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Total Phenols, Flavonoids And Sterols Of *Phlebodium Aureum* (L.) J. Smith And *Oeosporangium Viride* (Forrsk.) Fraser-Jenk. & Pariyar Gametophytes And Sporophytes Ethanolic Extracts And Their Antioxidant And Toxicity

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Abstract

Objective: The present study aimed to reveal the phenols, flavonoids, and sterols quantities of *Phlebodium aureum* (L.) J. Smith and *Oeosporangium viride* (Forrsk.) Fraser-Jenk. & Pariyar gametophyte and sporophyte ethanolic extracts and their antioxidant and cytotoxicity. **Methods:** Quantitative estimation of *P. aureum* and *O. viride* gametophyte and sporophyte ethanolic extracts was performed using the standard procedure. The scavenging properties of *P. aureum* and *O. viride* gametophyte and sporophyte ethanolic extracts were determined using DPPH. Cytotoxicity of *P. aureum* and *O. viride* gametophyte and sporophyte ethanolic extracts was determined using Brine Shrimp Lethal Bioassay. **Results:** Quantitative analysis confirmed the presence of phenol, flavonoid, and sterol in the gametophytes and sporophytes of *P. aureum* and *O. viride*. The phenol, flavonoid, and sterol content was highest in the gametophytes of the studied ferns. The highest antioxidant activity can be observed in the gametophytes of *P. aureum ethanolic* extracts with an IC_{50} value of 131.23 µg/ mL and followed by the sporophytes of *P. aureum ethanolic* extracts with an IC_{50} value of 724.63 µg/ mL.

Keywords: Phenols, Flavonoids, Sterols, Gametophytes, Sporophytes, Antioxidant, Toxicity

INTRODUCTION

In recent years, the plant kingdom has become the treasure of potential drugs with the consciousness of medicinal plants. There are fewer consequences for drugs from plants as they are accessible, safe, and efficient. By the world health organization WHO, 8o% of people rely on traditional medicine for primary health issues (Mir *et al*., 2013). For the synthesis of complex

chemical substances and their use in medicine, understanding chemical constituents is important (Parekh and Chandra, 2008). Health supplements have witnessed a sharp increase in the market due to the anxiousness about human health and eco-friendly lifestyle. Synthetic drugs have been reported for their side effects even if they show immediate efficacy (Branen, 1975). Therefore, increasing the inclination for natural drugs (Nakatani, 1992) after facing many stochastic changes, pteridophytes have adapted to the environmental changes (Wallace *et al.*, 1991). Hence, ferns are predicted to have various secondary metabolites. Also, metabolites are unique only to ferns (Wallace *et al*., 1991; Zeng-fu et al., 2008). The second largest groups of vascular cryptogams are Pteridophytes (ferns and fern allies) which consist of 12,000 species of which 1106 species are recorded in India (Alex and Johnson, 2021) and 351 in Western Ghats (Benniamin and Sundari, 2020). A plant during its life span produces various phytoactive compounds as secondary metabolites for its growth and survival. Identification and characterization of these active principles can be used in generating a species-specific fingerprint (McChesney *et al*., 2007). Chandra *et al*., (2017) revealed the phytochemical profile of *Adiantum latifolium, Angiopteris evecta*, and *Marattia fraxinea.* Vidyarani *et al.,* (2023) studied the functional groups through FTIR analysis in the gametophyte and sporophyte stages of *Phlebodium aureum*. Manickam et al (2003) optimized the *in vitro* propagation protocol for *Cheilanthes viridis*. Johnson (2003) optimized the in vitro propagation protocol for *Phlebodium aureum* using in vitro spore culture. Johnson and Manickam (2007) studied the influence of sucrose on the morphogenetic developments of *C. viridis* and *P. aureum*. Johnson and Manickam (2010) observed the influence of age, season, and pH on the developmental stages of *C. viridis* and *P. aureum*. Johnson and Manickam (2009, 2010) studied the influence of plant growth regulators on gametophytes of *P. aureum* and *C. viridis*. Johnson and Manickam (2012) studied the organogenic development of *C. viridis*. Johnson et al. (2020) revealed the toxicity of aqueous extracts and silver nanoparticles of *P. aureum*. To identify the chemical variation between gametophyte and sporophyte of *Asplenium ceterach*, phytochemical characterization, and antioxidant potential was employed (Suzana et al., 2017) Antioxidant potential of *Selaginella* ethanolic extracts was reported by Sivaraman et al (2013) Johnson et al., (2020) noted the scavenging potential and antioxidant properties of *Sphaerostephanos unitus*. Manivannan et al. (2022) determined the antioxidant potentials of *Tectaria paradoxa* and *Bolbitis appendiculata*. Janakiraman and Johnson (2016) reported the cytotoxic properties of three tree ferns in South India. Johnson *et al*. (2014) recorded the cytotoxic potential of *Asplenium aethiopicum*. But there is no report on the phytoprofiles, antioxidant, or cytotoxic activities of *Phlebodium aureum* (L.) J. Smith and *Oeosporangium viride* (Forrsk.) Fraser-Jenk. & Pariyar gametophytes and sporophytes ethanolic extracts. In the present study, an attempt is made to reveal the quantitative profile of secondary metabolites, their antioxidant potentials of the gametophyte and sporophyte extracts of *Phlebodium aureum* (L.) J. Smith and *Oeosporangium viride* (Forrsk.) Fraser-Jenk. & Pariyar ethanolic extracts through DPPH assays and cytotoxic activity.

