Synergistic Effect in Antimicrobial Activity of Microscopic Epidermal Glands of Two Thelypteroid Ferns from South India

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Abstract

Pathogenic diseases are widespread across the globe. Due to the emergence of new resistant pathogenic strains, as well as the formation of side effects by the continuous use of commercial antibiotics, there is a pressing need to identify new antimicrobial agents from natural resources. Several reports are available on the antimicrobial effects of whole plants or specific macroscopic parts of the plants. However, several microscopic parts are well recognized as containing potential antimicrobial agents, which in turn, are accountable for the antimicrobial activity of the entire plant or some part of it. Interestingly, very limited studies are available on the antimicrobial activity of any microscopic part of the plant. In the interim, several studies are present on the antimicrobial activity of an individual plant or part. But studies dealing with the synergistic effect of different species are very rare. The current study demonstrates the outstanding antimicrobial activity of the microscopic epidermal glands present in the leaves of two primitive terrestrial vascular plants, Thelypteris parasitica (L.) Fosberg and Cyclosorus interruptus (Willd.) H. Ito (Thelypteridaceae: Pteridophyta), and their significant synergistic effects.

Keywords: Epidermal glands; Ferns; Antimicrobial activity; Synergistic effect

Introduction

In the world today, innumerable previously unknown diseases and disorders continuously arise in response to the emergence of new and resistant pathogenic strains, produced by the increased environmental levels of various chemical pollutants, as well as the complete change in lifestyle patterns. The search for new drugs is actively and continuously on, throughout the world. Modern processes of drug discovery are most often based on the detection and characterization of the principal bioactive chemical agent from a particular natural source. But, such a newly-discovered single bioactive agent may not prove useful in the treatment of a particular disease, due to its single target action and its harmful side effects. An effective drug should be able to easily reach the target site and efficiently perform its curative function, avoiding any type of side effect. As a single agent cannot possibly accomplish all these functions, a mixture of agents performing different functions will prove to be a more valuable drug. This principle is adopted only by certain traditional medicinal systems like Ayurveda and Chinese Traditional medicines, which normally include more than two ingredients for their synergistic effect [1,2]. In the future, drug development using natural products will not necessarily rely solely on the discovery and analysis of new structures from the extremely rich biodiversity available in nature, but can systematically explore combinatory drug regimens [2].

Ferns and fern-allies, together termed Pteridophytes, are primitive, terrestrial, seedless vascular plants with several well-developed adaptive mechanisms to cope with both the physical and biological factors in the new terrestrial environment. Most terrestrial vascular plants contain several bioactive chemical compounds present throughout the whole plant, whereas some plants synthesize and store several bioactive compounds in the secretory glandular epidermal trichomes present in the leaves. Thus, the pure extracts of such epidermal glands may reveal more effective bioactivity when compared with the extracts from the whole plant or from a specific macroscopic part of the plant, like the leaf, stem, root and seed. Although several reports proving the antimicrobial activities of the whole plant or aerial parts of the pteridophytes are available [3–6], only a few reports on the antimicrobial activities of the microscopic epidermal glands of ferns are present [7,8]. An antibacterial study on the macroscopic and microscopic parts of the sporophyte and the in vitro cultured gametophyte of the fern Cyclosorus interruptus (Willd.) H. Ito has revealed the maximum antibacterial effect of the sterile leaves, in which the epidermal glands play a key role [9]. There are also a few reports on the antimicrobial activity in the fern gametophytes [3,9]. In the interim, studies on the synergistic effect related to the antimicrobial activity of plant extracts from various species are very rare and all of them deal with angiosperms [10,11]. A considerable number of studies are available, focusing on the synergistic effect of plant extracts with commercial antibiotics [10–15]. In several cases an enhanced effect of the antibiotic is reported when combined with the plant extract. Thus, the combination of extracts from different plants or with commercial antibiotics has been demonstrated to augment the antimicrobial efficacy. The present study aims at proving the presence of antimicrobial agents in the microscopic epidermal glands of the two ferns Thelypteris parasitica (L.) Fosberg, and Cyclosorus interruptus (Willd.) H. Ito, and their synergistic effects in antimicrobial activity, including a phytochemical study.

Materials and Methods

Plant specimens were collected from their natural habitats. Two morphotypes, both the glandular and eglandular forms were found in Thelypteris parasitica (L.) Fosberg (=Christella parasitica) and the plants of the glandular form were collected from the Upper Kodayar Range

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dried leaves were separated from the plant. Vouchers (XCH 23364, XCH 23365) are stored in St. Xavier’s College Herbarium, Palayamkottai, India (Figures 1E and 1F). The aerial parts of the plant specimens were shade dried and the dried leaves were separated from the plant.

