Glucosidase Inhibitory and Radical Scavenging Properties of Lichen Metabolites Salazinic Acid, Sekikaic Acid and Usnic Acid

Liken Metabolitleri Salazinik, Sekikaik ve Usnik Asitin Glikosidaz Engelleyici ve Radikal Süpürücü Özelliği

Research Article

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ABSTRACT

This paper reports α- and β-glucosidase inhibitory and radical scavenging potential of the metabolites; salazinic acid, sekikaic acid and usnic acid isolated from three terrestrial natural lichen species *Ramalina celastri, Ramalina nervulosa* and *Ramalina pacifica* of the genus *Ramalinaceae*. Lichen metabolites showed both glucosidase inhibitory and radical scavenging capacity. Half-inhibiting concentration (IC_{50}) values of the lichen metabolites; salazinic acid, sekikaic acid and usnic acid are 13.8 to 18.1, 13.8 to 14.6 and 17.7 to 18.9 μg/mL for the α- and β-glucosidase inhibition. Scavenging of radicals are found with an IC_{50} values of salazinic acid, sekikaic acid and usnic acid are 17.2 to 20.5, 13.7 to 17.4 and 18.8 to 25.5 μg/mL respectively. Inhibition kinetic studies of the metabolite towards α- and β-glucosidase showed sekikaic acid and salazinic acid have competitive inhibition towards α-glucosidase activity and noncompetitive inhibition for β-glucosidase activity. Usnic acid showed noncompetitive type of inhibition for α-glucosidase and uncompetitive for β-glucosidase activity.

Keywords:
Lichen metabolite, glucosidase inhibition, radical scavenging activity.

ÖZET

Bu makale, *Ramalinaceae ailesine* ait *Ramalina celastri, Ramalina nervulosa* ve *Ramalina pacifica* isimli üç doğal kara likeninden izole edilen salazinik, sekikaik ve usnik asit metabolitlerinin α- ve β-glukosidaz engelleyici ve radikal süpürücü potansiyelini anlatmaktadır. Liken metabolitleri her iki glukosidaz engelleme ve radikal süpürücü kapasiteye sahiptir. α- ve β- glukosidaz inhibisyonu için liken metabolitlerinin yarı inhibisyon derişimi (IC_{50}) değerleri 13.8-18.1, 13.8-14.6 ve 17.7-18.9 μg/mL'dir. Salazinik, sekikaik ve usnik asitin IC_{50} radikal süpürme değerleri sırasıyla 17.2-20.5, 13.7-17.4 ve 18.8-25.5 μg/mL olarak bulunmuştur. Metabolitlerin α- ve β-glukosidaza yönelik inhibisyon kinetik çalışmaları sekikaik ve salazinik asitin α-glukosidaz aktivitesine yarışmalı ve β-glukosidaz aktivitesine yarışmasız inhibisyon yaptıklarını göstermektedir. Usnik asit α-glukosidaz aktivitesi için yarışmasız ve β-glukosidaz aktivitesi için yarışma dışı inhibisyon göstermiştir.

Anahtar Kelimeler
Liken metabolit, glukosidaz inhibisyonu, radikal süpürme aktivitesi

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INTRODUCTION

Alpha-glucosidase (EC 3.2.1.20) is a key enzyme involved in the digestion of dietary carbohydrates in humans. This enzyme hydrolyzes the oligosaccharides and disaccharides into glucose in small intestine, which is absorbed through the gut wall to become the blood glucose [1,2]. Inhibition of α- glucosidase activity reported to be involved in the decrease of glucose levels in plasma, as a result suppression of postprandial hyperglycemia [3]. Therefore, decreasing of glucose level in plasma has been considered as one of the most effective therapeutic approach for type 2 diabetes. Many animal viruses including human immunodeficiency virus (HIV-1), human hepatitis B virus (HBV), human cytomegalovirus virus (HCMV) and influenza virus contains an outer envelope composed of glycoproteins. These glycoproteins are important to complete the virus life cycle and their infectivity [4]. Beta-glucosidase (EC 3.2.1.21) is another enzyme of hydrolases group, reported to be involved in the processing of glycoproteins [5]. Therefore, the study of inhibitors for both α- and β- glucosidase enzymes are of great importance for the treatment of type 2 diabetes and the treatment of viral diseases [6].

Lichens are stable and self supporting symbioses between fungi (the mycobiont) and photoautotrophic algal partners (the photobiont). The natural thalli of lichens have been found to contain a variety of secondary lichen metabolites with strong antioxidant activity along with antibiotic, antimycobacterial, antiviral, anti-inflammatory, antioxidative, analgesic, antipyretic, antiproliferative and cytotoxic effects [7]. The cumulative research evidences with regards to diabetes suggested that diabetic patients are fall under oxidative stress, when an imbalance between the free radical generating and radical scavenging capacities occurs in the body. The increased free radical production and reduced antioxidant defense in the system may partially mediate the initiation and progression of diabetes associated complications [8-10]. Again, many reports show that the phenolic compounds having antioxidant activity have the potential to inhibit the glucosidase activity [11]. In search of natural antioxidant compound from the lichens we have screened many natural lichens and their derivative cultures or their compounds in the laboratory for the antioxidative potential along with various biological activities. All our experimental results on lichens/their metabolites indicated that they can be used as novel bioresource for natural antioxidants [12-20].

Our extensive literature survey with respect to glucosidase inhibition potential of lichens or their metabolites, we found hardly one or two reports. It implies that there were not much work done in this aspects of lichens or their metabolites. Therefore, we have undertaken this study to find out lichens or their metabolites having glucosidase inhibitory activity along with radical scavenging potential of three terrestrial lichen species of *Ramalina* eg. *Ramalina celastri*, *Ramalina nervulosa, Ramalina pacifica* and their metabolites salazinic acid, sekikaic acid and usnic acid.