MATERIALS AND METHODS

Sample preparation

One gram of *in vitro* cultured gametophyte and sporophyte of *Phlebodium aureum* and *Oeosporangium viride* (Fig. 1) was taken with 60 ml of ethanol and soaked for 72 h in a shaker at

40℃. The extract was filtered through Whatman No. 1 filter paper. The filtered extracts are stored in tightly sealed bottles at 7℃ and further employed for the analysis (Murugan and Parimelazhagan, 2014)

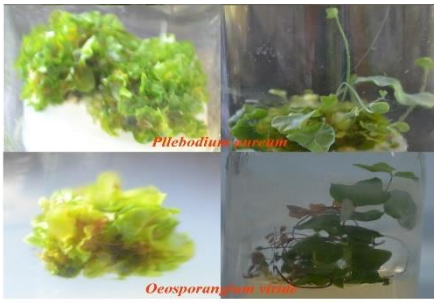

Fig 1: Gametophyte and Sporophyte of *Phlebodium aureum* and *Oeosporangium viride*

Total phenolics

The total phenolics content of *Phlebodium aureum* and *Oeosporangium viride* gametophytes and sporophytes ethanolic extract was determined according to the method described by Sidduraju and Becker, (2003). 100 µg of *Phlebodium aureum* and *Oeosporangium viride* gametophytes and sporophytes ethanolic extract was taken in the test tube and dissolved in 100 µl of ethanol and made up to 1ml by using distilled water. Then 500 µl of Folin - ciocalteu reagent (1:1 with dist. H_2O) was added and incubated for 5 min. 2.5 ml of sodium carbonate (20%) was added sequentially in each test tube. All the test tubes were vortexed well and incubated in the dark for 40 min and the absorbance was recorded at 725 nm against blank. The analysis was performed in triplicates and the results are expressed as mg GAE/g

Total flavonoids

The total flavonoid content of *Phlebodium aureum* and *Oeosporangium viride* gametophytes and sporophytes ethanolic extract was quantified using the method described by Zhishen *et al.* (1999) 100 µg of *Phlebodium aureum* and *Oeosporangium viride* gametophytes and sporophytes ethanolic extract was taken in the test tubes dissolved in 100 µl of ethanol and made up to 2 ml by using distilled water. Then 150 µl of 5% $NaNO_2$ was added to all the test tubes and then the mixtures were incubated for 6 min at room temperature. After incubation, 150 µl of 10% $AlCl_3$ was added to all the test tubes including the blank. Then 2 ml of 4% NaOH was added and made up to 5 ml with distilled water. All the test tubes were vortexed well and they were allowed at room temperature for 15 min. The pink color was developed. The absorbance of the mixtures was measured at 510 nm. The analysis was performed in triplicates and the results are expressed as mg QE/g.

