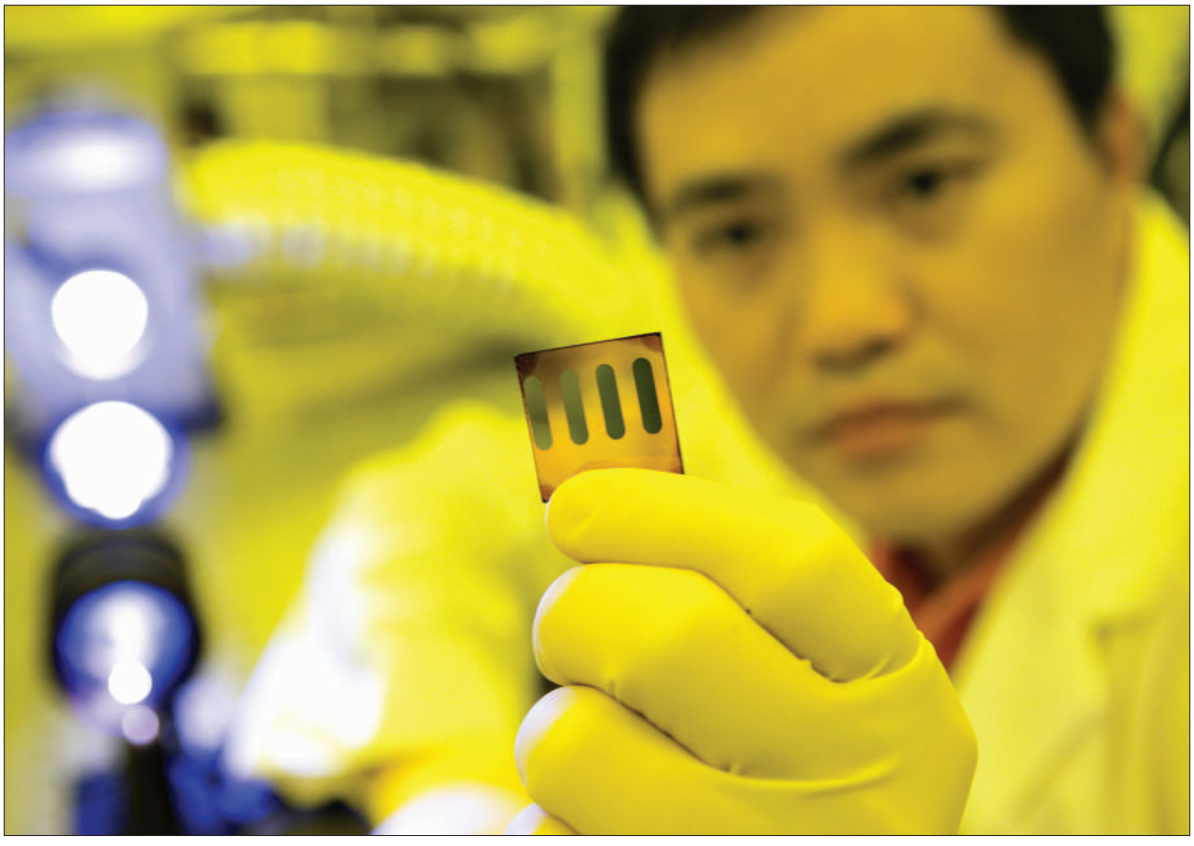

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Research Article
Free Radical Scavenging Activity of Chromatography Fractions of *Dirinaria picta* on Two Host Trees

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Abstract

Background and Objective: Free radicals and related reactive intermediates are the leading cause of oxidative stress associated with the progression and complication in diseases of ageing, degeneration, cancer, obesity, hypertension, gout and rheumatism among others. This study aimed at investigating the free radical scavenging activity of *Dirinaria picta* epiphytic on two host trees: *Elaeis guineensis* and *Cocos nucifera*. **Materials and Methods:** The crude ethanol extract of *D. picta* was obtained by exhaustive cold maceration. Phytochemical screening was done using standard reagents, while quantitative antioxidant activity was determined using the spectrophotometric 2, 2-Diphenyl-1-picrylhydrazyl (DPPH) model at 517 nm. Ascorbic acid was used as reference antioxidant standard for comparison. The crude ethanol extracts were respectively fractionated using column chromatography and the chromatography fractions obtained pooled together based on similarities after thin layer chromatography examination. **Results:** Pooled chromatography fractions: seven (CPF1-CPF7) for the crude ethanol extract (LEC) from *D. picta* epiphytic on *Cocos nucifera* and ten (PPF1-PPF10) for the crude ethanol extract (LEP) from *D. picta* epiphytic on *Elaeis guineensis* were obtained. Two fractions CPF2 and CPF6 from LEC and four fractions: PPF5, PPF6, PPF8 and PPF9 from LEP were the most active. **Conclusion:** This study supports the use of metabolites from lichens for the development of drugs for the management of pathologic diseases due to oxidative stress and the antioxidant activity of *D. picta* could vary with the host tree.

