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Proximate Composition, Antioxidant, Anthelmintic and Insecticidal Activity of a Macrolichen *Ramalina conduplicans* Vain. (Ramalinaceae)

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Abstract: In the present study, we have investigated proximate composition, antioxidant, anthelmintic and insecticidal efficacy of methanolic extract of a macrolichen *Ramalina conduplicans* Vain. (Ramalinaceae). Rich carbohydrate content was found to be present in the lichen material. The fat and crude fibre contents were not so high. The high ash content is suggestive of a rich mineral content. The methanol extract exhibited marked antioxidant activity by scavenging DPPH* (free radical) and converting into DPPHH in a dose dependent manner. In anthelmintic study conducted using adult Indian earthworms, the methanol extract exhibited a dose-dependent inhibition of spontaneous motility. The insecticidal activity of different concentrations of methanolic extract of *R. conduplicans* was studied on second instar larvae of *Aedes aegypti*. It was found that the larval mortality increased with increase in the concentration of extract. Thin layer chromatography revealed the presence of usnic acid, salanizic acid and sekikaic acid. Preliminary phytochemical analysis of methanol extract showed the presence of tannins and steroids. The presence of appreciable amounts of carbohydrates, crude fibre and others is suggestive of the possible use of the lichen studied as diet. The antioxidant, anthelmintic and insecticidal activity of the methanol extract may be due to the presence of these metabolites. Further studies are to be carried to isolate the active constituents and determine their biological efficacy.

Key words:*Ramalina conduplicans* vain · Proximate composition · DPPH free radical assay · Fe^{+3} reducing assay · *Aedes aegypti* · *Pheritima pasthuma*

INTRODUCTION

Lichens are self-supporting symbiotic associations of a fungus and one or several algal or cyanobacterial components. India is a rich center of lichen diversity, contributing nearly 15% of the 13,500 species of lichens so far recorded in the world [1]. Lichens and lichen products have been used in traditional medicines for centuries and still hold considerable interest as alternative treatments in various parts of the world. In various systems of traditional medicine worldwide, including the Indian system of medicine, these lichen species are said to effectively cure dyspepsia, bleeding piles, bronchitis, scabies, stomach disorders and many disorders of blood and heart [2-4]. They produce characteristic secondary metabolites that are unique with respect to those of higher plants [5]. Lichen metabolites exert a wide variety of biological actions including antibiotic, antimycobacterial, antiviral, anti-inflammatory, analgesic, antipyretic, antiproliferative and cytotoxic effects [6]. The utility of lichens is due of range of secondary compounds produced by them. A wide range of secondary metabolites of lichens were characterized. According to their chemical structure, most lichen substances are

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phenolic compounds, dibenzofuranes, Usnic acids, depsidones, depsones, lactones, quinines and pulvunic acid derivatives [7].

Free radicals are found to be a product of normal metabolism. Although oxygen is essential for aerobic forms of life, oxygen metabolites are highly toxic. As a consequence, reactive oxygen species (ROS) are known to be implicated in many cell disorders and in the development of many diseases including cardiovascular diseases, atherosclerosis, chronic inflammation etc [8,9]. Although organisms have endogenous antioxidant defences produced during normal cell aerobic respiration against ROS, other antioxidants are taken both from natural and synthetic origin [10]. Antioxidants that can inhibit or delay the oxidation of an oxidizable substrate in a chain reaction, therefore, appear to be very important [11]. Synthetic antioxidants are widely used but their use is being restricted nowadays because of their toxic and carcinogenic effects. Thus, interest in finding natural antioxidants, without any undesirable effect, has increased greatly [10].

Mosquitoes are the most important single group of insects acting as vector for many tropical and subtropical diseases such as dengue fever, yellow fever, malaria, filariasis, Japanese encephalitis and others [12]. The large-scale use of chemical pesticides in agriculture and public health leads to adverse effects such as development of pesticide resistance, frequent pest out breaks, emergence of new pests, pollution and health hazards. In order to search an environmentally safe alternative, scientists considered the pesticides of biological origin (biopesticides) in the place of synthetic insecticides. Throughout history, plant products have been successfully exploited as insecticides, insect repellents and insect antifeedants [13]. The approach to combat these diseases largely relied on interruption of the disease transmission cycle by either targeting the mosquito larvae through spraying of stagnant water breeding sites or by killing the adult mosquitoes using insecticides [14]. Helminthic infections are among the most common infections in man, affecting a large proportion of the world's population. Today, the principal mode for control of gastrointestinal parasites is based on the commercial anthelmintics. Because of the increasing anthelmintic resistance and the impact of conventional anthelmintics on the environment, it is important to look for alternative strategies against gastrointestinal nematodes [15].