MATERIAL AND METHODS

Chemicals

Linoleic acid, 1,1-diphenyl-2-picryl-hydrazil (DPPH), α-glucosidase, β-glucosidase, p-nitrophenyl-α-D-glucopyranoside, p-nitrophenyl-β-D-glucopyranoside, potassium hexacyanoferrate (III), folin-ciocalteu reagent, coomassie brilliant blue G–250, peroxidase, nitroblue tetrazolium (NBT), nicotinamide adenine dinucleotide (NADH), phenazine methosulphate (PMS), sodium nitroprusside, sulfanilamide and napthylethylenediamine dihydrochloride were procured from Hi-Media Chemicals, India. 2,2′-azinobis (3-ethylbenzothiazoline-6-sulfonate (ABTS), 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox), ascorbic acid, 2,2′-azobis,2-amidineopropane dihydrochloride (AAPH), butylated hydroxyanisole (BHA), butylated hydroxytoluene (BHT), acarbose, castanospermine and quercetin were purchased from Sigma-Aldrich Chemical, USA. All other routine chemicals used were of analytical grade.

Lichen material

Fresh thallus of natural lichen *Ramalina celastri* (voucher no. AMH 94.51) producing usnic acid, *Ramalina nervulosa* (voucher no. AMH 09.514) producing sekikaic acid & usnic acid, *Ramalina*

pacifica (voucher no. AMH 09.513) producing salazinic acid & usnic acid under natural condition were collected from Bandipur National Park in Chamarajanagar District of Karnataka State, India. The GPS location was N11^{0}40.861′ E076^0 37.658′ Elevation 3132 ft. A part of the material of lichen species used for the studies has been preserved as specimen in the Ajrekar Mycological Herbarium (AMH) at Agharkar Research Institute, Pune, India.

Identification of lichen substances by TLC and HPLC

Lichen substances present in the species *R. celastri, R. nervulosa* and *R. pacifica* were identified with routinely used standardized method of TLC [21] by using standard solvent system TDA (toluene: 1-4-dioxane: acetic acid; 180 mL: 45 mL: 5 mL, total volume 230 mL). The identification of lichen substances was made by comparison with the standard lichen substances and sample of several species containing salazinic acid, sekikaic acid and usnic acid and corresponding natural thallus. As these lichen metabolites are not available commercially, therefore, these metabolites were isolated manually from the lichen species by several round of preparative TLC. The purity of the isolated metabolites was checked by TLC and HPLC [22] before made into standard concentration. The HPLC analysis was carried out on Agilent 1100 system with autosampler, Zorbax Eclipse XDB-C8 (4.6 mm x 150 mm, 5 μm) column and UV detector, at 28^{0}C with solvent methanol : water : phosphoric acid (80:19:1 v/v/v). The detection wavelength was 245 nm and the injection volume was 10 μl with a flow rate of 1 mL/ min. Lichen metabolites were identified by their peak symmetry and their retention time (r_t), by comparison with authentic substances made to standard concentration and also comparison with the literature for the ultraviolet spectra of the chromatographic peaks [23-25] confirmed our isolated compounds are salazinic acid, sekikaic acid and usnic acid. The retention times (r_t) were recorded 5.077 min for salazinic acid, 8.792 for sekikaic acid and 7.469 min for usnic acid respectively (Scheme 1). These pure compounds were used for further experiments to explore their biological activities potential.

