Phenolic Constituents from the Lichen *Parmotrema stuppeum* (Nyl.) Hale and Their Antioxidant Activity

Guddadarangavvanahally K. Jayaprakasha*,[a] and Lingamallu Jaganmohan Rao[b]

[a] Human Resource Development
[b] Plantation Products, Spices and Flavour Technology Central Food Technological Research Institute, Mysore-570 013, India. Fax: +91–0821–517233. E-mail: gkjp@cscftri.ren.nic.in; gkjp@yahoo.com

* Author for correspondence and reprint requests

Z. Naturforsch. **55 c**, 1018–1022 (2000); received July 28/August 30, 2000

Phenolic Acids, Antioxidant Activity, β-Carotene-linoleate Model System

Lichen, *Parmotrema stuppeum (P. stuppeum)* was successively extracted with benzene and acetone. Both the extracts were fractionated on 1% oxalic acid impregnated silica gel column to obtain four phenolic compounds. The structures of compounds were identified by ^{1}H and ^{13}C NMR spectra as methyl orsenillate, orsenillic acid, atranorin and lecanoric acid respectively. Antioxidant activity of benzene extract, acetone extract and isolated compounds were evaluated in a β-carotene-linoleate model system. The pure compounds showed moderate antioxidant activity. This is the first report on the isolation and characterisation of compounds from the lichen *P. stuppeum* as well as on their antioxidant activity.

Introduction

Lichens constitute a class of small perennial plants, which are a combination of two organisms-a fungus and an alga-growing together in symbiotic association. Lichens are widely distributed from the arctic to the tropics and are found on soil, barren rocks and tree trunks. Several lichens possess medicinal properties and a few are consumed as delicacies. Certain lichens containing volatile oil were used in perfumery and cosmetic industries. Lichens were formerly used as sources of fermentable sugars for the production of ethyl alcohol. *Parmotrema stuppeum is* abundantly growing foliose lichen in South India (The Wealth of India, 1962).

Antioxidants protect the quality of foods by retarding oxidative breakdown of the lipid components (Shahidi *et al.*, 1994). Commercial antioxidants are generally synthetic compounds and there has been a growing interest in replacing them with natural ingredients (Chang *et al.*, 1977). Due to the possible toxicity of synthetic antioxidants there has been an increasing interest in preparing antioxidants from natural sources. The use of natural antioxidants in food is limited due to lack of knowledge about their molecular compositions, the content of active compounds in the raw materials and the availability of relevant toxicological data. Hence, evaluation of the antioxidative activity of naturally occurring substances has been of interest in recent years (Amarowicz, 1996). This study was carried out to identify the major constituents of the lichen *P. stuppeum* and the antioxidant activity of crude extracts and purified compounds. This is the first report on the isolation and characterization of compounds **1–4** from *P. stuppeum* and their antioxidant activity as well.

Materials and Methods

Materials

All solvents / chemicals used were of analytical grade and obtained from Merck, Mumbai, India. β-Carotene, linoleic acid and butylated hydroxyanisole were obtained from Sigma Chemical Co., (St. Louis, MO, USA). Visible spectra were recorded using Genesys-5-UV-visible Spectrophotometer (Milton Roy, NY, USA). ^{1}H and ^{13}C NMR spectra were recorded at 400 and 100 MHz, respectively, on a Bruker AMX 400 FT instrument (Bruker, Rheinstetten, Germany). ^{13}C NMR spectral assignments were given on the basis of spin-echo fourier transform spectra. Tetramethyl silane was used as internal standard.

Source of lichen

The lichen sample was collected from a local market. The species was identified by International Mycological Institute (Egham, Surrey, U. K.) as *Parmotrema stuppeum* (Nyl.) Hale. A voucher specimen was deposited in the reference collection centre (International Mycological Institute, Egham, Surrey, U. K.) (IMI No. 367183).