Key words: *Dirinaria picta*, lichen, host effect, antioxidant, oxidative stress, pathologic diseases

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Competing Interest: The authors have declared that no competing interest exists.

Data Availability: All relevant data are within the paper and its supporting information files.

INTRODUCTION

Free radical scavengers are compounds which can impede oxidation process by reacting with free radicals, chelating catalytic metals as well as scavenging oxygen in biological systems[1]. They are therefore antioxidants and play crucial role in the maintenance of health, well-being and in the prevention and management of oxidative stress. Oxidative stress occurs when free radicals and related reactive intermediates are generated in amounts that exceed the system's ability to neutralize and eliminate them[2]. Oxidative stress has been associated with the progression and complication in diseases of ageing, degeneration, cancer, gout and rheumatism, obesity and hypertension among others.

Lichen is a symbiotic form of life consisting of an ascomycetous fungus (the mycobiont) and an alga or blue-green alga (photobiont). The photobiont manufactures the food by virtue of chlorophyll present in cells, while the mycobiont provides the physical structure for the relationship. Fossil records have shown that lichens are the earliest colonizers of terrestrial habitats[3]. They are abundant as epiphytes on trees, rocks, walls, gravestones and on exposed soil surfaces. Lichens can survive some extreme environment and thus can secrete biologically active metabolites that enable them survive otherwise stressful environment. Native Americans and Europeans used lichens in ancient medicine in the treatment pulmonary tuberculosis, wounds and skin disorders. They have also been used in the treatment of diverse diseases like arthritis, alopecia, constipation, kidney diseases, leprosy, pharyngitis rabies, cold infection, fever, jaundice, convulsion and cough[4]. A numerous number of secondary metabolites in lichens are currently known. These secondary metabolites fall into various chemical classes including; diterpenes, triterpenes, dibenzofuran, depsides, depsidones, anthraquinones, xanthones, usnic acid and pulvinic acid derivatives[5]. *Dirinaria picta* is foliose lichen. It is widespread in pan- tropical and sub-tropical regions growing on barks of trees, woods and rocks[6]. The cortex gives a positive yellow colouration to paraphenylenediamine (P) and potassium hydroxide (K) and is negative with calcium hypochlorite (C), potassium hydroxide and calcium hypochlorite (KC). Its medulla gives no reaction with the reagents. Atranorin and divaricatic acid derivatives, carbohydrates and hopane-type triterpenoids are common metabolites present in *Dirinaria* species[6]. As a follow-up to our bioprospection for bioactive metabolites from the Nigeria lichens and related mycoflora[7-12], present study evaluated the *in vitro* free radical scavenging activity of the lichen *Dirinaria picta* from two host plants *Elaeis guineensis* and *Cocos nucifera* using the 2, 2-Diphenyl-1-picrylhydrazyl (DPPH) assay.

MATERIALS AND METHODS

Study area: The investigations were carried out in the laboratories of the Department of Pharmacognosy and Phytotherapy during the period July, 2016-December, 2016.

Sample collection, identification and preparation: The lichen *Dirinaria picta* used for this study was collected in the month of July 2016 from the bark of *Elaeis guineensis* (Arecaceae) and *Cocos nucifera* (Arecaceae) growing in the campus of the University of Port Harcourt, Rivers state and identified by Dr Andre Aptroot, ABL Herbarium (Netherlands) and Ms. Louise Olley, Royal Botanic Garden (Edinburgh). Standard lichen spot test were also used for the identification. Voucher specimens (LC 001 for *D. picta* epiphytic on *Cocos nucifera* and LC 002 for *D. picta* epiphytic on *Elaeis guineensis*) were deposited at the Herbarium of Pharmacognosy and Phytotherapy, University of Port Harcourt, Nigeria. Just after collection, sorting of the lichen was done to remove foreign matter, followed by shade drying at room temperature (24-26°C) after which the dried sample were pulverized into coarse powder prior to extraction.

