern state possesses a diverse medicinal flora, many species have yet to be adequately explored [6–8]. Many new drug specialists find natural medicines beneficial for a broad range of therapeutic reasons [9, 10]. Many people are moving their focus from modern medications [11, 12] to natural treatments as a result of the adverse effects of cutting-edge pharmaceuticals [13]. A novel approach to analyzing plants for conventional claims is being developed in the search for bioactive components in natural [14] flora. *Torenia crustacea (L.) Cham. and Schltdl.* is one such plant. This plant belongs to the *Linderniaceae* family. *Torenia crustacean (L.) Cham. and Schltdl.* is an efficient ethnomedicinal plant used worldwide for millennia. It is one of Indonesia and Malaysia's most extensively used medicinal plants. Traditionally, this herb is used for earaches, bruises, fevers, thrush, and anti-inflammatory skin disorders [15] like itching, boils, ulcers, dysentery, and ringworm in postpartum women [16]. The current research is based on the extraction and phytochemical analysis of *Torenia crustacea (L.) Cham. and Schltdl.* and investigate by in-vitro anticancer investigation employing cell lines based on the antioxidant assessment results. The study aims to determine the phytochemical profile of *Torenia crustacea (L.) Cham. and Schltdl.* and it's in-vitro pharmacological assessment through antioxidant and anticancer activities.

2 Materials and methods

2.1 Materials

Petroleum ether, toluene, chloroform, acetone, and hydroalcohol were obtained from Merck Specialties and Loba Chemicals, respectively. All of the solvents and all chemicals were of analytic grade. *Torenia crustacean (L.) Cham. & Schltdl.* plants were used in bulk amount. HCT116 Cell line was obtained from National Centre for Cell Science (NCCS), Pune, India.

2.2 Methods

2.2.1 Preparation of plant extract

Torenia crab plant material was cleaned with tap water to remove any dirt or other organic contaminants. The plant material was washed with deionized distilled water twice and dried in a tray dryer at 45 °C. The plants were dried with our hands after drying because cold maceration was used as the extraction method. Then, successive extractions were performed using solvents that ranged from non-polar up to polar. Solvents were used to extract *Torenia crustacean (L.) Cham. & Schltdl.* [17], based on the literature review [18]. The solvents used for extraction

were non-polar, polar and a combination of both. The solvents that were used were toluene, petroleum ether, acetone, chloroform and hydro alcohol. Cold maceration was the extraction method used in this study. We chose this method because we did not know what the chemical composition of the plants was. If any active thermolabile chemical component in the plant is present, then it could be denatured. To avoid this, cold maceration is used for the extraction process. The fractions were extracted over 7 days and then dried in order to eliminate excess solvent. The extracts underwent phytochemical [19] screening and were screened for % yield.

2.2.2 Phytochemical screening techniques

To increase the solubility of all extracts in the solvents used for the screening, they were converted to powder [20]. The results of several screening tests were used to determine the presence of bioactive compounds [21]. Specific identifying agents were used to partially identify components. The results of the spraying agents were then used as a basis for TLC.

2.2.2.1 Detection for carbohydrates *Molisch's test* Add 2–3 ml extract to a solution of alpha-naphthol in alcohol, stir, then add a few drops. From the sides of the test tubes, H_2SO_4. A violet ring appears at the junction of two liquids.

Fehling's test One minute is spent boiling 1 ml Fehling's A solution and 1 ml Fehling's B. We then add the same amount of test solutions and heat them for 5–10 min in boiling water. The precipitation appears yellow initially and then turns brick red.

Iodine test Combining a 3 ml test solution with a few drops of diluted iodine is a good way to check the colour. The solution changes to blue when it is boiled and then returns to normal once it has cooled [22].

2.2.2.2 Detection for steroids *Salkowski reaction:* Shake well. Mix 2 ml extract with 2 ml chloroform, 2 ml concentrated $H_2 SO_4$. The acid layer is greenish-yellow, while the chloroform layer is red.