Extract preparation

The glandular extract was prepared by adopting the procedure of Irudayaraj [16] who confirmed that solvent acetone was the best, after performing solubility tests using various solvents. The saturated acetone extract of the epidermal glands was prepared by soaking the dried leaves in acetone. The crude acetone extract was then filtered and concentrated. From the dried extract, 0.1% solution was prepared using acetone for further antimicrobial studies.

Antimicrobial studies

Bacterial samples (Escherichia coli-ATCC25922, and Staphylococcus albus–ATCC23) were procured from Scudder Microbiological Laboratory, Nagercoil, Kanyakumari district, Tamil Nadu, and the sample of Candida albicans (MTCC227) was obtained from the Entomology Research Institute, Loyola College, Chennai, and Tamil Nadu. The Kirby-Bauer disk diffusion method [17] was used for testing the antimicrobial activity. Nutrient agar medium and Sabouraud’s Dextrose Agar (SDA) medium were used for testing the bacteria and Candida, respectively. Solvent acetone represented the negative control. The antibiotics Nalidixic acid for bacteria and Fluconazole for Candida were used as the positive control.

GC-MS analysis

To identify the probable chemical compounds of the epidermal glands, the gland extracts of the two species were analyzed in GCMS, implementing the following conditions. Instrument-Shimadzu–GC 17. A., Column-OV–I, Detector -Mass Spectra, Carrier gas-Helium, Flow rate-0.6 ml/min. GC Parameters: Injector temperature -250°C, Oven: Initial temperature -70°C, Initial time, 2 min. Program: Rate 10°C/ min, Temperature 250°C, Time: 5 min. Interface: Temperature: 300°C, Run time: 29 min. At first, 10 mg of the sample was dissolved in 1 ml of hexane and filtered through the microfilter. The components eluted were detected by the mass selective detector and identified by matching them with the database available, referred from the Tutor library and Wiley 139 library.

Results and Discussion

In order to identify the solvent best suited to dissolve the epidermal glands, both the ferns were subjected to a solubility test by using various solvents such as water (at room temperature and at 100°C), Petroleum ether, Benzene, Ethanol, Acetone and Chloroform. The pale green-colored, spherical, sessile glands (Figures 1G and 1H) of Cyclosorus interruptus (Willd.) H. Ito was totally resistant to the extracts of the leaf with glands. The extract from the leaves without glands showed the least size (0-12 mm) of the inhibition zone. The bacterium Staphylococcus albus was totally resistant to the extracts of the leaf with glands and without glands. In contrast, it shows greater susceptibility to the pure epidermal gland extract, with the maximum inhibition zone

![Figure 1: Materials of the present study.](image-url)
of 26 mm (Figure 3). Thus, it is evident that the pure epidermal gland extract possesses greater antimicrobial efficacy when compared with the leaf tissue, with or without glands.

In the next step, the antibacterial efficacy of the epidermal gland extracts of two different species, both individually and in different combinations, was tested against two bacteria, namely Escherichia coli and Staphylococcus albus. The results (Figure 4) emphasize that the epidermal gland extracts of both Thelypteris parasitica and Cyclosorus interruptus exert a significant effect on both the bacteria when used individually. The inhibition zone produced by the extract of the T. parasitica epidermal glands is slightly larger than that of Cyclosorus interruptus. When the epidermal gland extracts of both the species were mixed in different ratios (1:1, 2:1 & 1:2) the antibacterial effect was altered slightly or drastically based upon the ratio. The 1:1 ratio of gland extracts of the two ferns produced results that were exactly identical to that of the individual extract. When double the amount of gland extract of Cyclosorus interruptus was present in the mixture (1:2) the antibacterial effect was slightly decreased in E. coli and the same mixture revealed no effect with Staphylococcus albus. Thus, when more quantity of the gland extract of Cyclosorus interruptus is present in the mixture, it neither expresses itself nor allows the expression of the antibacterial effect of Thelypteris parasitica. When the mixture contains twice the amount of extract of T. parasitica (2:1), a remarkable increase in the antibacterial efficacy is observed, both in E. coli and S. albus, particularly in the formation of clear zones. In the mixture, the result gets altered based on the species with greater amounts of gland extract. At higher concentrations, the gland extract of Cyclosorus interruptus suppressed or masked the effect of the gland extract of Thelypteris parasitica. Thus, it is concluded that between the two ferns in this study, the gland extract of Christella parasitica is more effective, whereas that of Cyclosorus interruptus is more influential. The difference observed in the antibacterial effect may be due to the difference in the chemical composition of the gland extracts.

The in vitro anticandidal study with Candida albicans revealed positive results with 14 mm and 16 mm diameter inhibition zones in Thelypteris parasitica and Cyclosorus interruptus, respectively. The mixture containing the epidermal gland extracts in 1:1 ratio of both the species resulted in the intermediate sized inhibition zone (15 mm) of 26 mm (Figure 3). Thus, it is evident that the pure epidermal gland extract possesses greater antimicrobial efficacy when compared with the leaf tissue, with or without glands.