Extraction and fractionation of lichen compounds

Dried *Parmotrema stuppeum* was powdered (50 g) and successively extracted in a soxhlet ex-

0939–5075/2000/1100–1018 $ 06.00 © 2000 Verlag der Zeitschrift für Naturforschung, Tübingen · www.znaturforsch.com · D

Dieses Werk wurde im Jahr 2013 vom Verlag Zeitschrift für Naturforschung in Zusammenarbeit mit der Max-Planck-Gesellschaft zur Förderung der Wissenschaften e.V. digitalisiert und unter folgender Lizenz veröffentlicht: Creative Commons Namensnennung-Keine Bearbeitung 3.0 Deutschland Lizenz.

This work has been digitalized and published in 2013 by Verlag Zeitschrift für Naturforschung in cooperation with the Max Planck Society for the Advancement of Science under a Creative Commons Attribution-NoDerivs 3.0 Germany License.

Zum 01.01.2015 ist eine Anpassung der Lizenzbedingungen (Entfall der Creative Commons Lizenzbedingung „Keine Bearbeitung") beabsichtigt, um eine Nachnutzung auch im Rahmen zukünftiger wissenschaftlicher Nutzungsformen zu ermöglichen.

On 01.01.2015 it is planned to change the License Conditions (the removal of the Creative Commons License condition "no derivative works"). This is to allow reuse in the area of future scientific usage.

tractor with benzene and acetone for 8 h each. The extracts were filtered and evaporated in vacuum yielded 1.25 and 0.8 g (w/w). TLC of benzene and acetone extracts showed four spots with different concentrations. Hence, both the extracts were mixed and loaded onto 1% oxalic acid impregnated silica gel column. Compounds **1–4** were eluted with hexane:benzene (3:1 v/v), benzene, 2%, 5% ethylacetate in benzene yielded 200, 150, 650 and 800 mg (w/w) respectively.

Identification of compounds

The melting points of compounds **1–4** were recorded as 139–40, 174–75, 187–88 and 173–74° C respectively. TLC of purified compounds with an acid-free developing solvent (benzene: EtOAc 95:05 v/v) on silica gel containing 1% oxalic acid was carried out and the compounds are visualised as yellow spots when sprayed with 10% sulfuric acid in methanol followed by heating at 110° C. The R_f values of compounds **1–4** were found to be 0.65, 0.31, 0.93 and 0.27 respectively. Further, the structures of isolated compounds were confirmed by ^1H and ^{13}C NMR spectra (Tables I and II).

Antioxidant assay by β-carotene system

The antioxidant activity of extracts, pure compounds was evaluated by the β-carotene-linoleate model system according to Hidalgo *et al.* (1994) with slight modification (Jaganmohan Rao *et al.*, 1998). 0.2 mg of the β-carotene in 0.5 ml of chloroform, 20 mg of linoleic acid and 200 mg of Tween-40 (polyoxyethylene sorbitan monopalmitate) were mixed together. The chloroform was removed at 40° C under vacuum using a rotary evaporator. The resulting solution was immediately diluted with 10 ml of triple-distilled water and the emulsion was mixed well for 1 min. The emulsion was further diluted with 40 ml of oxygenated water before use. 4 ml aliquots of this mixture were transferred into different tubes containing 0.2 ml of test extracts and pure compounds in ethanol butylated hydroxyanisole was used for comparative purposes. A control containing 0.2 ml of ethanol and 4 ml of the above mixture was prepared. Optical density (OD) at 470 nm were taken for the all extracts and pure compounds immediately (t = 0) at 15 min intervals for 1.5 h (t = 90). The tubes were incubated at 50° C in a water bath. All determinations were performed in triplicate. Measurement of OD was continued until the colour of β-carotene disappeared in the control (Figs 1 and 2). The antioxidant activity (AA) of the extracts was evaluated in terms of bleaching the β-carotene using the following formula of Hidalgo *et al.* (1994). $AA = 100[1-(A_o-A_t)/(A°_o - A°_t)]$ where A_o and $A°_o$ are the absorbance values (ODs) measured at zero time of the incubation for test sample and control, respectively. A_t and $A°_t$ are the absorbance measured in the test sample and control, respectively, after incubation for 90 min.