Preparation of extract for the testing of biological activities

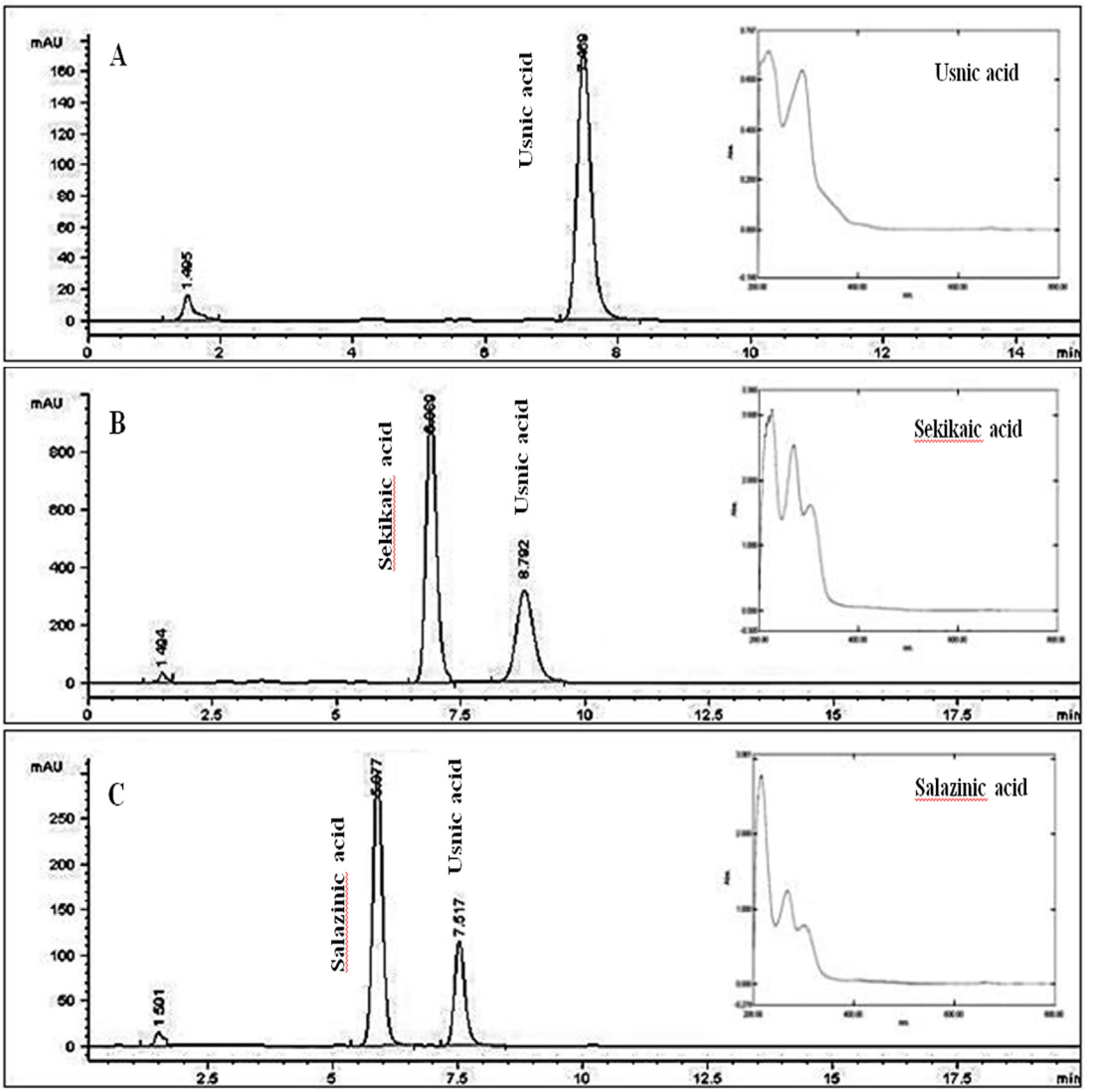
The thallus of lichen *R. celastri, R. nervulosa* and *R. pacifica* were separated from the tree bark and washed by keeping over night under flow of tap water and further by distilled water. The cleaned thallus was cut in to small pieces and then placed in the Soxhlet apparatus for the extraction. Different organic solvents; acetone, ethanol, methanol of 10% concentration were used for successive fractionation extraction and resulted extracts were filtered by Whatman filter paper (No.1) and then dried in vacuo. Only in the case of extraction with dimethyl sulphoxide (DMSO), the lichen thallus was soaked in 10% DMSO over night and resulted extract was filtered and dried as above. The dry extract obtained from different solvents acetone, ethanol, methanol, DMSO of *R. celastri* (23.93 mg, 67.4 mg, 95.7 mg, 270.9 mg); *R. nervulosa* (100.3 mg, 135.2 mg, 180.6 mg, 304.9 mg) and *R. pacefica* (17.3 mg, 21.1 mg, 29.6 mg, 67.4 mg) were stored at 4^{o}C for further work.

TLC bioautography for rapid determination of glucosidase inhibition and scavenging of radicals

Thin layer bioautography method [6] was used for rapid determination of glucosidase inhibitory activity in lichen *R. celastri, R. nervulosa* and *R. pacifica*. Radical scavenging property of above lichen species have been determined by the thin layer bioautography method [18,26].

Glucosidase inhibitory activity of lichens Alpha- Glucosidase inhibition assay

The α- glucosidase inhibitory activity of solvent extracts of lichen *R. celastri, R. nervulosa* and *R. pacifica* were determined according to the spectrophotometric method [27] by monitoring the release of p-nitrophenol from p-nitro phenyl-α-D-glucopyranoside subs-trate in UV visible spectrophotometer (UV-1601, Shimadzu, Japan). Briefly, the reaction mixture containing 20 mU of α- glucosidase in 50 mM acetate buffer (pH 6) and lichen extract (200 μl) was incubated at 37^{0}C for 10 min. 0.5 mL of 2 mM p-nitro phenyl-α-D-glucopyranoside was added to the mixture and again incubated for 10 min at 37^{0}C. The reaction was terminated by adding 0.9 mL of 0.2 M sodium carbonate. The release of p-nitrophenol during the



Scheme 1. HPLC chromatogram of lichen compounds of (A) *R. celastri*, (B) *R. nervulosa*, (C) *R. pacefica* and UV spectrum of usnic acid, sekikaic acid and salazinic acid.

reaction was measured at 405 nm. The reaction mixture containing α- glucosidase, acetate buffer in place of lichen extract and substrate p- nitro phenyl-α-D-glucopyranoside is served as positive control. The negative control, 0.2 M sodium carbonate was added at the beginning of the reaction to block enzyme activity. Acarbose a commercially available α-glucosidase inhibitor was used for comparison with the inhibition potential of the lichen extracts. The percentage inhibition was calculated using the following formula:

$$\text{Inhibition (\%)} = 1 - \frac{\text{Final (Abs test sample} - \text{Abs negative control)}}{\text{Abs positive control}} \times 100$$

Beta- Glucosidase inhibition assay

The β- glucosidase inhibitory activity of solvent extracts of lichen *R. celastri, R. nervulosa* and *R. pacifica* were measured spectrophotometerically [5]. Briefly, a 2 mM p-nitro phenyl-β-D-glucopyranoside (0.5 mL), 0.2 mL lichen extract (20μg/mL), 50 mM potassium phosphate buffer (0.3 mL) pH 5 were placed in a test tube and incubated at 37°C for 10 min in a water bath. A 20 mU of enzyme β-glucosidase was added in the mixture and incubated at 37°C for 30 min. After completion of incubation period, the enzymatic reaction was terminated by addition of 2.6 mL of potassium phosphate buffer (50 mM) pH 10. Reaction mixture containing 50 mM potassium phosphate buffer pH 5 in place of lichen extract

Table 1. Glucosidase inhibitory and radical scavenging activity; phytochemical content: polysaccharide content, protein/polysaccharide ratio and total polyphenol content of lichen extracted in various solvents. Data presented are the average of three parallel readings of the extract (20 μg/mL) in each assay system.