2.2.2.3 Detection for glycosides *Legal's test:* Add 1 ml of pyridine to 1 ml of sodium nitroprusside to an aqueous extract or alcohol. The colour changes from pink to red.

Keller-Killiani test: Add glacial acetic acid, one drop of 5 percent $FeCl_3$, and conc. H_2SO_4 to 2 ml of extract. The junction of the two liquid layers appears reddish brown, while the top layers seem blue-green.

Bontrager's test Add dilute H_2SO_4 to the 3 ml extract. Boil and filter. The filtrate is then added to an equal amount of chloroform or benzene. Separate the organic solvent by shaking vigorously. Ammonia is then added. This layer is then colour pink or red.

Foam test Shake the dry powder or drug extract vigorously with water. The foam is stable and continuous.

Haemolytic test One drop of blood is mixed with the dry powder or drug extract on a slide. It is possible to see the haemolytic area [23].

2.2.2.4 Detection for flavonoids *Shinoda test* Then, add 5 ml of 95 percent ethanol and a few drops of concentrated HCl. Add 0.5 g magnesium powder or turn it into an extract. The colours range from pink, orange, purple, and red.

Sulphuric acid test The addition of sulphuric (66–80%) acid dissolves flavones into a yellowish solution. Chalcones, aurones, and flavones all produce the same colour: red to blue [23].

2.2.2.5 Detection for tannins and phenolic compounds *5% $FeCl_3$ solution* The colour deep blue-black is produced.

Lead acetate solution The precipitation is white.

Bromine water Bromine water decolourization.

2.2.2.6 Detection for alkaloids *Dragendorff's test* Add a few drops to 2- or 3-ml filtrate. A precipitate of brown-orange colour is produced.

Wagner's test Wagner's Reagent is added to 2–3 ml extract filtrate, resulting in a brownish-red precipitate.

Mayer's test For precipitation, combine 2–3 ml extract filtrate with Mayer's Reagent [23].

2.2.3 Cell line studies

Cell lines are cultures that have existed for some time. They will multiply forever if they receive enough fresh medium and room [24]. Cell culture involves the removal of cells from animals or plants and their growth in a controlled atmosphere. The HCT116, a colorectal carcinoma cell line (obtained from National Centre for Cell Science (NCCS), Pune, India) [25], was used for in-vitro study. The mutation is in codon 13 of the proto-oncogene ras. Cells are epithelial and adherent.

2.2.3.1 MTT assay The MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide] assay is a colorimetric assay for assessing cell viability. The assay is based upon the ability of NADPH-dependent cellular enzymes, oxidoreductase, to reduce tetrazolium (MTT) to its insoluble formazan form (Fig. 1). An MTT test was used to investigate the cytotoxic activity of plant extracts [26]. The assay measures the mitochondrial dehydrogenase's reduction of MTT into a violet formazan, which is indicative of mitochondrial function. Plant extracts were dissolved into 10 ml DMSO at a concentration of 300 µg/ml Cells are placed in the 96 well plates at least 24 h prior to the test. MTT in PBS is then added the next day, and these wells are incubated at 37 °C. Plant extracts at concentrations of 120,150, 180,210,240,270,300 µg/ml respectively were used to test the time after 72 h of incubation and dose responsiveness of cells (as shown in Table 2). The reduced MTT concentration was measured by measuring the absorbance of 570 nm using a microplate readout [27]. By comparing the optical densities of treated and untreated cells, the cytotoxic effect of the extract was determined. The cytotoxicity of the extract relative to control cells was determined using this formula: % Cytotoxicity = [(Ac-At)/Ac)] × 100. The mean absorbances of control and test wells are Ac and At, respectively.