Total sterols

The total sterols content of *Phlebodium aureum* and *Oeosporangium viride* gametophytes and sporophytes ethanolic extract was determined using a modified Liebermann - Burchard colorimetric assay (2015) 100 µg of *Phlebodium aureum* and *Oeosporangium viride* gametophytes and sporophytes ethanolic extract was taken in the test tubes dissolved in 100 µl of ethanol and made up to 1 ml using distilled water and 2 ml of acetic anhydride (1.25 ml of Con.

H_2SO_4 to 50 ml of acetic anhydride reagent) were added. The mixture was stirred for 1 min and incubated at room temperature (26 °C) for 13 minutes. The absorbance of the mixture was measured at 650 nm.

DPPH radical scavenging activity

The antioxidant activity was determined in terms of hydrogen donating or radical scavenging ability using the stable radical DPPH (1958). Ethanolic extracts of *Phlebodium aureum* and *Oeosporangium viride* gametophytes and sporophytes with various concentration (125, 250, 500, 750, and 1000 µg/ml) was added to 5 ml of 0.1 mM ethanolic solution of DPPH and allowed to stand for 20 min at 27°C. The absorbance of the sample was measured at 517 nm. Ascorbic acid was used as standard and DPPH solution without extract was served as negative control. Radical scavenging activity was expressed as the inhibition percentage of free radical by the sample and was calculated using the formula:

% of Inhibition = [(Control OD - Sample OD) / Control OD × 100].

Toxicity

Hatching of Brine shrimp

Artificial seawater (38 gm NaCl/1000ml tap water) was taken in a small tank and shrimp eggs were allowed to grow for 48 h to hatch and mature as nauplii. During this period constant O_2 supply, temperature (37^{o}C), and light supply were maintained. The hatched shrimps were taken for bioassay.

Ethanolic extracts of *P. aureum* and *O. viride* gametophytes and sporophytes were taken in different concentrations viz., 10, 25, 50, 75, and 100 µg/ml. Every experiment was repeated thrice and 5 tubes were kept as control (each concentration). With the help of a Pasteur pipette, 15 living shrimp's nauplii were dropped into each test tube (Mclaughlin et al., 1998)

Preparation of control group

The control group was added for cytotoxic activity to validate the test method and the results obtained due to the cytotoxic activity of the test agents. In this case, only 50 µl of acetone was added to control tubes containing 5 ml of mother solution and 15 shrimp nauplii were added. Plant extracts were not added to the control solution. If the brine shrimp in these tubes show a rapid mortality rate, then the test was considered as invalid as the nauplii died due to some reason other than the cytotoxicity of the studied plant extracts.

Counting of nauplii

After 24 h the tubes were inspected using a magnifying glass and the number of survived nauplii in each vial was counted.

The quantification of metabolites, the average and standard deviation of the metabolites was measured using MS EXCEL 2007, and the IC_{50} of the extracts was calculated using Microsoft Excel 2007. The correlation and significance between the gametophytes and sporophytes ethanolic extracts metabolites concentration and scavenging activities was determined using SPSS 20.0. The LC_{50}, 95 % confidence limit was calculated for each extract using SPSS 20.0.