	Radical scavenging activity					Glucosidase inhibitory activity		Phytochemical content		
Solvent extracts of natural lichens	DRS (%)	ALP (%)	SAS (%)	NOS (%)	TEAC (mM)	α- GI (%)	β- GI (%)	Polysaccharide content (mg/g dry wt)	Protein/ polysaccharide ratio	Total polyphenol content (mg/g dry wt)
Ramalina celastri										
Acetone	50.26	33.8	25.9	29.2	0.8	31.93	42.32	0.56	0.78	1.83
Ethanol	48.04	35.5	34.8	36.1	1.1	47.13	48.75	0.85	1.06	2.91
Methanol	52.43	53.9	53.3	66.8	2.8	69.97	61.96	0.95	1.58	7.68
DMSO	46.03	41.6	41.8	40.2	1.7	56.29	53.93	1.10	1.87	1.43
Ramalina nervulosa										
Acetone	61.53	40.9	39.8	53.4	0.9	51.33	49.51	0.64	1.01	5.43
Ethanol	60.46	54.9	47.9	69.9	0.3	63.66	67.54	0.77	1.28	8.05
Methanol	81.24	77.8	87.7	85.7	4.9	87.88	89.21	1.10	1.92	17.85
DMSO	62.26	49.6	54.8	79.8	1.8	68.25	68.23	2.66	3.02	9.33
Ramalina pacifica										
Acetone	56.87	48.3	36.4	30.3	0.4	47.65	45.19	0.57	1.06	5.99
Ethanol	50.37	51.7	52.4	51.5	2.2	54.43	51.41	0.59	1.18	6.71
Methanol	79.85	61.6	67.4	77.8	3.8	76.84	69.38	1.36	2.01	12.80
DMSO	53.54	56.9	59.6	65.6	1.1	58.32	58.12	1.91	2.38	6.92
Standard antioxidants										
BHA	76.0	35.4	58.2	45.1	-					
BHT	85.4	47.0	66.9	46.0	-					
Trolox	-	55.3	-	-	3.6					
Quercetin	63.0	40.8	43.1	40.6	-					
Melatonin	57.1	46.5	12.4	31.2	-					
Ascorbic acid	51.0	46.0	10.8	32.0	-					
Standard glucosidase inhibitors										
Acarbose						67.80				
Castanospermine							59.98			

DRS: Free radical-scavenging activity, ALP: Anti-linoleic acid peroxidation, SAS: Superoxide anion-scavenging activity, NOS: Nitric oxide-scavenging activity, TEAC: Trolox-equivalent antioxidant capacity, α- GI: Alpha- glucosidase inhibitory activity, β- GI: Beta- glucosidase inhibitory activity, DMSO: Dimethylsulphoxide, BHA: Butylated hydroxyanisol and BHT: Butylated hydroxytoluene.

was used as positive control and for negative control, phosphate buffer of pH 10 was added at the beginning of the reaction to block enzyme activity. Afterwards, absorbance was read at 410 nm. Castanospermine a commercially available β- glucosidase inhibitor used for comparison of β- glucosidase inhibitory potential of the extracts. The percentage inhibition was calculated using the same formula described above for the calculation of α- glucosidase inhibitory activity.

Radical scavenging potential of lichens

The radical scavenging activity of solvent extracts of lichen species *R. celastri, R. nervulosa* and *R. pacefica* were determined by DPPH radical scavenging assay (DRS) [28], anti-linoleic acid peroxidation assay (ALP) [29], superoxide anion-scavenging assay (SAS) [30], nitric oxide-scavenging assay (NOS) [31] and Trolox-equivalent antioxidant capacity (TEAC) assay [32]. The details of the minor modifications in the above procedures are reported earlier by us [18]. Butylated hydroxyanisole (BHA), butylated hydroxytoluene (BHT), Trolox, a water-soluble vitamin E analogue, quercetin, melatonin and ascorbic acid were used as standard antioxidants.

Phytochemical content estimation

Lichens in nature produce many primary and secondary compounds for their different metabolic activity. In order to know their quantity, we have estimated the phenols, polysaccharide and proteins content of three lichen species. The total soluble phenolics in the lichen extracts were determined with folin-ciocalteu reagent using pyrocatechol as a standard [33]. The total polysaccharide content was determined, using the phenol-sulfuric acid method [34]. The protein content was determined by the coomassie-dye binding method [35]. The details of the above procedures are reported in our previous report [18].

Isolation and purification of lichen metabolites

Methanolic extract of three lichen species *R. celastri, R. nervulosa*, *R. pacifica* showed moderate to high glucosidase inhibitory and radical scavenging activity in comparison to other solvent extracts. Extracts contains many compounds along with lichen substances. Based on the above results we have only isolated three lichen substances salazinic acid, sekikaic acid and usnic acid from three lichen species by preparative TLC. Purification of these lichen metabolites were done by eluting several time using standard solvent system as described in the section of identification of lichen substances by TLC and HPLC. Purity of these lichen metabolites were checked by HPLC.

