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Original Article

IMMUNOMODULATORY, ANTICANCER AND ANTIOXIDANT ACTIVITIES OF *CYCLEA PELTATA* (LAM.) HOOK. F. AND THOMSON

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ABSTRACT

Objective: The present study was performed to evaluate the immunomodulatory, anticancer, and antioxidative properties of the fraction (CP_2) isolated from *Cyclea peltata*.

Methods: Immunomodulation was evaluated in lymphocytes by lymphocyte proliferation assay and in THP-1 macrophage cell lines by MTT assay. The nitrite production by the macrophages was also measured by the nitrite assay using griess reagent. The anticancer activity of the fraction was determined by MTT assay. The antioxidant activity was evaluated by DPPH assay and total antioxidant assay by phosphomolybdenum method. It is expressed as number of gram equivalent of ascorbic acid.

Results: The plant fraction showed the presence of flavonoids which induced lymphocyte proliferation rate of 4.29±0.007 at 100 µg/ml. It was not toxic to THP-1 macrophage cells and also could induce nitrite production at 1 mg/ml. It also exhibited good anticancer activity at 100 µg/ml after 48h of incubation. The DPPH activity was found to be low since 100 µg/ml showed only an inhibition rate of 22±0.026. The total antioxidant activity at 1000 µg/ml of CP_2 was found to be equivalent to 79±0.03 µg/ml of ascorbic acid exhibiting moderate antioxidant activity.

Conclusion: The fraction CP_2 containing flavonoid, isolated from *Cyclea peltata* has good immunomodulatory, antioxidant and anticancerous property.

Keywords: Immunomodulation, Anticancer, *Cyclea peltata* (Lam.) Hook. f. and Thomson, Antioxidant, Lymphocyte, Macrophage

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INTRODUCTION

A number of currently available drugs have been developed from medicinal plants making them a regular source of medicine. Plants act as a source of a wide variety of secondary metabolites with potent pharmacological activity [1]. Plant-based drugs are popular due to their limited side effects and low cost. Mostly available chemotherapeutic drugs in the market have immunosuppressive effects along with a variety of side effects [2]. Immunomodulators derived from plants are gaining attention as they can overcome the side effects exerted by synthetic drugs [3]. Herbal immunostimulators are effective as nutrient supplement providing resistance as well as acting as immune strengtheners in immunocompromised conditions [4]. Phytochemicals modulate the immune system in different ways by acting on the different mechanisms of immune system [5].

Cancer is one of the leading causes of death worldwide. Reports from WHO states that death due to cancer are predicted to rise to 13.1 million by 2030 [6]. Among cancers, stomach cancer causes about 800,000 deaths worldwide per year. Eating salty, spicy and pickled food, excessive alcohol consumption, and infection by *Helicobacter pylori* and smoking may be associated with stomach cancer [7]. Unfortunately in more than 90% of cases, the disease would have reached an advanced stage by the time it is diagnosed. Hence, there is an urgent need to characterize these cancers through identifying potential biomarkers and novel therapeutic targets [8]. Nature serves as a never-ending source of anticancer drugs and almost 60% of this are from plants, marine organisms and microorganisms [9]. The oxidative damage caused by free radicals to biomolecules has been implicated in diseases like cancer, diabetes, arthritis, and neurodegenerative disorders. Occasionally, the natural antioxidant mechanisms fail to overcome the damage caused by free radicals, making antioxidant supplements necessary [10].

Cyclea peltata (Lam.) Hook. f. and Thomson (Family: Menispermaceae) has a wide distribution in India. The National Medicinal Plant Board of India has termed this plant as "medicinal plant species in high trade sourced from tropical forests." It is a known blood purifier and IQ enhancer [11]. *Cyclea peltata* roots have been reported to have antioxidant, antihyperlipidemic, antilithiasis and hepatoprotective activity. The leaves of the plant have antioxidant and antidiuretic activity [12]. The crude extract of this plant has been proven to have antimicrobial activity [13]. Their roots have been reported to contain alkaloids like fangchinoline, tetrandrine, cycleapeltine, cycleacurine, cycleadrine and cycleanorine [14]. Our study aims to isolate new biomolecules with immunomodulatory, anticancer and antioxidant properties from the plant.

