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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 the 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 pteridophyes 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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(Figures 1A and 1B), Western Ghats, Kanyakumari District, India. Plant samples of the fern *Cyclosorus interruptus* (Willd.) H. Ito, were collected from an open marshy habitat in Marthandam, Kanyakumari District, India (Figures 1E and 1F). Vouchers (XCH 23364, XCH 23365) are stored in St. Xavier's College Herbarium, Palayamkottai, India. 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.

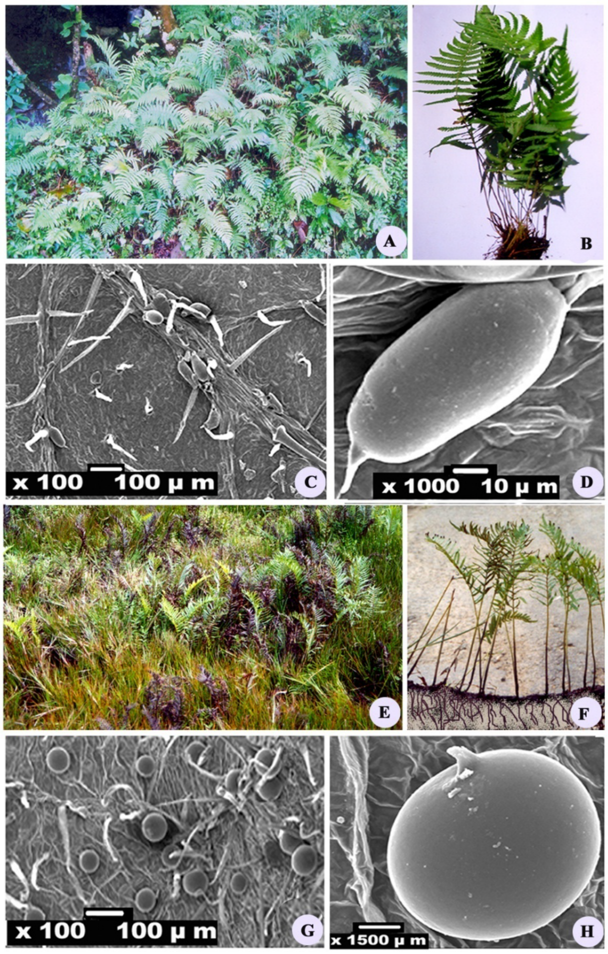
GC-MS analysis

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

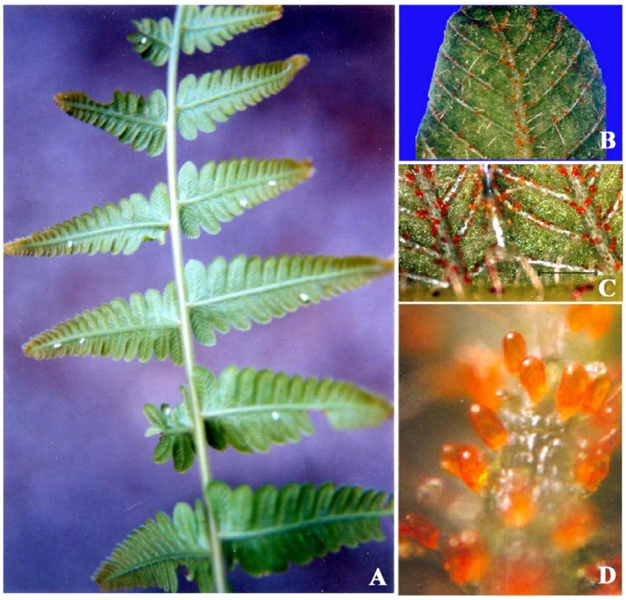
In order to identify the solvent best suited to dissolve the epidermal glands, both the ferns were subjected to a solubility test by using various solvents such as water (at room temperature and at 100°C), Petroleum ether, Benzene, Ethanol, Acetone and Chloroform. The pale green-colored, spherical, sessile glands (Figures 1G and 1H) of *Cyclosorus interruptus* (Willd.) H. Ito easily dissolved only in the Acetone and remained insoluble in all the other solvents tested. In contrast, while the orange-colored, elongated, stalked epidermal glands (Figures 1 and 2) of *Thelypteris parasitica* (L.) Fosberg were insoluble in water and mildly soluble in Petroleum ether, Benzene, Ethanol and Chloroform and they easily dissolved in Acetone. Therefore, Acetone was used as the solvent to dissolve the epidermal glands of both the ferns. In a prior study [16] on the preliminary phytochemical screening of acetone extract prepared by soaking the dried leaves of both the glandular and eglandular forms of *Thelypteris parasitica,* completely different results were reported, with the glandular extract alone, showing the presence of the triterpenoids.