Glucosidase inhibitory and radical scavenging activity of salazinic acid, sekikaic acid and usnic acid

The glucosidase inhibitory and radical scavenging activity of the lichen metabolites salazinic acid, sekikaic acid and usnic acid were determined using the same *in vitro* assays descried as above. The half inhibitory concentration (IC_{50}); the concentration requires for 50% glucosidase inhibitory and radical scavenging activity of lichen metabolites, commercial glucosidase inhibitors, and standard antioxidants were calculated by extrapolation from concentration/effect regression lines obtained from 3 to 4 different concentrations (2, 5, 10 or 20 μg/mL).

Inhibition kinetics of α- and β- glucosidase enzymes by lichen metabolites

The mechanism of inhibition of α- and β-glucosidase by lichen metabolites were studied. We measured the enzyme activity in the absence or presence of four different concentrations (5, 10, 15 and 20 μg/mL) of each lichen metabolite (salazinic acid, sekikaic acid and usnic acid) with increasing concentration (0.5, 1.0, 1.5 and 2.0 mM) of the respective enzyme substrates p-nitrophenyl-α-D-glucopyranoside and p-nitrophenyl-β-D-glucopyranoside. The type of inhibition of lichen metabolites against the activity of glucosidases were analyzed using double reciprocal Lineweaver-Burk plots.

Glucosidase inhibitory and radical scavenging potential of lichen metabolites under temperature and incubation time period

Glucosidase inhibitory and radical scavenging activity of lichen metabolites at various temperatures or incubation time period were

carried out. Sekikaic acid, salazinic acid, usnic acid were incubated at 4°C, 40°C for 1 h, for 30 days at 20°C and then their glucosidase inhibitory and radical scavenging activities were measured using the same in vitro assays as described above.

Statistical analysis: All the results expressed as the mean ± SD of the three parallel experiments (*n = 3*). Statistical significance was evaluated by Sigma Stat Version 9.0.

RESULTS

In the present study, extracts and the secondary metabolites isolated from the lichen species *Ramalina celastri, Ramalina nervulosa* and *Ramalina pacifica* have been tested for the glucosidase inhibition and scavenging of radicals using various *in vitro* assays.

Glucosidase inhibitory activity

Glucosidase inhibitory potential of various solvent extracts of lichen species are presented in Table 1. Methanol extract of *R. nervulosa, R. pacifica* and *R. celastri* showed high α-glucosidase inhibition (α-GI) activity 87.88%, 76.84%, 69.97% in comparison to other solvent extracts acetone, ethanol, DMSO (31.93% to 68.25%). The two species *R. nervulosa* and *R. pacifica* inhibited α-glucosidase more than 70% and found to be higher than the standard α-glucosidase inhibitor acarbose (67.80%). However, *R. celastri* had shown α-GI activity almost equivalent to the acarbose. As far as inhibition of β- glucosidase (β-GI) is concerned, the methanol extract of *R. nervulosa, R. pacifica* and *R. celastri* showed 89.21%, 69.38% and 61.96% β- GI activity, which was higher than the standard β- glucosidase inhibitor castanospermine (59.98%). However, other solvent extracts showed β- GI activity from 42.32% to 68.23%. The results indicates that, the extract of lichen species studied here have glucosidase inhibition potential.

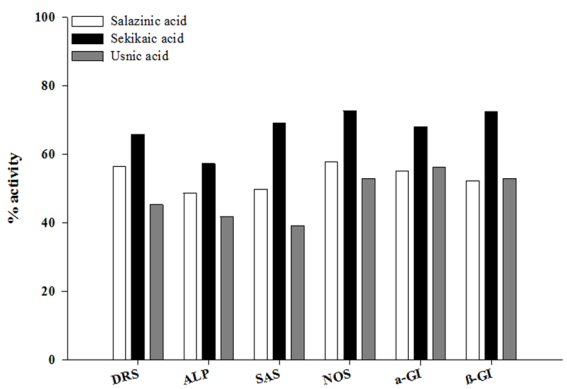


Figure 1. Glucosidase inhibitory and radical scavenging activity of lichen metabolites salazinic acid, sekikaic acid and usnic acid (50 μg/mL).

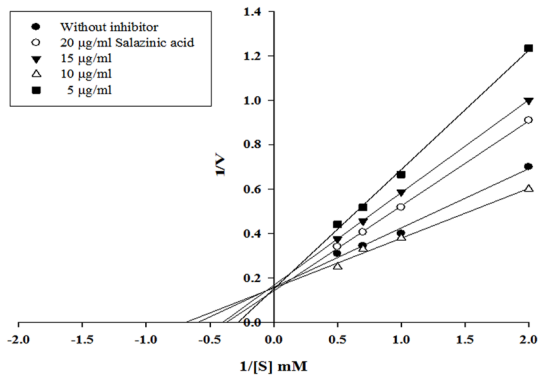


Figure 2. Lineweavr-Burk plot of the α- glucosidase activity in the presence of various concentration of lichen metabolites salazinic acid.

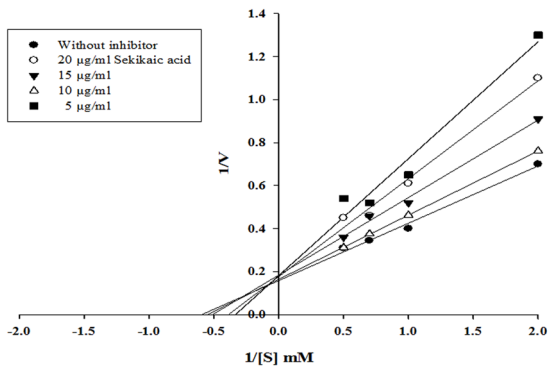


Figure 3. Lineweavr-Burk plot of the α- glucosidase activity in the presence of various concentration of lichen metabolites sekikaic acid.