MATERIALS AND METHODS

Chemicals

Cell-culture media was purchased from Invitrogen and all other reagents from Himedia. All chemicals and solvents used were of analytical grade.

Collection and preparation of plant sample

Cyclea peltata (Lam.) Hook. f. and Thomson (CP) was collected from different locations in Kannur District, Kerala and taxonomically identified from Dept. of Botany, Govt. Brennen College, Thalassery, Kannur. The plant was deposited in their herbarium with voucher number 3952. Only the leaves of the plant were taken for the study. It was washed with tap water, rinsed with distilled water and blotted gently between the folds of filter paper and shade dried. The dried plant material was powdered using mixer grinder and stored in an airtight container.

Extraction and isolation

Plant powder was subjected to successive soxhlet extraction using hexane, chloroform, and ethyl acetate and methanol-based on the increasing order of polarity for 24 h each. The ethyl acetate fraction, which exhibited the highest immunomodulatory property, was further subjected to successive column chromatography using silica gel of 60-120 mesh size and followed by 200-400 mesh size respectively. Elution was carried out using solvents of increasing polarity starting from n-hexane followed by ethyl acetate, methanol and water. The fractions

were collected and pooled based on their immunomodulatory activity and TLC pattern. The fractions were then dried and stored. The percentage yield of the fractions was determined.

Phytochemical screening

The fraction was screened for the presence of phytochemicals including phenols, flavonoids, tannins, saponins, alkaloids, glycosides, terpenoids and phytosterols by using standard protocols [15].

HPTLC analysis

5 µl of the sample was applied on the TLC Silica gel 60 F_{254} aluminium plate using Camag Linomat automatic sample applicator using microsyringe. The sample was loaded onto the plate and was separated in one dimension using mobile solvent ethyl acetate: methanol: water (5:1:5 v/v/v) at room temperature. After the development of the plate, it was air-dried and visualized using Camag TLC Visualizer.

Analytical HPLC analysis

For HPLC, Agilent 1200 High-Pressure Liquid Chromatographic system equipped with prep pump, a Rheodyne injector, Diode Array Detector in combination with Chem32 was used. The injection volume of sample was 20 µl with a flow rate of 1.0 ml/min using the methanol: water solvent system in the ratio 20:80. The running time was for 10 min.

In vitro lymphocyte proliferation assay

The immunomodulatory activity was checked on isolated human lymphocytes from blood [16]. Fresh human blood samples collected were layered on equal volumes of Hi-Sep LSM solution and centrifuged at 800 x g for 25 min at 18˚C. The thin white middle lymphocyte layer was collected and washed with RPMI-1640 media twice and centrifuged at 100 x g for 10 min at 18˚C. After discarding the supernatant, the cells were suspended in 10% RPMI-1640. The cell number was counted with a hemocytometer. Cells were seeded at a concentration of 20,000 cells/well in a 96-well plate and 100 µl of each extract at various concentrations (1-1000 µg/ml) was added to the wells. The plates were then incubated for 72 h in a CO_2 incubator. After incubation, 20µl MTT (5000µg/ml) was added to each well and incubation was continued for an additional 4 h. The insoluble formazan crystals formed were solubilized by the addition of 100µl MTT lysis buffer (SDS, dimethylformamide and distilled water) followed by an incubation of 2 h and the absorbance measured at 570 nm using a Biorad iMark microplate reader. The proliferation rate (PR) was calculated.

$$\text{Proliferation Rate} =< \text{Absorbance of test} \div \text{Absorbance of control} >\times 100$$

$$\text{Proliferation Index} = \text{Proliferation Rate} \div 100$$

Macrophage assays

THP-1 cells were obtained as a gift from Centre for DNA Fingerprinting and Diagnostics, Hyderabad. It was cultured in RPMI 1640 medium supplemented with 10% FBS, Penicillin (100U/ml), Streptomycin (100µg/ml) and Amphotericin B (250µg/ml) in a humidified atmosphere at 37°C with 5% CO_2. The cell viability was determined using Trypan blue. The cells were differentiated into macrophages using 0.1 µg/ml PMA. 4 x 10^4cells were seeded in 96 well plates containing complete medium with PMA. After 72 h, the medium was removed and washed with prewarmed PBS. 1-1000 µg/ml of CP_2 extract was added to the plates. MTT assay was performed 24 and 48 h after the addition of extract, as mentioned in the protocol above.