Reagents/chemicals: All the reagents/chemicals used were of analytical grade and re-distilled where applicable.

Extraction of lichen: The powder lichen samples epiphytic on *Elaeis guineensis* (98 g) and *Cocos nucifera* (16 g) were separately extracted with absolute ethanol (400 and 70 mL, respectively) by cold maceration, with fresh ethanol replacement every 48 h for a period of 8 days to ensure exhaustive extraction of the lichen metabolites. After filtration using a Whatman No. 1 filter paper, the combined filtrates were concentrated using a rotary evaporator to obtain the crude ethanol extract LEP and LEC from the lichen epiphytic on *Elaeis guineensis* and *Cocos nucifera* respectively. A pale yellow precipitate coded PPT-1 was obtained from the concentrated LEP on standing which was collected by filtration.

Phytochemical screening method: Preliminary phytochemical test were carried out on the crude lichen sample from each host using standard phytochemical screening reagents[13-14].

Antioxidant screening method: Antioxidant screening was done using the 2, 2-diphenyl-1-picrylhydrazyl (DPPH) spectrophotometric assay[15] with suitable modifications. Briefly, a preliminary antioxidant assay was carried out on the lichen crude ethanol extracts LEP and LEC at concentrations 1000 and 100 µg mL^{-1}, with methanol used as solvent. Two milliliter of each preparation was mixed with equal volume of 5% DPPH in methanol. After 30 min of incubation in the dark at room temperature, absorbance was measured at 517 nm. The experiment was performed in triplicate, with the control sample containing all the reagents except the extract. The percentage inhibition was calculated using the following equation[15]:

$$\text{Inhibition (\%)} = \frac{\text{Ac} - \text{As}}{\text{Ac}} \times 100$$

where, Ac is the absorbance of control, As is the absorbance of sample.

Thereafter, a further concentration dependent free radical scavenging assay was similarly done as outlined above on the crude ethanol extracts at concentrations 800-12.5 µg mL^{-1} following a two-fold serial dilution approach. As a reference standard for comparison, ascorbic acid at concentration 80-0.625 µg mL^{-1} was similarly evaluated. The half maximal inhibitory concentration (IC_{50}) was determined from the regression curve of a plot of DPPH inhibition (%) against concentration.

Chromatographic fractionation of the LEC and LEP: The column used for the lichen epiphytic on *Elaeis guineensis* had a length and diameter of 29.3 and 4 cm, respectively. The column used for host *Cocos nucifera* had a length and diameter of 20.3 and 2.8 cm, respectively. The dry packing method was used with silica gel adsorbent (200-400 mesh size) as stationary phase. A mobile phase gradient of: n-hexane, n-hexane: dichloromethane [1:1], dichloromethane, ethyl acetate, ethyl acetate: methanol [1:1] and methanol were used for elution. The fractions were collected after every 100 mL. The various fractions obtained, were then pooled together according to similarity in retardation factor (R_f) and characteristic colour under daylight and fluorescence on exposure to UV light of short (254 nm) and long (365 nm) wavelengths from thin layer chromatography. Pooled fractions CPF1-CPF7 and PPF1-PPF10 were obtained from LEC and LEP, respectively.

Quantitative DPPH-radical scavenging assay of pooled column fractions and PPT-1: The PPT-1, PPF1-PPF9 and CPF1-CPF7 were assayed for scavenging activity using the method outlined above at the IC_{50} of their respective parent crude ethanol extract. All fractions were dissolved in methanol as solvent for the assay except PPT-1 which was first dissolved in DMSO due to solubility issues. Fractions with >50% inhibition were considered active and their IC_{50} similarly determined.

Statistical analysis: Values expressed as mean±standard deviation were subjected to regression analysis and student t-test for significance (p-value = 0.05).