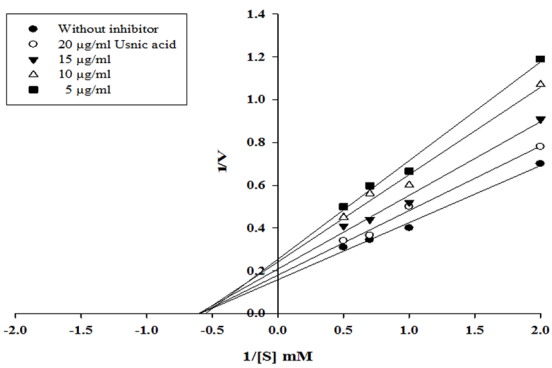


Figure 4. Lineweavr-Burk plot of the α-glucosidase activity in the presence of various concentration of lichen metabolites usnic acid.

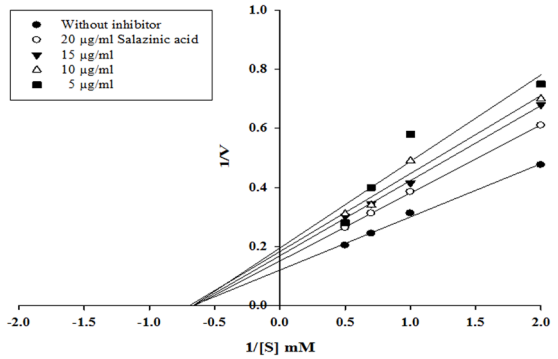


Figure: 5. Lineweavr-Burk plot of the β- glucosidase activity in the presence of various concentration of lichen metabolites salazinic acid.

Radical scavenging potential

Radical scavenging potential of solvent extracts of lichen species; *R. nervulosa, R. celastri, R. pacefica* were evaluated in terms of measuring the scavenging of nitric oxide (NOS), superoxide (SAS), DPPH radical (DRS), Trolox-equivalent antioxidant capacity (TEAC) and anti-linoleic acid peroxidation (ALP) activity. The results are presented in Table 1.

The methanol extract of *R. nervulosa* showed 81.24% DRS activity, which was higher than the activity of lichen *R. celastri, R. pacefica* and standard antioxidants quercetin, melatonin, ascorbic acid, BHA but found lower than the activity shown by BHT (85.4%). The methanol extract of *R. pacifica* has shown 79.85% DRS activity which was also higher or equivalent to the activity of standard antioxidants quercetin, melatonin and ascorbic acid. However, the DRS activity in *R. celastri* was found 50.26% which is lower than the other two species. Other solvent extracts of three lichen species showed the DRS activity lower than the methanol solvent extract. The methanol extract of *R. nervulosa, R. pacifica* and *R. celastri* showed high ALP activity 77.8%, 61.6%, 53.9% respectively in comparison to other solvent extracts (acetone, ethanol, DMSO). The ALP activity of *R. nervulosa* and *R. pacifica* were found to be higher than the activity from 35.4% to 55.3% shown by the standard antioxidants (BHA, BHT, Trolox, quercetin, melatonin and ascorbic acid). Furthermore, the methanol extract of the three lichen species showed superoxide anion scavenging (SAS) activity 53.3% to 87.7%, which was higher than the activity of other solvent extracts acetone, ethanol, DMSO. The methanol extract of *R. nervulosa* showed highest SAS activity (87.7%) and found to be higher than the activity of other lichen species *R. celastri, R. pacifica* and standard antioxidants (BHA, BHT, quercetin, melatonin and ascorbic acid). Scavenging of nitric oxide (NOS) by the three lichen species extracted by the solvent; methanol, acetone, ethanol, DMSO were recorded as 29.2% to 85.7%.

The methanol extract of *R. nervulosa, R. pacifica, R. celastri,* showed 85.7%, 77.8% and 66.8% NOS activity, which were also higher than the other solvent extracts and the standard antioxidants (BHA, BHT, quercetin, melatonin, ascorbic acid). As far as the Trolox-equivalent antioxidant capacity (TEAC) of lichen species extract is concerned, the methanol extract of *R. nervulosa* showed highest TEAC 4.9 mM, which was found to be higher than the other solvent extracts of lichens (*R. pacifica*, *R.*

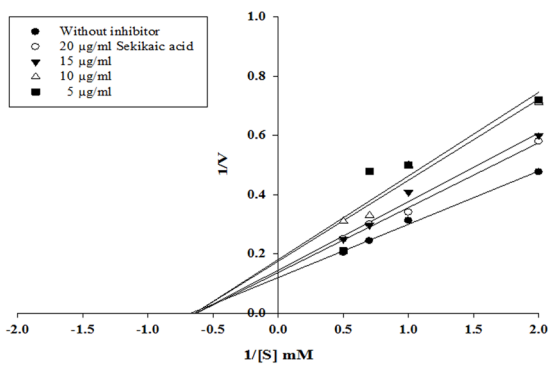


Figure 6. Lineweavr-Burk plot of the β- glucosidase activity in the presence of various concentration of lichen metabolites sekikaic acid.

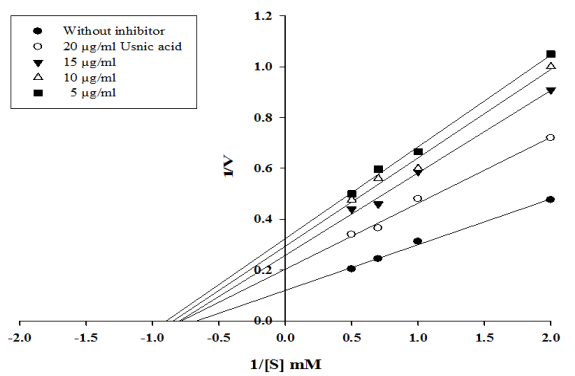


Figure 7. Lineweavr-Burk plot of the β- glucosidase activity in the presence of various concentration of lichen metabolites usnic acid.

celastri) and the standard water soluble vitamin-E analogue Trolox. As far as antioxidative potential of the three lichen species examined here is concerned, *R. nervulosa, R. pacifica, R. celastri* have indicated that they have high or equivalent antioxidative potential as compared to the commercially available standard antioxidants.