In the next step, the antibacterial efficacy of the epidermal gland extracts of two different species, both individually and in different combinations, was tested against two bacteria, namely Escherichia coli and Staphylococcus albus. The results (Figure 4) emphasize that the epidermal gland extracts of both Thelypteris parasitica and Cyclosorus interruptus exert a significant effect on both the bacteria when used individually. The inhibition zone produced by the extract of the T. parasitica epidermal glands is slightly larger than that of Cyclosorus interruptus. When the epidermal gland extracts of both the species were mixed in different ratios (1:1, 2:1 & 1:2) the antibacterial effect was altered slightly or drastically based upon the ratio. The 1:1 ratio of gland extracts of the two ferns produced results that were exactly identical to that of the individual extract. When double the amount of gland extract of Cyclosorus interruptus was present in the mixture (1:2) the antibacterial effect was slightly decreased in E. coli and the same mixture revealed no effect with Staphylococcus albus. Thus, when more quantity of the gland extract of Cyclosorus interruptus is present in the mixture, it neither expresses itself nor allows the expression of the antibacterial effect of Thelypteris parasitica. When the mixture contains twice the amount of extract of T. parasitica (2:1), a remarkable increase in the antibacterial efficacy is observed, both in E. coli and S. albus, particularly in the formation of clear zones. In the mixture, the result gets altered based on the species with greater amounts of gland extract. At higher concentrations, the gland extract of Cyclosorus interruptus suppressed or masked the effect of the gland extract of Thelypteris parasitica. Thus, it is concluded that between the two ferns in this study, the gland extract of Christella parasitica is more effective, whereas that of Cyclosorus interruptus is more influential. The difference observed in the antibacterial effect may be due to the difference in the chemical composition of the gland extracts.
but the zone was clearly visible when compared with the diffusive zone in the individual extracts of both the species (Figure 5). The positive control Flucanazole showed a very diffusive inhibition zone, 14 mm in diameter. The combination of the extracts of the epidermal glands of the two ferns showed a qualitative, though not quantitative, synergistic effect in the antifungal activity.

The results of preliminary phytochemical screening performed on the epidermal gland-extract of both the species following the standard methods [18,19] showed the exact similar results with the presence of steroids, triterpenoids, alkaloids, phenolic group, flavonoids, saponins, tannins and the absence of sugar, catechins, anthraquinones, amino acids, mucilage and reducing compounds. The GC-MS analysis (Tables 1 and 2) shows the presence of 6 and 11 different chemical compounds in *T. parasitica* and *C. interruptus*, respectively, with one unknown compound in the former species and two in the latter species. The combination of different types of chemicals present in the epidermal glands of both the ferns may be responsible for the synergistic effect of the two extracts in antimicrobial activity.

The present study on the antimicrobial activities of the epidermal gland extracts of two thelypteroid ferns indicates that the epidermal glands of these ferns contain several antimicrobial compounds which act as chemical defense agents against several pathogenic microbes in order to protect the plants. The GC-MS analysis reveals that the epidermal glands of these ferns contain mostly lipophilic substances along with various other bioactive compounds, particularly organometallic compounds, triterpenoids, alkaloids etc. Organosilicon derivatives are the active principle in cosmetic or pharmaceutical and particularly dermatological compositions. These are particularly useful in treating alopecia [20]. In the present study, the organosilicon compounds such as...
as silane and silamime are commonly present in the epidermal gland extracts. In the gland-extract of *Thelypteris parasitica* Distannoxane Ditributyl Tin Oxide, an active ingredient of a common pesticide Fenbutatin-oxide [21], is present. It is suggested that at least some of the 17 different chemical compounds, 6 in *Thelypteris parasitica* and 11 in *Cyclosorus interruptus*, may perform the synergistic function with a few, like triterpenoids, alkaloids etc., acting as the active ingredient; some of the other compounds may act as enhancing agents, while many of the compounds, particularly the lipophilic ones, may perform other functions related to the antimicrobial activity. It is well recognized that several types of metallic ions are used as chelating agents in antibiotics [22]. The mixture of the epidermal gland extracts of the two ferns with different bioactive compounds to perform major functions, along with other additional compounds to perform secondary functions, may be an efficient antimicrobial agent. Thus, the plant skin or epidermis, combined with several antibacterial and antifungal compounds, may be useful in the treatment of human pathogenic skin diseases. Further, High Throughput Screening (HTS) studies are required to understand the precise positive and negative role of every single chemical compound present in the epidermal glands of these two ferns.