In order to confirm the antimicrobial efficacy of the epidermal glands, the extract of the leaf tissue with the glands, the extract of the leaf tissue without glands and pure gland extract from *Thelypteris parasitica* (L.) Fosberg, were tested for antimicrobial activity by using one Gram-negative bacterium (*Escherichia coli*), one Gram-positive bacterium (*Staphylococcus albus*) and one fungus (*Candida albicans*). A 20 mm inhibition zone was obtained for *E. coli* in the pure gland extract, 16 mm in the leaf extract with glands and 12 mm in the leaf extract without glands. In the case of *Staphylococcus albus* a 20 mm inhibition zone was observed in the pure gland extract and in the other two extracts the bacterium was resistant. For *Candida albicans* 10, 8 and 7 mm sized inhibition zones were obtained. In general, the maximum size (10-26 mm) of the inhibition zone has been observed in the pure gland extract followed by a medium sized (0-16 mm) inhibition zone in the leaf extract 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



A-D: *Thelypteris parasitica* (L.) Fosberg. E-H: *Cyclosorus interruptus* (Willd.) H. Ito; A and E: Colony in natural habitat, B and F: Habit; C-D and G-H: Scanning Electron Microscopic views of epidermal glands.

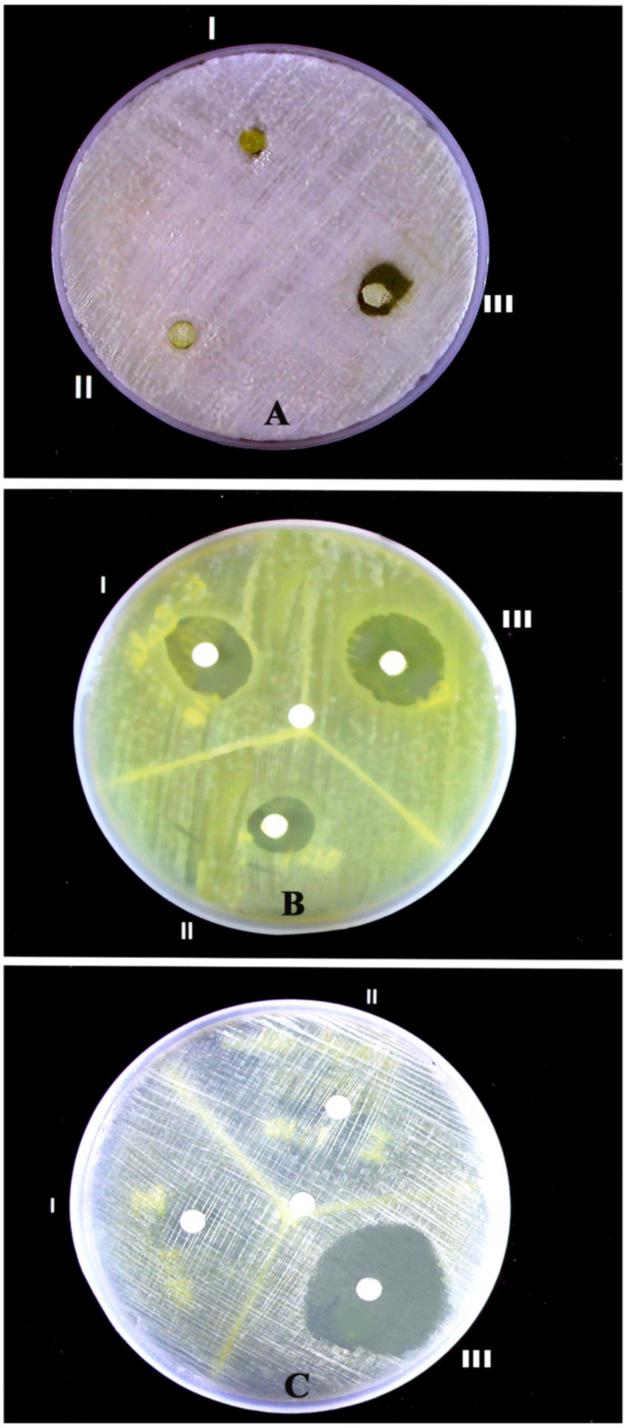
Figure 1: Materials of the present study.