Nitrite assay

The nitrite present in the supernatant of the treated cells was assayed by the Griess reaction. An equal volume of supernatant and Griess reagent was mixed. It was covered and subjected to shaking for 10 min. The absorbance was measured at 550 nm on Bio-Rad iMark microplate reader. 1 to 10µg/ml sodium nitrite was used as the standard [17].

Anticancer activity by MTT assay

The ant cancerous assay was carried out using HCT-116 human colon carcinoma cells maintained as monolayer cultures in RPMI medium supplemented with 10% Fetal Calf Serum, Penicillin G (100 U/ml), Streptomycin (100 µg/ml) and Gentamycin (50 µg/ml). Briefly, 1×10^4 cells ml^{-1} was seeded in each well of a96 well plate and incubated overnight at 37 °C in a humidified 5% CO_2/95% air atmosphere. When the cells in the 96 well plates were 80% confluent, the medium was removed from each well and the extracts at different concentrations (1-1000 µg/ml) were added. The plates were then kept for 24, 48 and 72 h of incubation. After incubation, the culture medium was discarded followed by the addition of 100µl of MTT solution. MTT treatment of live cells produced a dark blue formazan product, which was solubilized by the addition of 100µl of SDS lysis buffer after 4 h of incubation. Absorbance was measured after 2 h of incubation at 570 nm [18]. The Inhibitory Rate (Ir) was calculated from the equation:

$$\text{Inhibition Rate} = 100 - \text{Proliferation Rate}$$

DPPH radical scavenging assay

DPPH (1, 1-Diphenyl-2-picrylhydrazyl) is a stable free radical used for quantification of natural antioxidants [19]. Due to the presence of a free-electron it gives a strong absorbance at 517 nm. On accepting hydrogen from a corresponding donor, the solution loses the characteristic deep purple colour and the discoloration was proportional to the concentration and scavenging activity of the compound. The reaction mixture contained 2.8 ml of 100µM DPPH dissolved in methanol and various concentrations (1-1000µg/ml) of compounds. This mixture was incubated at room temperature for 30 min. After shaking, absorbance was measured at 517 nm. The percentage of DPPH scavenging activity was calculated. Ascorbic acid served as positive control.

Total antioxidant assay (TAA)

The total antioxidant assay is based on the reduction of molybdenum (VI) to molybdenum (V) to form a green phosphate complex [20]. Briefly, 0.3 ml of different extracts ranging from 1-1000 µg/ml concentration was mixed with 3 ml of reagent solution (0.6 M sulfuric acid, 28 mM sodium phosphate and 4 mM ammonium molybdate). Reaction mixture was incubated at 95˚ C for 90 min underwater bath. Absorbance at 695 nm was measured after cooling to room temperature. Ascorbic acid was used as the standard.

TAA is expressed as the number of equivalents of ascorbic acid.

Statistical analysis

Data were expressed as means±standard deviations (SD) of the triplicate values. The significance of the data was analyzed by one-way analysis of variance (ANOVA) using SPSS Statistics 20 software. The P value<0.05 was considered statistically significant.

RESULTS

The ethyl acetate extract (crude) of *Cyclea peltata* recorded the highest immunomodulatory potential and was selected for column chromatography. The active fraction obtained from 60-120 mesh size silica using ethyl acetate: methanol (2:1 to 1:5) was further subjected to column chromatography using silica gel of 200-400 mesh size. A yellow active water-soluble fraction CP_2 (Ethyl acetate: Methanol– 1:4) was obtained. The percentage yield of the fraction was 0.48%.