Results and Discussion

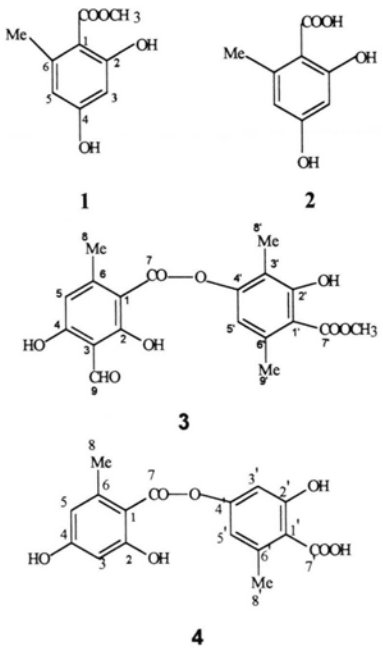
Parmotrema stuppeum was successively extracted using benzene and acetone. Fractionation of benzene and acetone extracts on oxalic acid impregnated silica gel column chromatography yielded four crystalline compounds. The compounds showed a single spot on TLC. It was noticed that without using acidic medium, tailing of spots was observed on the TLC plates and pure compounds could not be obtained using silica gel column chromatography. Therefore silica gel containing 1% oxalic acid was used for TLC and column chromatography. The compounds **1–4** were characterised and identified as methyl orsenillate, orsenillic acid, atranorin and lecanoric acid respectively, using ^1H NMR and ^{13}C NMR spectra (Tables I and II, Scheme I). Chemical shifts of compounds were compared with reported values (Witiak *et al.*, 1967; Devlin *et al.*, 1971; Sundholm and Huneck, 1980, 1981).

The antioxidant activity of benzene extract, acetone extract and isolated compounds **1–4** at 200 and 500 μg/ml concentrations were compared with butylated hydroxyanisole is presented in Figs. 1 and 2. It shows the decrease in absorbance of β-carotene in the presence of lichen extracts/pure compounds and BHA with the coupled oxidation of β-carotene and linoleic acid. The addition of extracts compounds **1–4** and butylated hydroxyanisole at 200 and 500 μg/ml concentrations prevents the bleaching of β-carotene to different degrees. β-Carotene in this model system undergoes rapid discoloration in the absence of an antioxi-

Table I. ^{1}H NMR spectral data of compounds **1, 2, 3**, and **4** (400 MHz).

H	**1***	**2***	**3****	**4***
1	3.79 ($COOCH_3$)	10.11 (COOH)	–	–
2	10.73 (OH)	13.40 (OH)	12.50 (OH)	10.48 (OH)
3	6.15 (H)	6.12 (H) d (2.0)	–	6.22 (H)
4	9.98 (OH)	12.13 (OH)	11.95 (OH)	10.47 (OH)
5	6.17 (H)	6.17 (H) d (1.5)	6.52 (H)	6.62 (H)
6	2.28 (CH_3)	2.39 (CH_3)	–	–
8			2.54 (CH_3)	2.35 (CH_3)
9			10.36 (CHO)	–
1′			–	–
2′			12.55 (OH)	10.33 (OH)
3′			–	6.60 (H)
4′			–	–
5′			6.40 (H)	6.61 (H)
6′			–	–
7′			3.98 ($COOCH_3$)	10.01 (COOH)
8′			2.68 (CH_3)	2.37 (CH_3)
9′			2.09 (CH_3)	–

Chemical shifts are followed by coupling constants J (in Hz): values in parentheses.
*: DMSO-d_6.
**: $CDCl_3$.
d: doublets.



Scheme I.
Compounds determined in the *Parmotrema* extract
(1) Methyl orsenillate
(2) Orsenillic acid
(3) Atranorin
(4) Lecanoric acid

Table II. ^{13}C NMR spectral data of compounds **1, 2, 3,** and **4** (100 MHz).