RESULTS AND DISCUSSION

The percentage yield of crude ethanol extract of *D. picta* epiphytic on *Elaeis guineensis* and *Cocos nucifera* were 4.32 and 2.91% w/w, respectively. Carbohydrate, phenolic compounds (Ferric chloride test), triterpenoids and cardiac glycosides were detected as metabolites. This corroborated similar reports on the presence of triterpenoids[6] and phenolics[6-7] in the *Dirinaria* genus of lichens. Flavonoids, phlobatannins, alkaloids, anthraquinones, saponins, cyanogenic glycosides and free reducing sugars were however absent. Although carbohydrates based on the Molisch test were present, however, the inability to reduce copper (II) ion to copper (I) in Fehling's test indicated the absence of reducing sugars. Also, the formation of an iron-phenol complex with a characteristic greenish or blue-black colour is typical of phenolics compounds (which include depsides, flavonoids, tannins, coumarins and related phenolic compounds) using ferric chloride test[13,14]. Lichen depsides and depsidones would give a positive result with the ferric chloride test because they all have the enol moiety typical of phenolic compounds. Flavonoids are phenolic compounds which are distinguished using the specific Shinoda test. Their absence indicated that the phenolic compounds present are unlikely to be flavonoids. Generally, the presence of phenolic compounds mostly depsides and depsidones[6,7,16,17] in several lichen species have been documented. Some of the depsides and depsidones includes: Lasallic acid, from

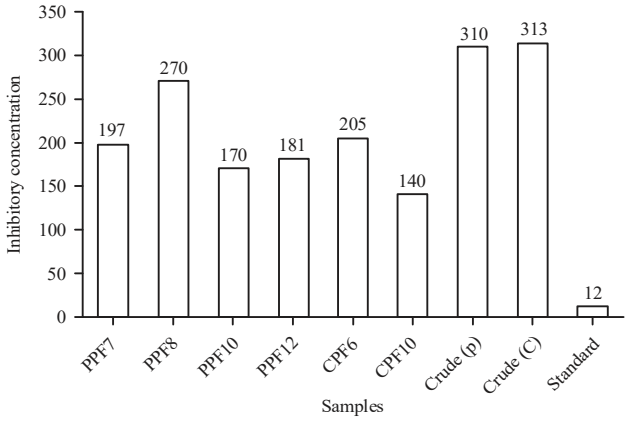

Fig. 1: Half maximal inhibition concentration (IC_{50}) of reference, crude extracts and pooled column fractions
Crude (p): Crude ethanol extract (Host: *Elaeis guineensis*), Crude (C): Crude ethanol extract (Host: *Cocos nucifera*), Standard: Ascorbic acid

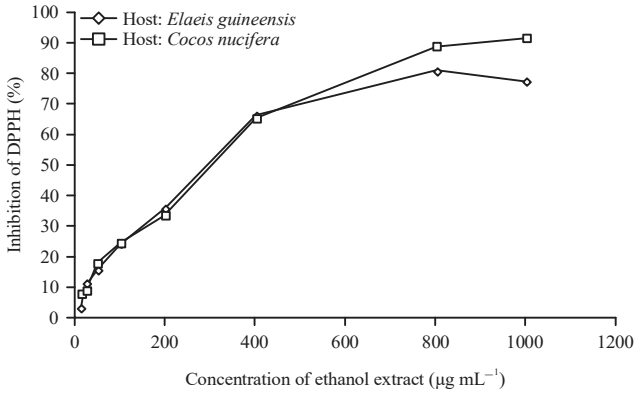

Fig. 2: Concentration response free radical scavenging activity curve for the crude ethanol extracts from *Dirinaria picta* epiphytic on two host trees

Table 1: Preliminary assay for free radical scavenging activity

Host trees	Concentration (µg mL^{-1})	Inhibition of DPPH (%)±SD
Elaeis guineensis (Arecaceae)	1000	77.23±1.64
	100	24.04±0.90*
Cocos nucifera (Arecaceae)	1000	91.16±0.00
	100	24.60±0.08*

*Not significantly different (p = 0.3435>0.05, n = 3)