Phytochemical screening

Phytochemical screening of CP_2 confirmed the presence of flavonoids. Results for all the other phytochemicals including alkaloids, terpenoids, steroids, polysaccharides, proteins, tannins, saponins, and glycosides were negative.

HPTLC analysis

Following HPTLC analysis, the fraction was visualized at 254 nm. Two compounds were visible with Rf values of 0.67 and 0.40 (fig. 1) thus confirming the presence of two compounds in the fraction.

HPLC analysis

After HPLC analysis, the fraction indicated the presence of two peaks. Peak 1 was obtained at retention time 1.662 with a peak area of 38.331% and peak 2 was obtained at retention time 3.010 with a peak area of 61.668% (fig. 2). The HPLC analysis further confirms the presence of two compounds in the fraction.

Fig. 1: HPTLC profile of CP_2 at 254 nm

***In vitro* lymphocyte proliferation assay**

Different concentrations of CP_2 ranging from 1µg/ml-1000µg/ml were used for performing the *in vitro* lymphocyte proliferation assay. A concentration-dependent increase in proliferation was seen. PHA (1-10µg/ml) was used as positive standard. The proliferation index of 100 µg/ml of fraction is 4.29±0.007. 1000 µg/ml of CP_2 induced four times more proliferation when compared to 100 µg/ml of the fraction (fig. 3). No toxicity to the cells was observed. 1 µg/ml of CP_2 was found equivalent to 1 µg/ml of PHA. So the fraction has shown to have very good proliferative capacity which can be made use in the case of immunosuppressed patients.

Macrophage viability assay

The viability of macrophages upon treatment with CP_2 was checked using MTT assay. The fraction was nontoxic to macrophages even up to a concentration of 1000µg/ml. This proves that the fraction did not inhibit normal cells and can be effectively developed into a potent drug upon purification.

Nitrite assay

The concentration of the stable nitrite in the supernatant of the macrophages was assessed using Griess reaction. Sodium nitrate (0.1-0.75 µg/ml) was used as a positive standard. The amount of nitrite generated was quantified from the calibration curve of sodium nitrate.24 h after addition, 1 µg/ml concentration of the sample did not induce nitrite production. 1000 µg/ml of CP_2 induced 0.617±0.01 µg/ml of nitrite concentration after 48h incubation (fig. 4). This indicates the role of CP_2 in activating macrophages and leading to the generation of nitrites in the treated cells.