A: Abaxial side of young leaves with epidermal glands with light orange color; B, C: Portion of leaf under light microscope showing epidermal glands on veins; D: Enlarged view of clustered, elongated, orange colored glands on vein.

Figure 2: Epidermal glands in *Christella parasitica* (L.) H. Lev.

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.



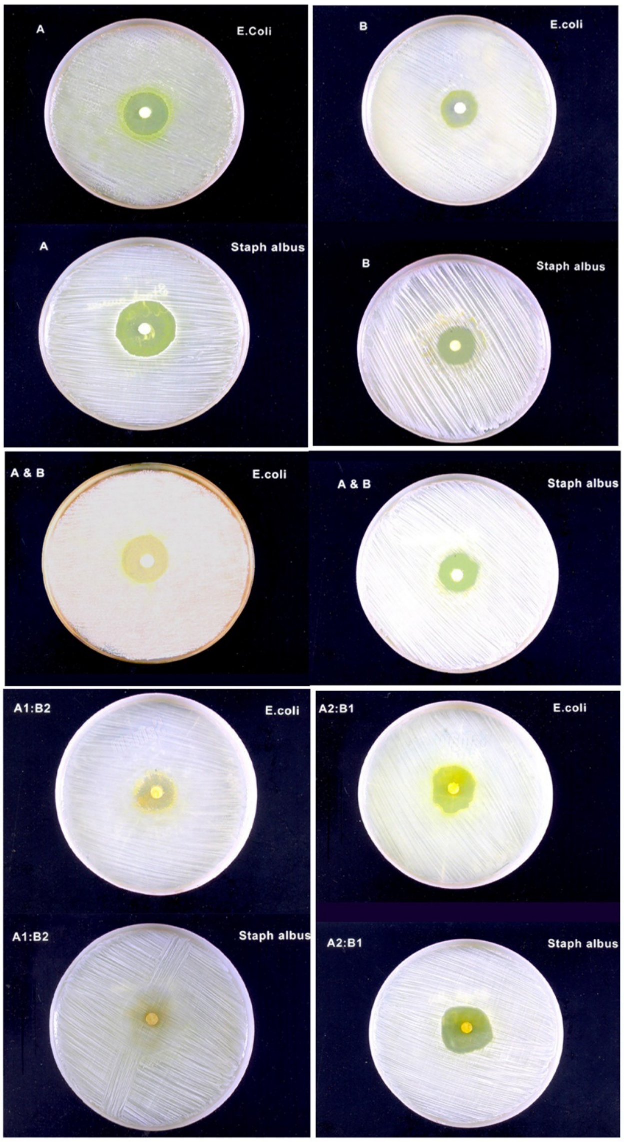
I: Extract from leaves with glands; II: Extract from leaves without glands; III: Extract from glands only.
A: *Candida albicans*, B: *Escherichia coli*, C: *Staphylococcus albus*.

Figure 3: Antimicrobial activity in different parts of *Thelypteris parasitica* (L.) Fosberg.

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.