Phytochemical content

The total protein, polysaccharide and polyphenol content in the extract of lichen species; *R. nervulosa, R. pacifica* and *R. celastri* are presented in Table 1. The ratio of protein/polysaccharide was found 0.78 to 3.02. The polysaccharide 0.56 mg to 2.66 mg/gm dry wt and polyphenol 1.43 mg to 17.85 mg/gm dry wt, were obtained from *R. nervulosa, R. pacifica, R. celastri*. Only in methanol extract had high polyphenol content in the three species.

Bioactive components in the lichen extract

The TLC bioautography results of the extracts of three lichen species *R. celastri, R. nervulosa* and *R. pacifica* confirms the presence of α- and β-glucosidase inhibitory and radical scavenging compounds along with the presence of monohydroxy and trihydroxy phenolic groups including lichen substances. Furthermore, HPLC and UV spectrophotometric analysis results also confirm the same.

Glucosidase inhibitory and radical scavenging potential in relation to phytochemical content

Several components, such as phenols, polysaccharides and proteins have been isolated from lichens and are reported for various biological activities [36]. Secondary metabolites produce by lichens are phenolic in nature. In very recent reports, Ramkumar et al. [11] reported the glucosidase inhibitory activity in relation to polyphenols present in the plant leaf extract of *Gymnema montanum*. Keeping this in view, we have also tried to determine the correlation between the protein/polysaccharide ratio and polyphenol content with respect to the glucosidase inhibitory and radical scavenging activities. A significant correlation was found between the total polyphenol content and α-GI activity' R^2 = 0.7901; β- GI activity' R^2 = 0.8121; DRS activity' R^2 = 0.7806; ALP activity' R^2 = 0.8765; SAS activity' R^2 = 0.857; NOS activity' R^2 = 0.7216 and with TEAC' R^2 = 0.6033. Protein/ polysaccharide ratio in the three species did not show any significant relationship either with the glucosidase inhibitory or with radical scavenging activities.

Effect of lichen metabolites on glucosidase inhibition and scavenging of radicals

Inhibition of glucosidase and scavenging of radicals by the lichen metabolites salazinic acid, sekikaic acid and usnic acid have been evaluated and the results are presented in Figure 1. Salazinic acid, sekikaic acid and usnic acid showed α- glucosidase inhibition (55.1%, 68.1%, 56.3%) and β- glucosidase inhibition recorded as 52.3%, 72.4%, 52.9% respectively. As far as the antioxidative potential of lichen metabolites are concerned, at 50 μg/mL concentration of salazinic acid showed 48.7% to 57.9%, sekikaic acid 57.3% to 72.7%, usnic acid 39.1% to 53% radical scavenging activity (Figure 1).

Furthermore, IC_{50} value of the lichen metabolites for the observed activities is concerned, the sekikaic acid was found most active (13.7 μg to 17.4 μg/mL), followed by the salazinic (17.2 μg to 20.5 μg/mL) and usnic acid (18.8 μg to 25.5 μg/mL) for 50% radical scavenging activities. 50% glucosidase (α- and β) inhibition was obtained by sekikaic acid at 13.8 μg to 14.6 μg/mL followed by the salazinic acid 13.8 μg to 18.1 μg/mL and usnic acid 13.8 μg to 25.5 μg/mL respectively. IC_{50} concentrations of these lichen metabolites for radical scavenging and glucosidase inhibition activities were found more or less equal with the standard antioxidants (12 μg to 28.2 μg/mL) and 14.7 μg to16.6 μg/mL for standard glucosidase inhibitors (Table 2).

In order to find out the influence of scavenging of radicals on glucosidase inhibition, we have correlated both the activities. Significant correlation was found between the inhibition of glucosidase (α- and β) and radial scavenging activity with the presence of lichen metabolites; sekikaic acid showed correlation (R^2 = 0.9106); salazinic acid (R^2 = 0.9082) and usnic acid (R^2 = 0.9709).

Table 2. Half-inhibiting concentration (IC_{50}) of lichen metabolites usnic acid, sekikaic acid and salazinic acid for the glucosidase inhibitory and radical scavenging activities. Data presented are the mean of three consecutive readings of the sample in assay performed.

	IC_{50}, μg/mL					
	DRS	ALP	SAS	NOS	α-GI	β-GI
Lichen metabolites						
Salazinic acid	17.6	20.5	20.0	17.2	18.1	13.8
Sekikaic acid	15.1	17.4	14.4	13.7	14.6	13.8
Usnic acid	21.8	23.9	25.5	18.8	17.7	18.9
Standard antioxidants						
BHA	12.9	28.2	16.4	22.1	-	-
BHT	12.0	23.0	15.7	21.7	-	-
Quercetin	15.8	24.9	24.0	24.6	-	-
Standard glucosidase inhibitors						
Acarbose					14.7	
Castanospermine						16.6

DRS: DPPH radical-scavenging activity, ALP: Anti-linoleic acid peroxidation, SAS: Superoxide anion-scavenging activity, NOS: Nitric oxide-scavenging activity, α- GI: Alpha- glucosidase inhibitory activity, β- GI: Beta- glucosidase inhibitory activity, BHA: Butylated hydroxy anisol and BHT: Butylated hydroxytoluene.