Good nutrition is often a major problem in most developing countries of the world and consequently the cases of under-nutrition are increasing in these countries. To be able to reduce the adverse effect of hunger and or starvation, it is pertinent that some lesser-known sources are investigated for their nutritive value in human or non-ruminant nutrition. Lichens are one of the lesser-known nutritive sources to reduce the malnourishment problems in most of the countries. Bhadra reserve area 75°15'-75°50' E and 13°25'-13°50' N latitude. The area comprises the forests of Western Ghats and its fringes. Sanctuary being situated in the south interior Karnataka, with cool climate throughout the year and affords pleasant days during the hot months. *Ramalina conduplicans* Vain. (Ramalinaceae) is fruticose lichen with thallus corticolous 3-5 cm long, decumbent, greenish grey colour and branched [16]. In the present study, we have investigated proximate composition, antioxidant, anthelmintic and insecticidal efficacy of methanolic extract of *R. conduplicans*.

MATERIALS AND METHODS

Collection and Identification of Lichen Material: The lichen *R. conduplicans* was collected from the trees of Bhadra wildlife sanctuary, Karnataka. The voucher specimen of lichen (Voucher no. KSV/KU01130) was deposited in the Department of Applied Botany, Shankaraghatta for future reference. The dried lichen material was identified based on morphological, anatomical and color tests [16]. Thin layer chromatography in solvent A (180 ml toluene: 60 ml 1,4, dioxine: 8 ml acetic acid) was performed to detect secondary metabolites [17,18].

Extraction and Phytochemical Analysis: For extraction, 20g of powdered lichen material was added to 100 ml methanol, sonicated for 30 minutes and left at room temperature overnight. The extract was filtered over Whatman No 1 filter paper and the filtrate was concentrated under reduced pressure to pasty mass [19]. The condensed solvent extract was subjected to phytochemical screening [20,21].

Determination of Proximate Composition: The nutritive composition of powdered lichen sample was carried out to determine the moisture, ash, fat, crude fibre, protein, carbohydrate and mineral content. The moisture content was determined by drying in an oven at 100°C until constant weight, ash by incineration in a muffle furnace at 550°C for 48 h, Proteins by nitrogen determination using the Kjeldahl method and conversion of nitrogen to

proteins by the factor 6.25. Fat was by Bligh dyer technique, crude fibers by successive digestion of the defatted sample with 0.26 N sulphuric acid and 0.23 N potassium hydroxide solutions. Percentage carbohydrate was calculated using the formula: 100 - (percentage of ash + percentage of moisture + percentage of fat + percentage of protein). Nutritive value was finally determined by: Nutritive value = 4 xpercentage of protein + 9 x percentage of fat + 4 x percentage of carbohydrate [22-24].

Antioxidant Activity of Methanolic Extract by Dpph Radical Scavenging Assay: The antioxidant activity of different concentrations, namely 0.125, 0.250, 0.5 and 1.0 mg/ml, of methanol extract and the Ascorbic acid was tested on the basis of the radical scavenging effect of the stable DPPH free radical activity. 0.002% of DPPH in methanol was used as the free radical. In clean and labeled test tubes, 2 ml of DPPH solution was mixed with 2 ml of different concentrations of methanol extract and standard separately. The tubes were incubated at room temperature in dark for 30 minutes and the optical density was measured at 517 nm using UV-Vis Spectrophotometer. The absorbance of the DPPH control (containing no sample) was also noted [25,26]. The scavenging activity of the extract against the stable DPPH* was calculated using the equation.

$$\text{Scavenging activity (\%)} = A - B / A \times 100$$

Where A was the absorbance of DPPH solution and B was the absorbance DPPH* solution with extract.

Anthelmintic Activity of Methanolic Extract: The anthelmintic assay was performed on adult Indian earthworm *Pheretima pasthuma* due to its anatomical and physiological resemblance with the intestinal roundworm parasite of human beings. Standard drug (Piperazine citrate, 1%) and different concentrations of methanol extract of lichen (5, 10, 15 and 20mg/ml) were prepared in normal saline (0.85%) and poured into respective labeled petriplates (50 ml). Six worms of nearly equal size were introduced into each of the plates. Observations were made for the time taken to paralysis and death of individual worm. Paralysis was said to occur when the worms were not able to move even in normal saline. Death was concluded when the worms lost their motility followed with fading away of their body colors [27]. Death was also confirmed by dipping the worms in slightly warm water. The mortality of parasite was assumed to have occurred when all signs of movement had ceased [15].