RESULTS

Quantitative phytochemical analysis

The ethanolic extracts of gametophytes and sporophytes *P.aureum* and *O. viride* showed varied amounts of metabolites (Fig. 2). The gametophytes possessed more amount studied metabolites than the sporophytes (Fig. 2). The phenol content was highest in the gametophytes of *Oeosporangium viride* followed by *Phlebodium aureum.* The highest amount of flavonoids was observed in the gametophytes of *Phlebodium aureum* ethanolic extracts next to that *of Oeosporangium viride*. In the total sterols, *O. viride* gametophytes ethanolic extracts showed the best results.

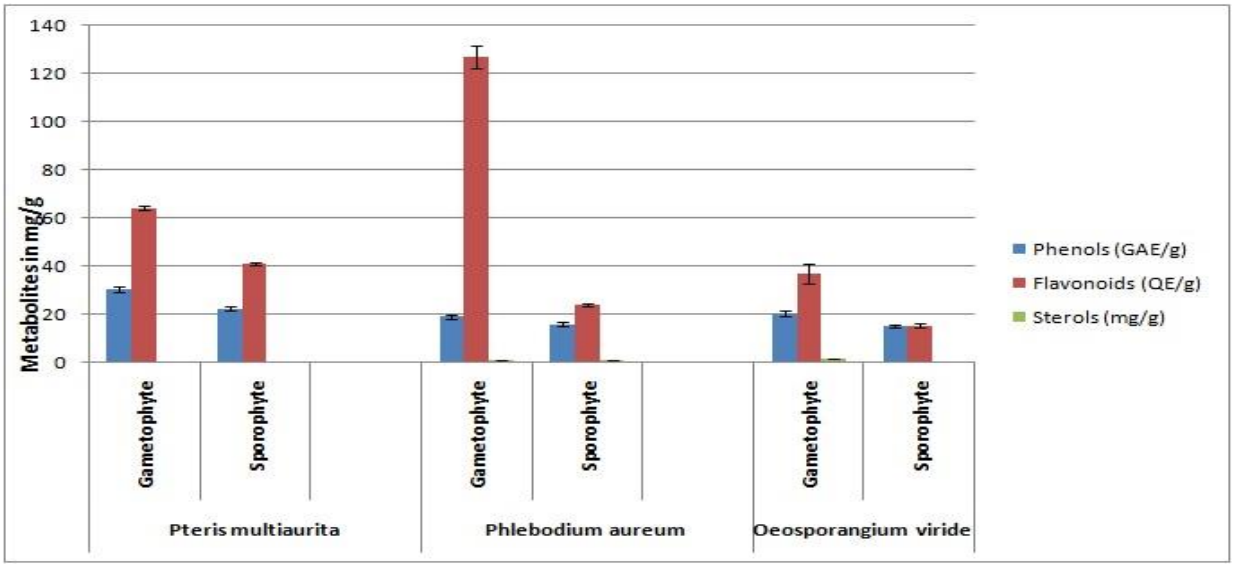

Fig. 2: Total Phenolics, Flavonoids, and Sterols of *Phebodium aureum and O. viride* Gametophytes and Sporophytes Ethanolic Extract

IC_{50} is the concentration of an antioxidant at which 50% inhibition of free radical activity is observed. The lower the IC_{50} values, the greater the overall effectiveness of the antioxidant activity. The free radical scavenging activity of *Phlebodium aureum* and *Oeosporangium viride* gametophytes and sporophytes ethanolic extracts were depicted in Fig. 3. DPPH is a stable free radical with a characteristic color of purple at the absorption 517 nm. They lose their color when they react with anti-oxidants. A dose-dependent scavenging activity was observed in the gametophytes and sporophytes of *Phlebodium aureum* and *Oeosporangium viride* ethanolic extract. The highest antioxidant activity can be observed in the gametophytes studied ferns than sporophytes (Fig. 2).