C	**1***	**2***	**3****	**4***
1	104.8	102.9	107.3	108.2
2	164.4	169.1	161.2	160.2
3	100.5	108.7	100.5	100.5
4	100.5	167.5	161.5	161.5
5	161.9	142.9	110.3	109.9
6			140.4	140.4
7			169.6	167.2
8			25.4	167.2
9			193.7	–
1′			116.8	116.4
2′			162.9	158.7
3′			110.4	107.4
4′			152.3	152.3
5′			116.0	114.8
6′			139.8	139.6
7′			172.1	170.7
8′			23.8	21.0
9′			9.3	
$COO\mathbf{C}H_3$	51.8	–		
COOH	–	173.2		
$\mathbf{C}OOCH_3$	170.3	–		
CH_3	22.1	23.4		

* DMSO-d_6.
** $CDCl_3$.

dant. This is because of the coupled oxidation of β-carotene and linoleic acid, which generates free radicals. The linoleic acid free radical formed upon the abstraction of a hydrogen atom from one of

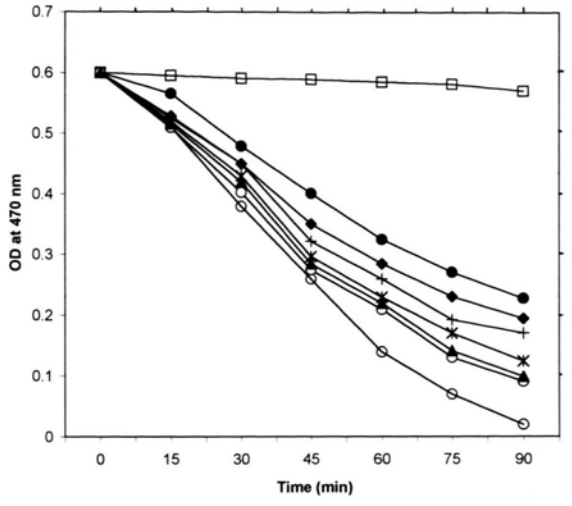


Fig. 1. Antioxidant activity of lichen extracts, compounds and butylated hydroxyanisole assayed by β-carotene-linoleate model system at 200 μg/ml concentration.

—○— Control —□— Butylated hydroxyanisole
—◆— Benzene extract —●— Acetone extract
—✳— Methyl orsenillate —+— Orsenillic acid
—○— Lecanoric acid —▲— Atranorin

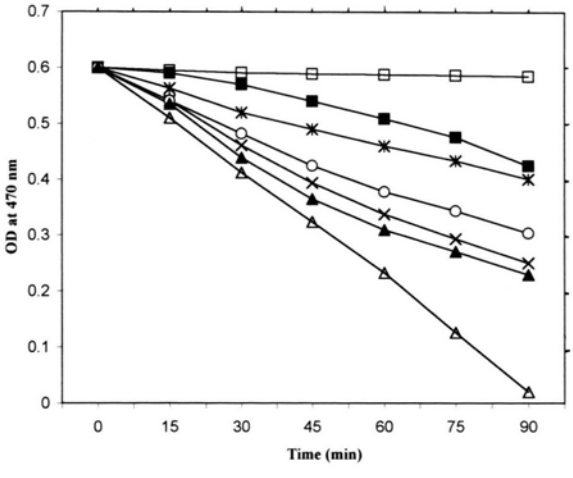


Fig. 2. Antioxidant activity of lichen extracts, compounds and butylated hydroxyanisole assayed by β-carotene-linoleate model system at 500 μg/ml concentration.

—△— Control —□— Butylated hydroxyanisole
—■— Benzene extract —✳— Acetone extract
—✳— Methyl orsenillate —○— Orsenillic acid
—▲— Lecanoric acid

Table III. Antioxidant activity (AA) of compounds **1–4** and different crude extracts evaluated from protection of β-carotene at 90 min.

Compounds/extracts	% AA at 200 μg/ml	% AA at 500 μg/ml
Butylated hydroxyanisole	93	96
Methyl orsenillate (**1**)	18	40
Orsenillic acid (**2**)	26	50
Atranorin (**3**)	14	*
Lecanoric acid (**4**)	12	36
Benzene extract	30	65
Acetone extract	35	68

* Not determined due to precipitation of compound at higher concentration.

its diallylic methylene groups attacks the highly unsaturated β-carotene molecules. As a result, β-carotene will be oxidised and broken down in part, subsequently the system looses its chromophore and characteristic orange colour, which can be monitored spectrophotometrically. The extracts and the isolated compounds **1–4** can hinder the extent of β-carotene bleaching by neutralising the linoleate free radical and other free radicals formed in the system. Extracts and compounds **1–4** showed 12–35% and 36–68% antioxidant activity at 200 and 500 μg/ml, respectively. The data (Table III) show that the extracts have a better antioxidant activity than the purified compounds **1–4**. Individual compounds showed less activity than the acetone extract. Hence, the antioxidant activity of benzene and acetone extracts may be due to a synergistic/cumulative effect of all the compounds.