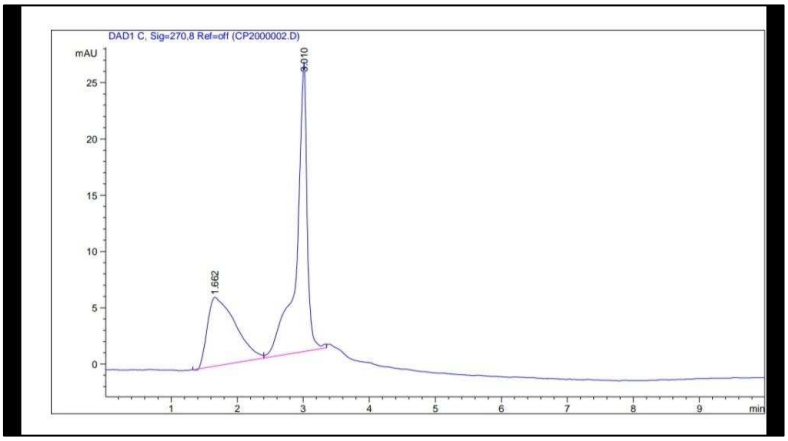

Fig. 2: HPLC profile of CP_2

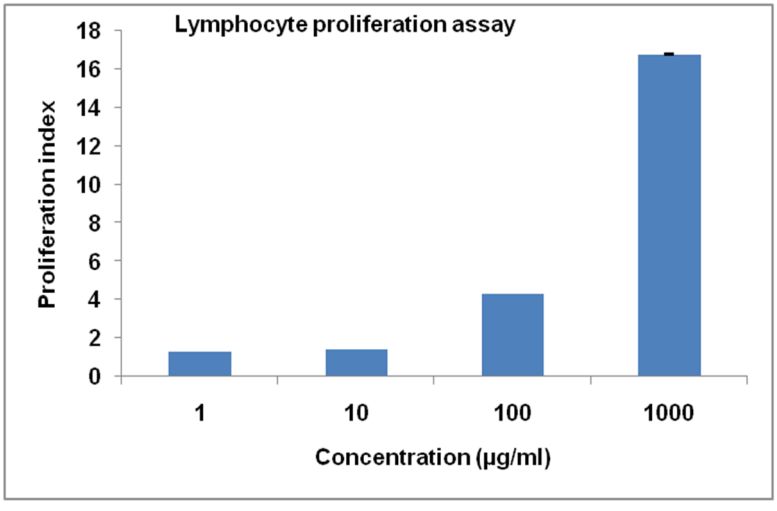

Fig. 3: Lymphocyte proliferation assay of CP_2 (n = 3; Data expressed as mean±SD; p<0.05)

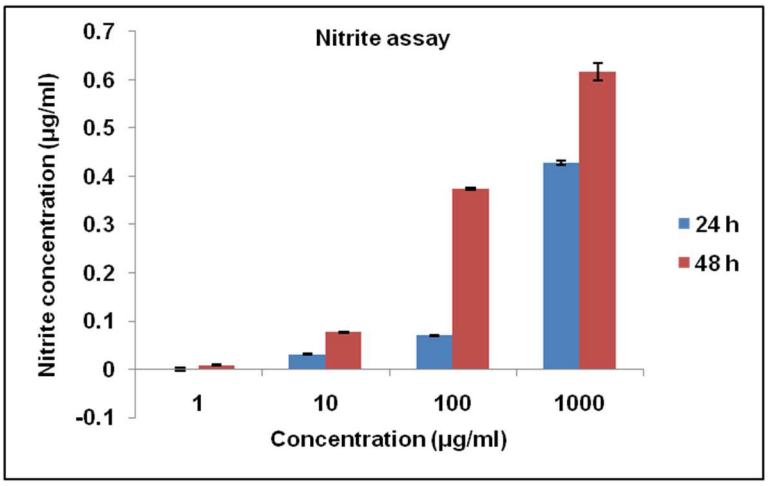

Fig. 4: Nitrite assay of macrophages at various concentrations (n = 3, data expressed as mean±SD; p<0.05)

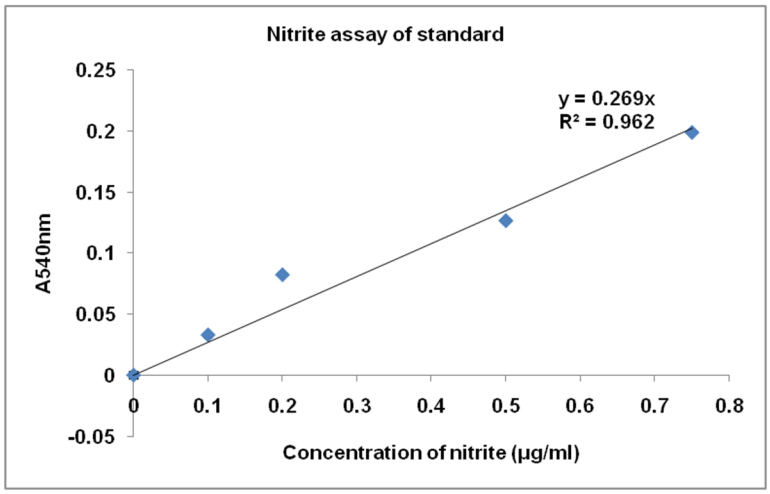

Fig. 5: Nitrite assay of standard (n = 3; data expressed as mean±SD)

Anticancer activity by MTT assay

The anticancer activity of *Cyclea peltata* was evaluated in human colon carcinoma cell line HCT-116 for 24, 48 and 72 h of incubation. There was an increasing inhibition rate with increasing concentration and time. At 1 µg/ml of fraction, no inhibition was seen at 24 and 48 h of incubation. Maximum inhibition of 75±0.0025 % was obtained after 72 h of incubation. The IC_{50} value was obtained at 800 µg/ml, 100 µg/ml and 50 µg/ml after 24, 48 and 72 h of incubation making the fraction a possible drug candidate against stomach cancers (fig. 6).