A positive correlation was observed between the metabolites concentration and scavenging activities of *Phlebodium aureum* and *Oeosporangium viride* gametophytes ethanolic extract with r = 0.963 and 0.982 respectively. The correlation is significant at the 0.01 level (2-tailed). A positive correlation was observed between the metabolites concentration and scavenging activities of *Phlebodium aureum* and *Oeosporangium viride* sporophytes ethanolic extract with r=0.971 and 0.984 respectively. The correlation is significant at the 0.01 level (2-tailed).

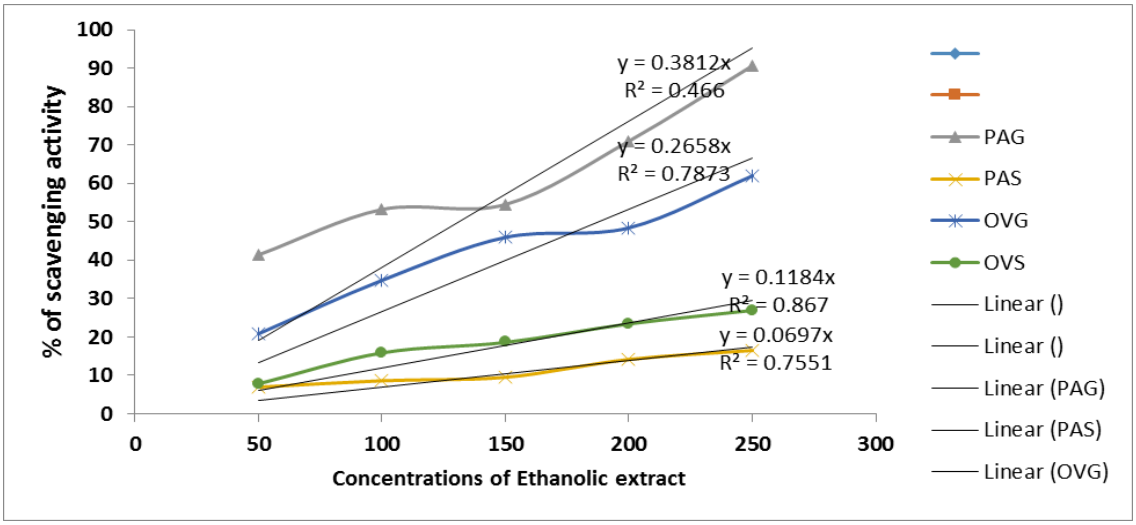

Fig. 3: DPPH Scavenging Activity of *P. aureum and O.viride* Gametophytes and Sporophytes Ethanolic Extract

The observed results of Brine Shrimps Lethality BioAssay (BSLB) suggest that ethanolic extracts of *Phlebodium aureum* and *Oeosporangium viride* gametophytes and sporophytes are more effective against the brine shrimps nauplii. A dose-dependent activity was observed for the gametophytes and sporophytes ethanolic extracts of *Phlebodium aureum* and *Oeosporangium viride. Phlebodium aureum* gametophytes and sporophytes ethanolic extract displayed toxicity with the LC_{50} values 122.03 µg/mL and 346.92 µg/mL respectively. A positive correlation was observed between the gametophytes and sporophytes metabolites concentration and their toxicity with r = 0.985 and 0.965 respectively. The correlation is significant at 0.01 level (2-tailed). The studied *Oeosporangium viride* gametophytes and sporophytes ethanolic extract displayed toxicity with the LC_{50} values 179.59 µg/mL and 263.54 µg/mL respectively. A positive correlation was observed between the gametophytes and sporophytes metabolites concentration and their toxicity with r = 0.997 and 0.992 respectively. The correlation is significant at 0.01 level (2-tailed).