3 Results and discussion

3.1 Plant extract materials

The percentage yield value of plant extract in petroleum ether extract was found to be 1.77%, and the percentage yield value in toluene, chloroform, acetone, and hydroalcoholic was found to be 1.07%, 1.91%, 2.24%, and 2.68%, respectively. The hydroalcoholic solvent yielded more extract (2.68%), and toluene yielded the lowest percentage of extract (1.07%). The order of % yield from increasing to decreasing order is shown below: Hydroalcoholic > Acetone > Chloroform > Pet roleum ether > Toluene.

3.2 Phytochemical screening techniques

The preliminary phytochemical screening exhibited that the carbohydrates, tannins, and phenols were found in the petroleum ether extract. Toluene extracts exhibited glycosides, tannins, and phenolic compounds. Carbohydrates, glycosides, and flavonoids were found in the chloroform extract. The acetone extract contained glycosides, flavonoids, and phenolics. Carbohydrates and tannins were found in the hydroalcoholic extract (All are demonstrated in Table 1). Alkaloids, coumarins and resins do not appear to exist. Except petroleum ether extract, the presence of flavonoids was

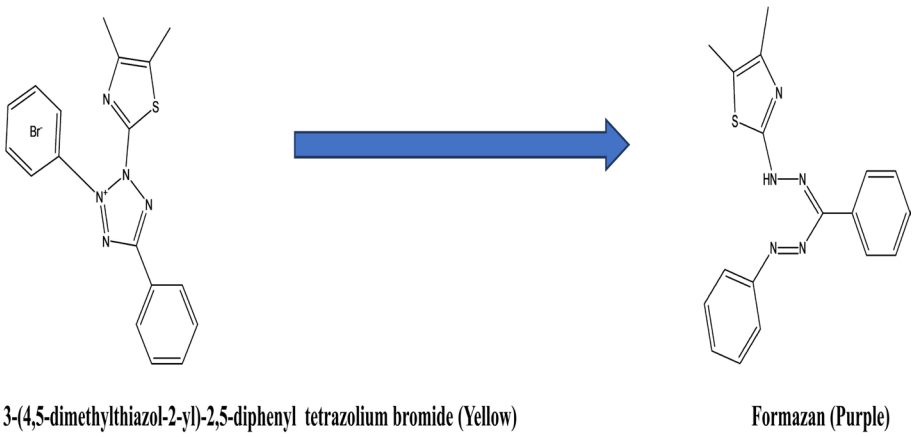


Fig. 1 Schematic diagram of MTT assay

Table 1 Phytochemical screening result for different extracts

Sl. No	Name of the test	Results					Effective substances
		Petroleum Ether extract	Toluene Extract	Chloroform Extract	Acetone Extract	Hydro-alcoholic Extract	
1	Molisch's test (for carbohydrates)	(+)ve	(−)ve	(+)ve	(−)ve	(+)ve	Positive for all except Toluene and Acetone Extract
2	Fehling's test (for reducing sugars)	(−)ve	(−)ve	(+)ve	(+)ve	(+)ve	Positive for all except Toluene and Petroleum Ether Extract
3	Iodine test (for non-reducing polysaccharides)	(+)ve	(−)ve	(−)ve	(−)ve	(−)ve	Positive for only petroleum Ether Extract
4	Salkowski reaction (for steroid)	(−)ve	(−)ve	(+)ve	(+)ve	(−)ve	Positive for only chloroform and Acetone Extract