Lasallia asiaeorientalis[18], gyrophoric acid isolated from *Punctelia microsticta*[17]. Table 1 has the preliminary assay results for free radical scavenging activity of the respective crude ethanol extract from *Dirinaria picta* epiphytic on each host. The crude ethanol extracts of *D. picta* elicited a statistically significant host dependent activity ($p<0.05$) at 1000 µg mL^{-1}. However, at the low concentration of 100 µg mL^{-1}, this was not significant ($p= 0.3435>0.05$). This trend was also observed in Fig. 1 for the concentration dependent inhibition of DPPH scavenging activity curve, with *D. picta* epiphytic on *Cocos nucifera* eliciting a greater activity at higher concentration above 800 µg mL^{-1} and activity between host varying only slightly with decreasing concentration. Thus, it could be said that at high concentrations, *Dirinaria picta* epiphytic on *Cocos nucifera* offers better scavenging activity and this may be due to metabolites secreted during host-lichen interaction. The observed effect of the host tree on the variation in activity in this study is in agreement with earlier reports on variation in biological activities of parasitic and epiphytic plants with host which has been attributed to metabolic and environmental variations due to the host plant[18]. The observed half maximal inhibitory concentration (IC_{50}) which is the minimum concentration required to produce a 50% inhibition of DPPH radical were comparable for the LEP and LEC respectively as seen from the concentration-response curve in Fig. 2 though not as active compared to the reference standard ascorbic acid (Fig. 2). A total of seven pooled chromatography fractions: (CPF1-CPF7) from the LEC while from the LEP, a total of ten pooled chromatography fractions: PPF1-PPF10 was obtained. Their free radical scavenging activity profiles are as shown in Table 2 and 3, respectively. Two fractions CPF2 and CPF6 from LEC and four fractions: PPF5, PPF6, PPF8 and PPF9 from LEP were the most active. Irrespective of the host, chromatography fractions eluted with moderately polar mobile phase gradients ethyl acetate and ethyl acetate: methanol (9:1 v/v) were generally the most active except for chromatography fraction CPF2 eluted with dichloromethane. This may infer that the active constituents of this lichen are more of moderate polarity and the variation in the degree of activity may be as a result of structure activity relationship of the active constituents and/or concentration. CPF6 showed higher inhibiting activity than PPF8 which was statistically significant ($p<0.05$). The precipitate PPT-1 obtained from the crude ethanol extract of *Dirinaria picta* epiphytic on *Elaeis guineensis* did not elicit free radical scavenging activity at the test concentration of 310 µg mL^{-1} which is the IC_{50} of its parent crude extract LEP. Table 4 showed the concentration-dependent free radical scavenging activity trend for the most active chromatography fractions from the LEC and LEP while Fig. 1 is a graphical representation of their respective IC_{50} from regression analysis compared to that obtained for their respective parent crude ethanol extracts and the reference standard ascorbic acid. The activity of the standard and fractions was of the trend; Ascorbic acid >CPF6>PPF8>PPF9>PPF5>CPF2>PPF6.

Table 2: Free radical scavenging activity of chromatography fractions from *D. picta* (Host: *Cocos nucifera*)

Pooled fractions	Yield (% w/w)	Eluting mobile gradient	Retardation factor (R_f)# @UV 254 nm	Inhibition of DPPH (%)±SD
CPF1	39.99	H:D (1:1-0:1 v/v)	0.91(p), 0.88 (p), 0.83 (p), 0.78 (p), 0.69 (p), 0.64 (p), 0.51 (p)	33.64±0.26
CPF2	2.15	D (100%)	0.70 (p), 0.66 (p), 0.54 (p),0.40 (p), 0.28 (p)	65.97±0.18*
CPF3	3.66	D (100%)	0.91 (r), 0.86 (r) , 0.70 (g), 0.55 (r)	13.09±0.00
CPF4	41.51	EA (100%)	0.97 (p), 0.75 (p)	46.41±0.14
CPF5	0.86	EA:M (9:1v/v)	0.97 (p), 0.86 (p)	14.72±0.00
CPF6	1.29	EA:M (9:1v/v)	0.90 (p)	85.92±0.14*
CPF7	2.56	EA:M (9:1-0:1 v/v)	0.94 (p)	43.15±0.00

*Most active fractions, CPF: Pooled column chromatography fractions from *D. picta* epiphytic *Cocos nucifera*, H: n-hexane, D: Dichloromethane, EA: Ethyl acetate, M: Methanol, #TLC condition: Stationary phase (silica gel HF_{254}), mobile phase (dichloromethane for CPF1-CPF3 and dichloromethane: methanol (9:1v/v) for CPF4-CPF7), development distance = 8 cm), p: Purple fluorescence, r: Red fluorescence, g: Green fluorescence, b: Blue fluorescence

Table 3: Free radical scavenging activity of chromatography fractions from *D. picta* (Host: *Elaeis guineensis*)