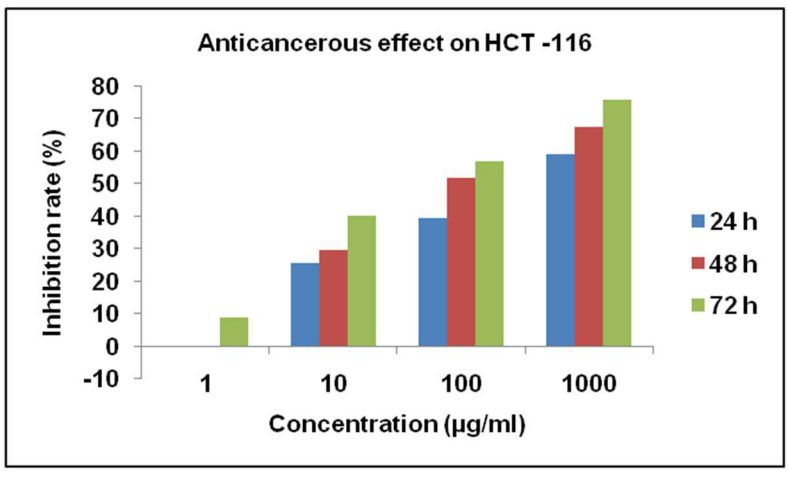

Fig. 6: Anticancer activity of CP_2 at different concentrations (n = 3, data expressed as mean±SD; p<0.05)

DPPH radical scavenging assay

DPPH assay evaluated the free radical scavenging capacity of CP_2. Ascorbic acid was used as standard. A range of 1-1000 µg/ml of the sample was used for the assay. The DPPH scavenging potential of the fraction was low. Even 1000 µg/ml of the fraction couldn't attain an IC_{50} value. The 1 mg/ml of the fraction was found to be equivalent to 0.064±0.007 mg/ml of ascorbic acid (fig. 7 and 8).

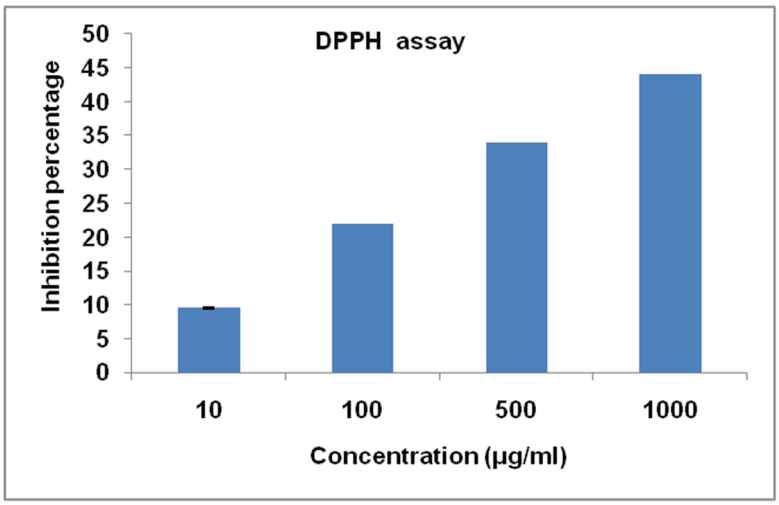

Fig. 7: DPPH scavenging activity of CP_2 at different concentrations (n = 3, data expressed as mean±SD; p<0.05)