Insecticidal Activity of Methanolic Extract: Insecticidal activity of methanolic extract of *R. conduplicans* was tested on second instar larvae of *Aedes aegypti* mosquito. Different concentrations of solvent extracts (5, 10, 15, 20 and 25 mg/ml) were prepared in 10% DMSO and added to sterile labeled beakers containing about 100ml of water. Twenty larvae were placed in each of the beakers containing extracts. A control was kept containing 10 % DMSO. After adding the larvae, the beakers were kept in the growth room maintained at room temperature. The insecticidal effect of extract was determined by counting the number of dead larvae after 24 hours. Dead larvae were identified when they failed to move after probing with a needle in siphon or cervical region. Each test was repeated thrice the percentage of larval mortality was determined [28].

RESULTS

Thin layer chromatography revealed the presence of usnic acid, salanizic acid and sekikaic acid. Preliminary phytochemical analysis of methanol extract showed the presence of tannins and steroids.

The results of proximate analysis showed an appreciable amount of ash, fibre, protein and carbohydrate content in the powdered lichen material (Table 1). The moisture content of lichen material was found to be 16.4%. The lichen material revealed a protein content of 9.1%. The fat (3.3%) and crude fibre (10.1%) content was not so high in the lichen material. The nutritive value was found to be 356 cal/100g of the lichen material. From the results, it is clear that the lichen is not a very good source for protein but is rich in carbohydrate. A high ash content of about 10% suggests that the lichen could contain a rich mineral content.

The result of antioxidant activity of different concentrations of methanolic extract and standard (ascorbic acid) is shown in Table 2. The extract exhibited marked antioxidant activity by scavenging DPPH* (free radical) and converting into DPPHH. The extract has shown concentration dependent radical scavenging activity. Over 50% scavenging activity was observed in all the concentrations of the extract. Radical scavenging activity of standard was higher than that of methanol extract.

The different concentrations of methanol extracts of *R. conduplicans* were evaluated for anthelmintic activity using adult Indian earthworm model. The extract exhibited a dose-dependent inhibition of spontaneous motility (paralysis). With higher doses (10 mg/ml and more) the effects were comparable with that of 1 % piperazine (Table 3).

Table 1: Proximate composition of *R. conduplicans*

Nutritive composition	Value
Moisture (%)	16.4
Ash (%)	10.0
Fibre (%)	10.1
Protein (%)	9.1
Fat (%)	3.3
Carbohydrate (%)	61.1
Nutritive value (cal/100g)	356.0

Table 2: DPPH radical scavenging activity of methanolic extract of *R. conduplicans*

	Radical scavenging activity (in %)	
Concentration (mg/ml)	Methanol extract	Ascorbic acid
0.125	58.19	81.69
0.250	63.88	88.01
0.500	72.61	91.66
1.000	85.41	97.08

Table 3: Anthelmintic activity of methanolic extract *R. conduplicans* and standard drug

Extract	Concentration	Paralysis time	Death time
Methanol extract	05 mg/ml	79	98
	10 mg/ml	59	86
	15 mg/ml	41	63
	20 mg/ml	25	38
Piperazine citrate	1%	78	96

Table 4: Insecticidal activity of methanolic extract *R. conduplicans*

Concentration (mg/ml)	% larval mortality
5	40.00
10	55.00
15	65.00
20	80.00
25	100.00

The insecticidal activity of different concentrations of methanolic extract of *R. conduplicans* is presented in Table 4. It is evident from the result that the extract has caused dose dependent mortality of larvae. At concentrations 1mg/ml and more, over 50% mortality of larvae was observed. The larval mortality was recorded as 100% at 25mg/ml concentration of extract.