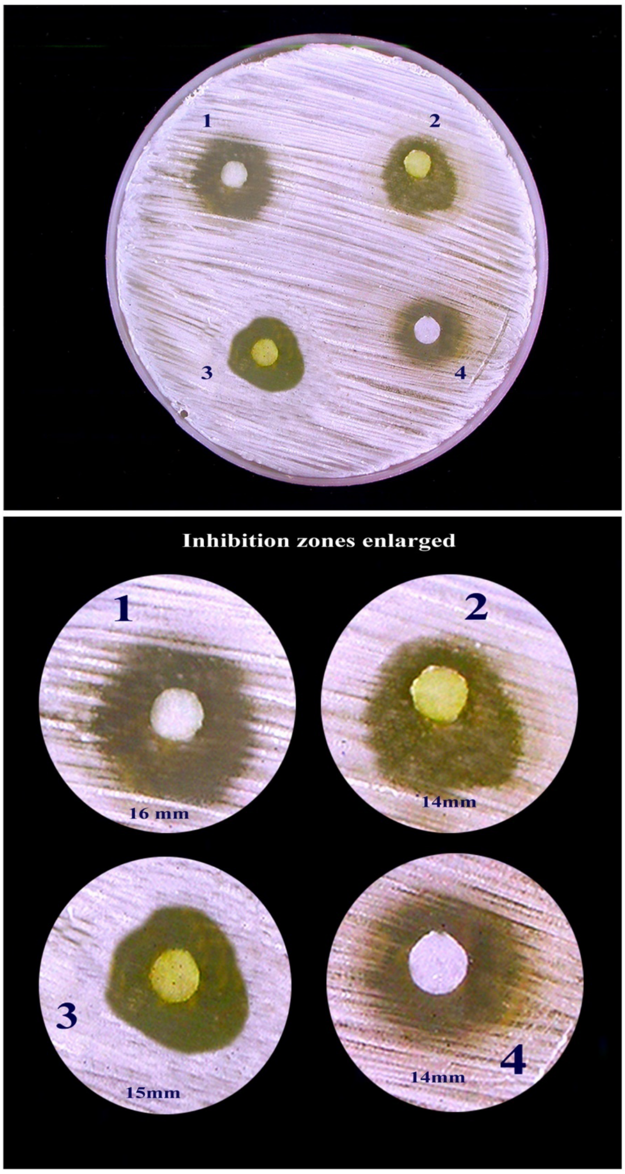
but the zone was clearly visible when compared with the diffusive zone in the individual extracts of both the species (Figure 5). The positive control Flucanazole showed a very diffusive inhibition zone, 14 mm in diameter. The combination of the extracts of the epidermal glands of the two ferns showed a qualitative, though not quantitative, synergistic effect in the anticandidal 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.



A: *Thelypteris parasitica* (L.) Fosberg; B: *Cyclosorus interruptus* (Willd.) H. Ito.

Figure 4: Individual effect and synergistic effect in antimicrobial activity of epidermal gland-extract from *Helypteris parasitica* (L.) Fosberg and *Cyclosorus interruptus* (Willd.) H. Ito.



1) *Cyclosorus interruptus* 2) *Thelypteris parasitica* 3) *Cyclosorus interruptus* and *Thelypteris parasitica* 4) Flucanazole.

Figure 5: Anticandidal activity in epidermal gland-extract of *Thelypteris parasitica* and *Cyclosorus interruptus.*

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

Sl. No.	Peak Number	Retention Time	Area Percentage	Matching Percentage	Probable Chemical Compounds
1	1	2.126	26.52	-	No hits
2	2	4.625	24.91	40	M-Quinquiphenyl
3	3	5.017	17.53	36	Glycyrrhiza chalcone
4	4	6.550	4.86	46	Trimethylsilyl ether of 17-alpha-hydroxy-5. alpha–pregnane-3, 20 alione
5	5	7.767	20.36	23	Distannoxane, Ditributyl Tin Oxide
6	6	11.517	5.82	23	Triacontane

Table 1: Results of GC-MS analysis on epidermal glands of *Christella parasitica.*

Sl. No.	Peak Number	Retention Time	Area Percentage	Matching Percentage	Probable Chemical Compounds
1	3	4.033	2.32	26	1-Oxyperethyl Homohexasilsesquioxane
2	5	5.033	5.91	-	No hits
3	6	5.517	12.39	44	18 Pentatriacontanone
4	7	6.133	8.46	48	Hexatria contane
5	8	6.575	3.07	25	Thiophene, 5-hexadecyl-3-methyl-2-pentadecyl
6	9	7.303	11.01	-	No hits
7	10	8.067	15.65	66	Octadeanoic acid
8	11	8.675	14.12	61	Hexatria contane (CAS/ n-Hexatria contane
9	12	9.225	8.67	33	Prelycopersene pyrophosphate
10	13	9.725	10.61	50	Lanosta-7,9(11),20-triene-3. beta., 18-diol, diacetate
11	16	11.567	2.36	46	Dimethyl derivative of Nalbuphine

Table 2: Results of GC–MS analysis on epidermal glands of *Cyclosorus interruptus*.

as silane and silamine 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.

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