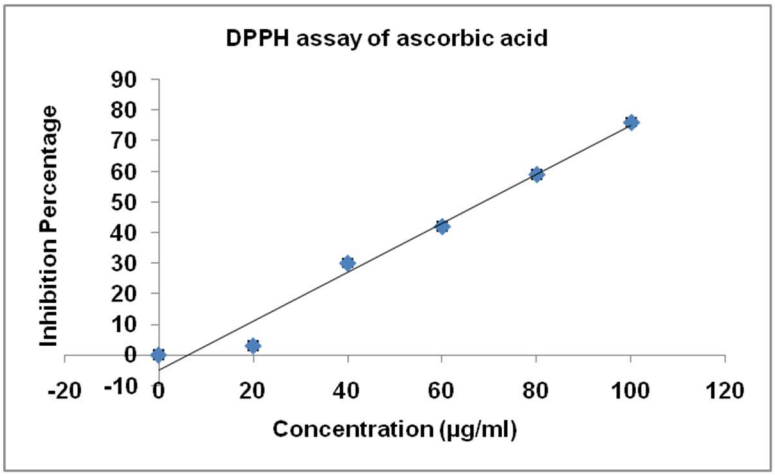

Fig. 8: DPPH scavenging activity of ascorbic acid at different concentration (n = 3, data expressed as mean±SD)

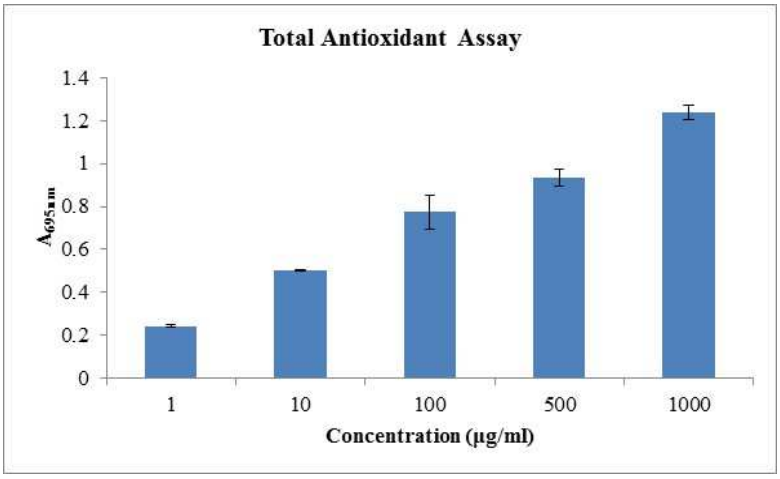

Fig. 9: Total antioxidant capacity of CP_2 at various concentration (n = 3, data expressed as mean±SD p<0.05)

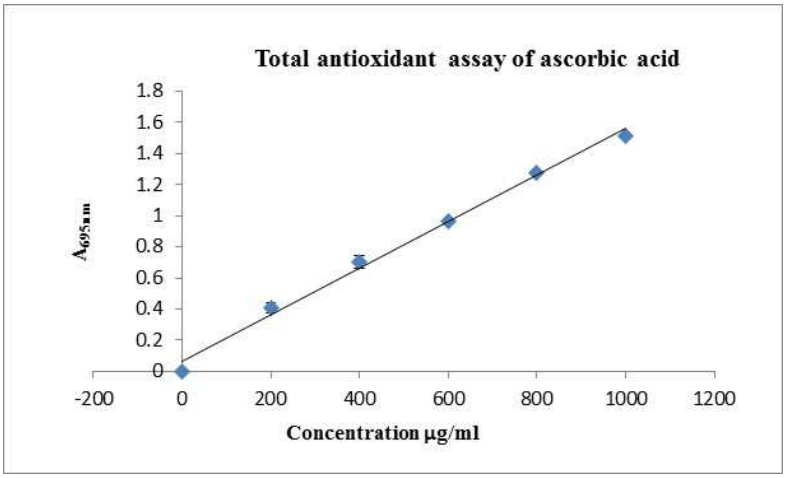

Fig. 10: Total antioxidant activity of ascorbic acid at various concentrations (n = 3, data expressed as mean±SD)

Total antioxidant assay

The phosphomolybdenum method is based on the reduction of molybdenum (VI) to molybdenum (V) by CP_2 extract. A concentration range of 1-1000 µg/ml of the fraction was used for the assay. Ascorbic acid was used as standard. 1 mg/ml concentration of CP_2 fraction was equivalent to 79±0.03 µg/ml of ascorbic acid (fig. 9 and 10).