**Acknowledgement**

The authors express their gratitude to Rev. Dr. A. Antonysamy S J., Rev. Dr. Alphonse Manickam, S J., Rev. Dr. A. Joseph, S J., and Rev. Dr. V. Gilburt Parihar P, Parihar L, Bohra A (2010) Synergistic effect of Traditional Chinese Medicine. Asian J Chem 19: 867-882.

**References**

1. Yinghong L, Ming KJ, Sai CL, Khang GN (2007) Synergistic effect of Traditional Chinese Medicine. Asian J Chem 19: 867-882.

2. Adwan G, Mhanna M (2008) Synergistic effects of plant extracts and antibiotics on *Staphylococcus aureus* strains isolated from clinical specimens. Middle East J Sci Res 3: 134-139.

3. Durasivasamy H, Nallaiyan S, Nelson J, Rathinasamy P, Johnson M, et al. (2010) The effect of extracts of Selaginella involvens and Selaginella inaequifoliala leaves on poultry pathogens. Asian Pac J Trop Med 3: 678-681.

4. John de Britto A, Gracelin DHS, Kumar PBJR (2012) *Pteris biurata* L.: A potential antibacterial fern against *Xanthomonas* and *Aeromonas* bacteria. J Pharm Res 5: 679-680.

5. Patric RD, Johnson M, Irudayaraj V, Janakiraman N (2012) Antimicrobial efficacy of selected ferns of Western Ghats, South India. JCPFRM 4: 58-60.

6. Manickam VS, Benniamin A, Irudayaraj V (2004) Antimicrobial activity of *Christella parasitica* (L.) H. Lev. Leaf glands. Indian Fern J 22: 87-88.

7. Paul RK, Irudayaraj V, Johnson M, Patric RD (2011) Phytochemical and antibacterial activity of epidermal glands extract of *Christella parasitica* (L.) H. Lev. Asian Pac J Trop Med 1: 8-11.

8. Paul RK, Irudayaraj V, Johnson M, Patric RD (2011) Phytochemical and antibacterial activity of epidermal glands extract of *Christella parasitica* (L.) H. Lev. Asian J Pharm Res 4: 1167-1172.

9. Ahmed Z, Khan SS, Khan M, Tanveer A, Lone ZA (2010) Synergistic effect of Salvedora persica extracts, tetracycline and penicillin against *Staphylococcus aureus*. AJBAS 2: 25-29.

10. Soare LC, Deliu I, Iosub I, Dobrescu CM, Ferdes M (2012) New therapeutic relevance. Int J Mol Sci 13: 8915-8932.

11. Abd El-Kalek HH, Mohamed EA (2012) *Acacia mearnsii* (De Wild.) with antibiotics against bacteria of clinical relevance. Int J Mol Sci 13: 8915-8932.

12. Adwan G, Mhanna M (2008) Synergistic effects of plant extracts and antibiotics on *Staphylococcus aureus* strains isolated from clinical specimens. Middle East J Sci Res 3: 134-139.

13. Olajuyigbe AO, Afolayan AJ (2012) *Acacia mearnsii* (De Wild.) with antibiotics against bacteria of clinical relevance. Int J Mol Sci 13: 8915-8932.

14. Adwan G, Mhanna M (2008) Synergistic effects of plant extracts and antibiotics on *Staphylococcus aureus* strains isolated from clinical specimens. Middle East J Sci Res 3: 134-139.

15. Pauline Vincent C, Irudayaraj V, Johnson M (2012) Antibacterial efficiency of macroscopic, microscopic parts of sporophyte and *in vitro* gameophyte of a fern *Cyclosorus interruptus* (Wild.) I. Ito (*Thelypteridaceae*-Pteridophyta). J Chem Pharm Res 4: 1167-1172.
17. Bauer AW, Sherris TM, Kirby WHM (1966) Antibiotic susceptibility testing by standardized single disc method. Am J Clin Pathol 45: 493-496.

18. Brindha P, Sasikala B, Purushothaman KK (1981) Pharmacognostic studies on Merugan kizhangu. BMEBR 3: 84-96.

19. Trease E, Evans WC (1987) Pharmacognosy (13th edn.) Billiare Tindall, London.

20. Stephane D (1999) Combinations of peroxide lipids and organosilicon compounds, cosmetic and dermatological compositions containing same, and uses thereof, in particular for treating alopecia. United States Patent 6001378.

21. Government of Canada (2016) Nineteen Substances on the Domestic Substances List Associated with Pesticidal Uses. Final Screening Assessment Chemical Abstracts Service Registry Numbers, Environment Canada, Health Canada.

22. Moon JH, Kim C, Lee HS, Kim SW, Lee JY (2013) Antibacterial and antibiofilm effects of iron chelators against Prevotella intermedia. J Med Microbiol 62: 1307-1316.