Compound **2** showed maximum antioxidant activity at 200 and 500 μg/ml compared to compounds **1, 3** and **4**. Compound **1** showed moderate antioxidant activity due to the presence of an electron-attracting group (-$COOCH_3$). In compounds **3** and **4** the electron-attracting property increases due to the two hydrogen bonds between 2′-OH and 1′-$COOCH_3$/COOH groups and 2-OH and 1-COO- groups and also due to the presence of the electron-attracting property of the COO- group that is conjugated with an aromatic ring. Hence, the antioxidant activity of these compounds decreases (Hong-Yu Zhang, 1999). Also, in compound **3** an additional hydrogen bond between the 4-OH and 3-CHO groups and the presence of an electron-attracting group (-CHO) in ortho position to -OH has no significant effect on the antiox-

idant activity compared to compound **4** (Table III). A similar trend of antioxidant activity of lichen compounds was observed by Hidalgo *et al.* (1994).

Acknowledgements

We wish to thank Dr. V. Prakash, Director and Dr. K. K. Sakariah, Head, Human Resource Development, Central Food Technological Research Institute, Mysore for their constant encouragement.

Amarowicz R., Wanasundra U. N., Karamac M. and Shahidi F. (1996), Antioxidant activity of ethanolic extract of mustard seed. Nahrung, **40,** 261–263.

Chang S. S., Ostric-Matyasevic B., Hsieh O. A. L. and Huang C. L. (1977), Natural antioxidants from rosemary and sage. J. Food Sci. **42,** 1102–1106.

Devlin J. P., Falshaw C. P. and Ollis W. D. (1971), Photochemical examination of the lichen *Lecanora rupicola* (L.) Zahlbr. J. Chem. Soc. (C), 1318–1323.

Hidalgo M. E., Fernandez E., Quilhot W. and Lissi E. (1994), Antioxidant activity of depsides and depsidones. Phytochemistry **37,** 1585–1588.

Hong-Yu Zhang (1999), Theoretical methods used in elucidating activity differences of phenolic antioxidants. J. Amer. Oil Chem. Soc. **76,** 745–748.

Jaganmohan Rao L., Jayaprakasha G. K. and Sakariah K. K. (1998), Antioxidant activity of natural flavidin. Submitted to Patent, Intellectual Property Management Division, CSIR, New Delhi. No. NF. 332/98, dt. 23/02/1998.

Shahidi F., Wanasundra U. N. and Amarowicz R. (1994), Natural antioxidants from low-pungency mustard flour. Food Res. Intern. **27,** 489–493.

Sundholm E. G. and Huneck S. (1980), ^{13}C NMR-Spectra of lichen depsides, depsidones and depsones 1. Compounds of the orcinol series. Chemica Scripta **16,** 197–200.

Sundhohn E. G. and Huneek S. (1981), ^{13}C NMR-Spectra of lichen depsides, depsidones and depsones 2. Compounds of the β-orcinol series. Chemica Scripta **18** 233–236.

The Wealth of India: A Dictionary of Indian Raw Material and Industrial Products (Thacker M. S., Lala Shri R., Krishnan M. S., Baini Prashad, Chopra R. N., Santapau H. and Sastri B. N., ed.). Publications and Information Directorate, Council of Scientific & Industrial Research, New Delhi 1962, **3,** p.81–90.

Witiak D. T., Patel D. B. and Lin Y. (1967), Nuclear magnetic resonance. Influence of substituents on the long-range spin-spin coupling constant between benzylic and ring protons in the orcinol series. J. Amer. Chem. Soc. **89,** 1908–1911.