Even though the CP_2 fraction did not show significant DPPH scavenging activity, it's capacity to reduce Mo (VI) to Mo (V) indicates the ability to scavenge free radicals, increased levels of which are the cause of various diseases. Hence, this fraction can be further purified, identified and developed into an active drug molecule for immunity-related diseases.

DISCUSSION

Secondary metabolites play an essential role in human health and drug discovery based on plant metabolites is increasing day by day [21]. A large number of medicinal plants have already been exploited for the pursuit of drug discovery. But still a large number of plants which are used in Ayurveda need scientific validation [22]. An attempt to evaluate the immunomodulatory, anticancer and antioxidant property of *Cyclea peltata* has been taken up in this study. We could isolate a fraction CP_2 with potent immunomodulatory, anticancer and antioxidant properties. It is a water-soluble flavonoid. Presence of flavonoids in the crude extract has been previously reported but only alkaloids have been isolated from *Cyclea peltata* till date [23]. Our immune system strictly controls and prevents various infections and diseases from affecting our body. Among the cells of the immune system, the monocytes play an unavoidable role in the innate immune response by migrating to infection sites and differentiating into macrophages and activating various cytokines initiating adaptive immune response [24]. CP_2 was also capable of proliferating lymphocytes, which are the major cells involved in adaptive immunity, effectively at100 µg/ml. Even at 1000 µg/ml, the fraction didn't show any toxicity; instead it stimulated lymphocytes to proliferate four times than compared to100 µg/ml. This proves that the fraction is capable of activating cell-mediated immunity [25]. NO plays a crucial role in the host defence mechanism. Low doses of NO have been proven to protect cells from apoptosis and also can induce the expression of cytokines like IL-1, IL-6, IL-8 Tumor Necrosis Factor (TNF) and IL-12 [26]. So CP_2 can help in regulating the immune mechanism by stimulating macrophages. Further purification of the compound can help in the identification and development of a lead drug molecule for the use by immune-compromised individuals.

Intervention of natural phenolic compounds has been reported at all the stages of cancer. They exert their effect by acting on DNA repair mechanisms, inhibition of cell cycle, downregulation of MMP's, induction of apoptosis and cell cycle arrest [27]. Phytocompounds like vinblastine, vincristine, topotecan and irinotechan are few examples of clinically used anticancer drugs [28, 29]. Its effect on HCT-116 colon cancer cell line proves it to be a good anticancer drug. 50 µg/ml of CP_2 with 72 h of incubation, showed an inhibition rate of 75%. So the combined action of two compounds in the fraction has a profound influence on their anticancer activity. Their purified form may be having more effective outcome on the cancer cell line even at low concentration and time. Antioxidants based drug formulations are used for the treatment of diseases like cancer, atherosclerosis, diabetes and stroke [30, 31]. CP_2 have exhibited total antioxidant activity, but DPPH activity was comparatively low. So they are capable of scavenging free radicals and thereby protecting from diseases. The further purification and characterization of the fraction into single compound will help in developing a drug with potent anticancer and immunomodulatory drug.

CONCLUSION

The current study evaluates the immunomodulatory, anticancer and antioxidant activity of a single fraction of *Cyclea peltata*. A yellow active fraction was isolated using Ethyl acetate: methanol (1:4). The fraction gave a good yield and is water-soluble. The HPTLC and HPLC analysis of the sample indicates the presence of two compounds in the fraction. Phytochemical analysis showed the presence of flavonoids. The fraction exhibits immunomodulatory potential since it stimulates the proliferation of lymphocytes and macrophages. The nitrite generation in macrophages is also indication of activation of macrophages. Even though the CP_2 fraction did not show significant DPPH scavenging activity, the capacity to reduce Mo (VI) to Mo (V) indicates the ability to scavenge free radicals which are the cause of various diseases. Hence, this fraction can be further purified, identified and developed into an active drug molecule for immunity-related diseases.

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AUTHORS CONTRIBUTIONS

Both the authors have contributed equally

CONFLICTS OF INTERESTS

The authors declare that there is no conflict of interest

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