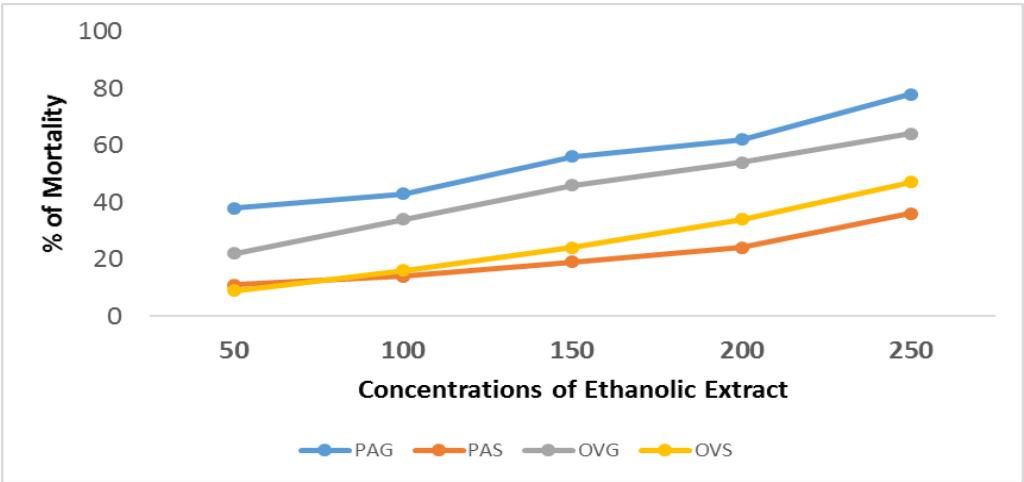

Fig. 4: Toxicity of Gametophytes and Sporophytes Ethanolic Extract of *P. aureum* and *O. viride*

DISCUSSION

The quantitative estimation of flavonoids confirmed the existence of flavonoids in the gametophytes and sporophytes of *Phlebodium aureum* and *Oeosporangium viride* and revealed that gametophytes possess more concentration than sporophytes. Flavonoids play an important role in natural remedies and protect the oxidative cell damage (Okwu and Josaiah, 2006). Flavonoids possess anti-inflammatory, anti-allergic, and anti-microbial properties (Amaraj et al., 2009). In the present study, flavonoids were found more amounts in gametophytes and revealed that they can protect gametophytes from predation. Flavonoids have antioxidant activity also (Jadhav et al., 2019). The scavenging activity observation also supported the previous observation and confirmed the role of flavonoids in oxidative cell damage. Phenols possess antioxidant, anti-inflammatory, anti-clotting, and wound-healing activity (Rieverc et al., 2009). The present study observation also supplemented and validated the previous reports and the phenols present the highest amount in the gametophytes of *Phlebodium aureum* and *Oeosporangium viride* displayed the highest scavenging and toxicity activity. Sterols are one of the sub-groups of triterpenoids (Sukumaran et al., 2012). They play an important role in herbal medicine and nutrients. Among the two stages tested, gametophytes of *P. aureum* and *O. viride* ethanolic extract showed the highest frequency of scavenging and toxicity activities compared to sporophytes. The display of the highest scavenging and toxicity activities may be due to the existence of the highest amount of metabolites. The correlation values obtained for the metabolites concentration and scavenging and toxic activities confirmed the relationship between the concentration of metabolites and scavenging activities. Jayanta et al (2017) evaluated the antioxidant activity of *Diplazium esculentum* and *Marsilea minuta* using a DPPH assay. The DPPH assay was greater in *Diplazium* than in *Marsilea* which may be due to the presence of phenol compounds. Similar observations were obtained in the present study also. The gametophytes possess more amount metabolites than sporophytes, these may be due to independent generation and to protect from the biotic and abiotic factors.

CONCLUSION

The quantitative analysis validated the occurrence of phenol, flavonoid, and sterol more amount in the gametophytes of *Phlebodium aureum* and *Oeosporangium viride* and showed more scavenging and toxicity activities, which indicates that the gametophytes of *Phlebodium aureum* and *Oeosporangium viride* have rich medicinal properties. Further studies may bring out active principles with antioxidant properties from *Phlebodium aureum* and *Oeosporangium viride*.

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