5	Legal's test (for cardiac glycoside—cardienolides)	(−)ve	(−)ve	(−)ve	(−)ve	(−)ve	Not positive for any
6	Keller-killiani test (for cardiac glycoside—deoxysugars)	(−)ve	(−)ve	(−)ve	(+)ve	(−)ve	Positive for only Acetone extract
7	Borntrager's test (for anthraquinone glycosides)	(−)ve	(−)ve	(+)ve	(−)ve	(−)ve	Positive for only chloroform Extract
8	Foam test (for saponin glycosides)	(−)ve	(+)ve	(+)ve	(+)ve	(−)ve	Positive for all except Petroleum Ether and Hydroalcoholic Extract
9	Shinoda test (for flavonoids)	(−)ve	(−)ve	(+)ve	(+)ve	(−)ve	Positive for Chloroform and Acetone Extract
10	Sulphuric acid test (for flavonoids)	(−)ve	(+)ve	(+)ve	(+)ve	(+)ve	Positive for all except Petroleum Ether Extract
11	5% $FeCl_3$ solution (for tannins and phenols)	(−)ve	(+)ve	(+)ve	(+)ve	(+)ve	Positive for all except petroleum Ether Extract
12	Bromine water (for tannins and phenols)	(+)ve	(−)ve	(−)ve	(−)ve	(−)ve	Positive for Petroleum ether Extract
13	Lead acetate solution (for tannins and phenols)	(+)ve	(+)ve	(−)ve	(+)ve	(−)ve	Positive for all except Chloroform and Hydroalcoholic Extract
14	Dragendorff's test (for alkaloids)	(+)ve	(−)ve	(−)ve	(−)ve	(+)ve	Positive for all Petroleum Ether and Hydroalcoholic Extract
15	Wagner's test (for alkaloids)	(−)ve	(−)ve	(+)ve	(−)ve	(−)ve	Positive for Chloroform Extract
16	Mayer's test (for alkaloids)	(−)ve	(−)ve	(−)ve	(−)ve	(−)ve	Not positive for any

Table 2 Cell cytotoxicity results

Control	Absorbance at 570 nm			% Cell viability		
	1.538	1.52	1.371	100	100	100
120 µg/ml	1.269	1.242	0.921	82.50975	81.71053	67.17724
150 µg/ml	0.794	0.801	0.832	51.62549	52.69737	60.68563
180 µg/ml	0.744	0.468	0.701	48.37451	30.78947	51.13056
210 µg/ml	0.589	0.567	0.672	38.29649	37.30263	49.01532
240 µg/ml	0.298	0.297	0.399	19.37581	19.53947	29.10284
270 µg/ml	0.201	0.192	0.195	13.06892	12.63158	14.22319
300 µg/ml	0.209	0.185	0.208	13.58908	12.17105	15.17141

Fig. 2 Percentage cell viability

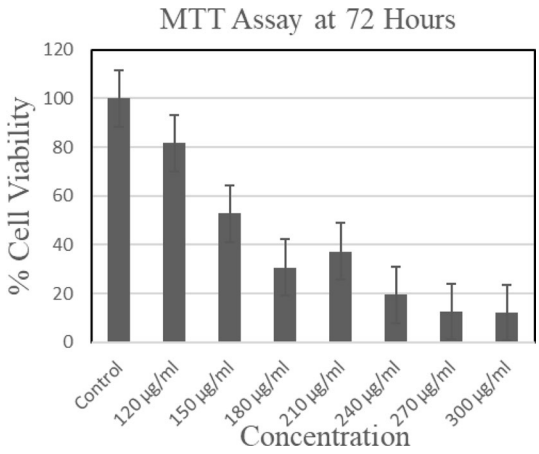
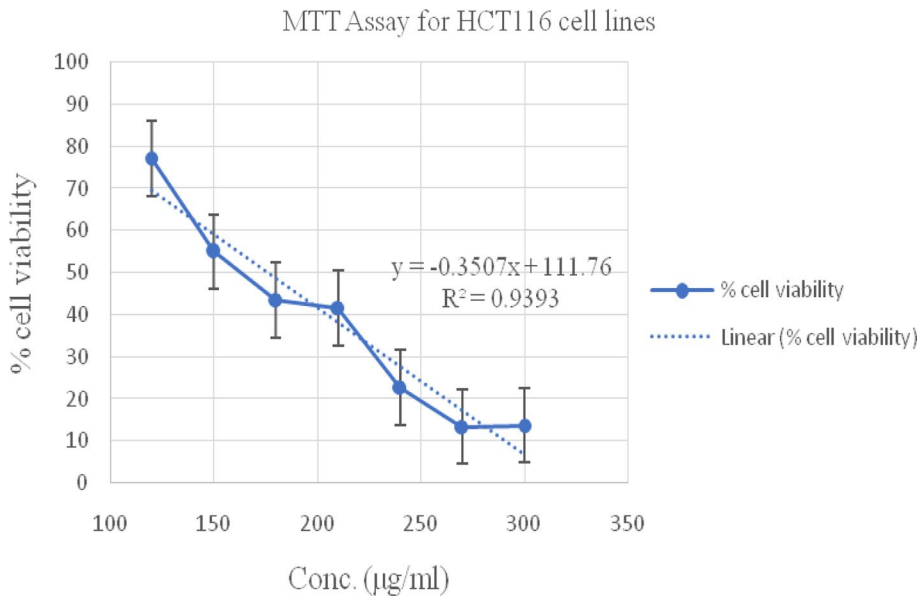