DISCUSSION

Free radicals are chemical species containing one or more unpaired electrons that makes them highly unstable and cause damage to other molecules by extracting electrons from them in order to attain stability [29]. Free radicals contribute to more than one hundred disorders in humans including atherosclerosis, arthritis, ischemia and reperfusion injury of many tissues, central nervous system injury, gastritis, cancer and AIDS [30,31]. In recent years much attention has been devoted to natural antioxidant and their association with health benefits [29]. There are several methods available to assess antioxidant activity of compounds. An easy, rapid and sensitive method for the antioxidant screening of plant extracts is free radical scavenging assay using 1,1, diphenyl-2-picryl hydrazyl (DPPH) stable radical spectrophotometrically. In presence of an antioxidant, DPPH radical obtains one more electron and the absorbance decreases [32]. In this study, the scavenging activity of methanol extract was found to be dose dependent i.e., higher the concentration, more was the scavenging activity. Though the DPPH radical scavenging abilities of the extracts were less than that of ascorbic acid, the study showed that the extract has the proton-donating ability and could serve as free radical inhibitors or scavenger, acting possibly as primary antioxidant. In the Fe^{+3} reducing assay, the reducing power of crude solvent extract was found to increase with the dose. The reducing capacity of compound may serve as significant indicator of its potential antioxidant activity [33]. The antioxidant activities have been reported to be the concomitant development of reducing power [34].

Helminth infections are among the most common infections in man, affecting a large proportion of the world's population. Parasitoses have been of concern to the medical field for centuries and the helminths still cause considerable problems for human beings and animals. During the past few decades, despite numerous advances made in understanding the mode of transmission and the treatment of these parasites, there are still no efficient products to control certain helminthes and the indiscriminate use of some drugs has generated several cases of resistance. Furthermore, it has been recognized recently that anthelmintic substances having considerable toxicity to human beings are present in foods derived from livestock, posing a serious threat to human health. Consequently, the discovery and development of new chemical substances for helminth control is greatly needed and has promoted studies of traditionally used anthelmintic plants, which are generally considered to be very important sources of bioactive substances [35]. In this study, the anthelmintic activity of methanol extract of *R. conduplicans* may be due to the presence of constituents.

Killing larvae of mosquitoes is a successful way of minimizing mosquito densities in breeding grounds before they reach adult stage. It largely depends on the use of synthetic chemical insecticides. But their repeated use has caused environmental problems and widespread development of resistance. Plants offer an alternative source of insect-control agents because they contain a range of bioactive chemicals, many of which are selective and have little or no harmful effect on non-target organisms and the environment. It is observed that the carbohydrates, saponins, phytosterols, phenols, flavonoids and tannins are having mosquito larvicidal activity [28]. Prenylated xanthones, tetracyclic phenols and saponins are reported to be effective in controlling mosquito *A. aegypti*, the vector of yellow fever [36]. The larvicidal activity of methanol extract of *R. conduplicans* could be mainly due to the presence of various constituents.

Relevant to human existence and living is good nutrition. All human beings require a number of complex organic compounds as added caloric requirements to meet the need for their muscular activities. Carbohydrates, fats and proteins form the major portion of the diet, while minerals and vitamins form comparatively a smaller part. The increasing populations of the world food demands have overwhelmed the available land resources. It has been reported that protein-calories malnutrition deficiencies is a major factor responsible in nutritional pathology [37]. The carbohydrates are main source and store of energy. They are the starting substances for biological synthesis of many compounds. The trace elements, together with other essential nutrients, are necessary for growth, normal physiological functioning and maintenance of life. They must be supplied in the food, since the body cannot synthesize them. Recommended intakes have been set for some trace elements and their deficiency can lead to disease [38,39]. Fibre is the portion that provides structural strength and form. Food fibres have been reported to aid absorption of trace elements in the gut and reduce absorption of cholesterol [40,41]. The dietary fibre plays an important role in decreasing the risks of many disorders such as constipation, diabetes, cardiovascular diseases, obesity etc [42]. Although crude fibre enhances digestibility in animals, the presence of high fibre levels in diet can cause intestinal irritation, lower digestibility and overall decreased utilization [43]. The nutrient composition of lichens varies widely between different species of lichens but they are generally high in carbohydrates and low in most other nutrients. It was found that calcium and iron levels are higher in lichens than cereals and are more comparable to green leafy materials [44]. The results of the present study showed a good carbohydrate composition of the lichen material. Low fat content and considerable crude fibre content is also a positive remark on possible use of the lichen as food. Though protein content is not as high as other sources, it could be used as a protein source. A rich mineral content in the lichen material could be expected as high ash content was detected.

CONCLUSION

The proximate composition of the lichen studied showed appreciable content of carbohydrates and others. The study revealed a marked antioxidant, anthelmintic and insecticidal activity of methanol extract of *R. conduplicans* which may be because of the presence of active constituents in the extract. Further studies on isolation of secondary metabolites and their biological activities *in vitro* and *in vivo* are to be carried out.

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