Pooled fractions	Yield (% w/w)	Eluting mobile gradient	Retardation factor (R_f)# @UV 254 nm	Inhibition of DPPH (%)±SD
PPF1	1.98	H:D (1:1 v/v)	0.95 (p)	6.60±0.00
PPF2	0.31	H:D (1:1 v/v)	0.88 (p)	19.57±0.04
PPF3	71.38	D:EA (1:0-0:1)	0.90 (p), 0.88 (b), 0.73 (g), 0.59 (p), 0.45 (p), 0.30 (p), 0.13 (p)	31.92±0.28
PPF4	10.42	EA (100%)	0.68 (p), 0.15 (p)	20.07±0.27
PPF5	2.24	EA (100%)	0.56 (r), 0.07 (r)	74.82±0.00*
PPF6	2.36	EA (100%)	0.89 (p)	56.62±0.07*
PPF7	0.14	EA:M (9:1 v/v)	0.96 (p), 0.91 (p)	25.31±0.00
PPF8	0.19	EA:M (9:1 v/v)	0.94 (p)	73.15±0.05*
PPF9	0.08	EA:M (1:1 v/v)	0.88 (p)	72.87±0.00*
PPF10	0.19	M (100%)	0.90 (p)	39.73±0.00
PPT-1	0.45	ND	ND	4.57±0.00

*Most active fractions; ND: None done, PPF: Pooled column chromatography fractions from *D. picta* epiphytic *Elaeis guineensis*, H: n-hexane, D: Dichloromethane, EA: Ethyl acetate, M: Methanol, #TLC condition: Stationary phase (silica gel HF_{254}), mobile phase (dichloromethane for PPF1-PPF5 and dichloromethane:methanol (9:1 v/v) for PPF6-PPF10), Development distance: 8 cm), p: Purple fluorescence, r: Red fluorescence, g: Green fluorescence, b: Blue fluorescence

Table 4: Concentration dependent DPPH inhibition of active column pooled fractions

Concentration	Inhibition (%)					
(µg mL^{-1})	PPF5 (P23-P26)	PPF6 (P27-P30)	PPF8 (P32)	PPF9 (P34-35)	CPF2 (C13-C15)	CPF6 (C25)
300	74.82	56.62	73.15	72.87	65.97	85.92
200	48.96	30.65	54.03	54.81	45.45	82.99
100	26.75	21.95	33.64	30.65	32.47	31.90
50	16.36	15.91	26.36	19.74	26.23	21.56
25	9.22	9.09	13.90	9.74	16.36	12.34
IC_{50} (µg mL^{-1})	197.00	270.00	170.00	181.00	205.00	140.00

The antioxidant properties of several lichen extracts and their metabolites have been reported with the phenolic constituents being the most active[19-20]. Thus, the presence of phenolics compounds as seen from the positive ferric chloride phytochemical test for the extracts could offer a plausible explanation for the observed trend in free radical scavenging activity of the *D. picta* from the two host trees in this study. Generally botanic derived phenolic compounds are known to be potent antioxidant agents[21-22] and several of these lichen phenolic compounds mostly the depsides and depsidones have been evaluated and found promising as leads for the development of drugs for the management of diseases associated with oxidative stress and inflammation[16,17,19,20].

CONCLUSION

The comparative free radical scavenging activity of the crude ethanol extract from *Dirinaria picta* epiphytic on *Elaeis guineensis* and *Cocos nucifera* was evaluated. Free radical scavenging activity of the lichen was influenced by its host, having statistically significant host-dependent activity ($p<0.05$) at higher concentrations. This study supports the use of metabolites from lichens for the development of drugs for the management of pathologic diseases caused by free radicals and that species of the host tree could play a role in the degree of the antioxidant activity of and the structural variations among the class of metabolites present in this species of lichen.

SIGNIFICANCE STATEMENT

This study discovered that the free radical scavenging properties and metabolites of the lichen species *Dirinaria picta* collected from Nigeria, could be influence by the host tree. This study will help the researcher to uncover the critical areas of chemical ecology of parasitic and epiphytic medicinal flora and their host trees in the discovery and development of drugs for the prevention and management of tissue damage due to inflammation that many researchers were not able to explore. Thus a new theory on the role host plant on the medicinal and bioactive chemical properties of parasitic and epiphytic medicinal flora may be arrived at.

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