Fig. 3 Percentage cell viability vs concentration (µg/ml) graph. * Each point denoting the average values of triplicates of % cell viability



detected during preliminary phytochemical screening which was directed for the evaluation of anticancer activity. Although phytochemical screening only provides an initial indication of the qualitative nature of active components found in plants.

3.3 Cell line studies

Cell line studies were done by using HCT116 cells. Cells seeded per well for the experiment were 5000. Cell cytotoxicity results are described in Table 2, and the percentage of cell viability and rate of cell viability vs concentration graph are described in Figs. 2, 3, respectively. At a maximum concentration of 270 µg/ml, the chloroform extract of *Torenia crustacea* (L.) Cham. & Schltdl demonstrated considerable anticancer activity against the HCT116 cell line. The IC50 was found to be 176.105 µg/ml. This suggests that the plant may contain a variety of secondary metabolites that are responsible for the anticancer effect. This opens up the prospect of isolating the biologically active components responsible for anticancer action from the chloroform extract of *Torenia crustacea* (L.) Cham. &Schltdl and commercializing the element as a viable option for anticancer drugs in the future.

4 Conclusion

Comparing impacts of plant *Torenia crustacea* (L.) extracts of five different solvents tested in this research and presence of different phytochemicals was confirmed. Chloroform extract was further showed invitro anti-cancer activity in MTT Assay. % Cell Viability got reduced in dose dependent manner (120 µg/ml–300 µg/ml). The IC50 was found to be 176.105 µg/ml. Further work should be carried out for the determination of the types of phytochemicals responsible for the chemical interaction which affects in anticancer activity.

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Author contributions D.N. Done methodology, investigation and writing-original draft. S.R.C.D. Done supervision, conceptualization and formal analysis. S.G: Writing—review and editing and data curation. TD: Supervision, conceptualization and methodology. S.C. Done conceptualization, investigation, writing—review and editing. R.M. Done data curation, conceptualization and writing—review and editing. All authors reviewed the manuscript.

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Data availability Data is provided within the manuscript. Any additional data could be available from the corresponding author upon request.

Declarations

Ethics approval and consent to participate *Torenia crustacean (L.) Cham. & Schltdl.* plants were used with national guideline in bulk from the Maligaon area (26.158664800483873, 91.70942620431127) in Guwahati, Assam, India. The plant was then authenticated and identified as *Torenia crustacea (L.) Cham. & Schltdl.* by a Taxonomist at the Department of Botany, Guwahati University, Guwahati, Assam-781014, India with authentication letter no. Herb. /GUBH/2021/012 dated 07/12/2021. HCT116 Cell line was obtained from National Centre for Cell Science (NCCS), Pune, India.

Consent for publication Not applicable.

Competing interests The authors declare no competing interests.

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