Enzyme kinetic studies on inhibition of glucosidase by lichen metabolites

Double reciprocal Lineweaver-Burk plots analysis showed that lichen metabolites; salazinic acid, sekikaic and usnic acid have variation in type of inhibition kinetics towards α- glucosidase enzyme (Figure 2, 3, 4). Salazinic acid and sekikaic acid had competitive type of inhibition and non competitive by usnic acid. The V_{max} and K_m value for each test were determined and presented in Table 3. There were no consistent trends observed in the V_{max} values of salazinic acid and sekikaic acid with the increase of metabolites concentration, where as the K_m values were increased with the increase of metabolite concentration. In the case of usnic acid, V_{max} value was found decreased with the increase of usnic acid concentrations (5 μg to 20 μg/mL) and the K_m value were remains almost equal indicating a noncompetitive type of inhibition. However, in β- glucosidase enzyme inhibition; salazinic acid and sekikaic acid showed noncompetitive type of inhibition, as no consistent trend was observed in the V_{max} and K_m values. However, usnic acid had uncompetitive type of inhibition (Figure 5, 6, 7). Consistent decrease in the V_{max} values showed with the increase of usnic acid concentration. The K_m values were found almost same which indicates an uncompetitive type of inhibition of β-glucosidase enzyme activity by the usnic acid.

Stability of lichen metabolites for glucosidase inhibition and radical scavenging potential

As far as the stability of the lichen metabolites towards the glucosidase inhibition and radical scavenging potential are concerned, it was observed that, decrease in the activities was found to be varied among the lichen metabolites salazinic acid, sekikaic acid and usnic acid depending on the temperature and incubation period. The metabolites incubated at lower temperature ≤4°C showed <2% to 3% losses in the activities. However, when we kept these lichen metabolites at ≥40°C showed 19% to 48% losses in their activities. The data of same activities observed at 60°C are not shown. Furthermore, 8% to 17% glucosidase inhibition and radical scavenging activities decreased when these metabolites kept at 20°C for 30 days (Table 4). The results indicated that these metabolites can only be preserved at <4°C for longer period to retain their bioactivities reported here.

DISCUSSION

Carbohydrate hydrolyzing enzymes such as α-glucosidase, β-glucosidase, α-galactosidase, β-galactosidase are involved in a variety of biochemical processes related to metabolic disorders and diseases such as diabetes, viral or bacterial infections, lysosomal storage disorders and cancer [37]. Inhibition of carbohydrate hydrolyzing enzyme α-glucosidase is most important therapeutic approaches to decrease hyperglycemia by retarding absorption of glucose [38]. Further, the effective role of β-glucosidase on virus induced diabetes mellitus has been reported by Yoon et al. [39]. However, most of the synthetic glucosidase inhibitors; acarbose, miglitol voglibose and castanospermine prescribed for the treatment of type 2 diabetis, viral diseases have certain adverse effects such as hypoglycemia at higher doses, liver problems, lactic acidosis and diarrhea [40,41].

Traditional medicinal plants are used as herbal drugs throughout the world as natural anti-diabetic agents to have limited side effects and cost effective compared to prescription drugs even though their biological active compounds and efficacy is unknown. Lichens are valuable plant resources and are used as remedies in folk medicines. In the Ayrvedic system of therapy they are useful in disease of blood, heart, bronchitis, scabies, leprosy, enlarged spleen, burning sensation, bleeding pile, thirst, vomiting, asthma; while in Unani system, lichens finds use in curing inflammations, stomach disorder, dyspepsia, vomiting, pain in liver, amenorrhea, vesicular calculus and many others [42].

Prior to the patent of novel α-glucosidase inhibitors form lichens for treating diabetes, viral infections, fungal infections, autoimmune faction disorders and obesity using compounds by Thadani et al. [43], there were no reports on the anti-hyperglycemic effects of the lichen metabolites. Till date 1050 secondary metabolites have been identified from the lichens but very few of them are reported for the biological activities [42]. Keeping this in view we have studied the α-glucosidase and β-glucosidase inhibitory

Table 3. Kinetic analysis of α- and β- glucosidase inhibition by lichen metabolites. Data presented are the mean of three parallel measurements. The Vmax and Km values were determined through double reciprocal Lineweaver-Burk plot analysis.

	Conc. (μg/mL)	V_{max} (mM/mg protein/min)	Km (mM)	V_{max} (mM/mg protein/min)	Km (mM)
Lichen metabolite		α-glucosidase		β-glucosidase	
Salazinic acid					
	0	0.31	0.56	0.20	0.62
	5	0.34	0.55	0.38	1.10
	10	0.35	0.64	0.31	0.63
	15	0.37	0.73	0.30	0.56
	20	0.34	0.72	0.26	0.62
Sekikaic acid					
	0	0.33	0.58	0.21	0.63
	5	0.54	0.63	0.31	1.60
	10	0.32	0.65	0.31	0.66
	15	0.36	0.66	0.25	0.70
	20	0.45	0.64	0.25	0.60
Usnic acid					
	0	0.33	0.57	0.23	0.64
	5	0.50	0.62	0.50	0.55
	10	0.45	0.61	0.47	0.48
	15	0.41	0.55	0.44	0.53
	20	0.34	0.62	0.34	0.57

Table 4. Biological activities shown by the lichen metabolites incubated under various temperature and time intervals.

Lichen Metabolites	Activities	Temp. 4°C/0 day	Temp. 40°C	Activity decrease (%)	30 day/20°C	Activity decrease (%)
Salazinic acid						
	DRS	56 ± 3.8	29 ± 1.2	27	48 ± 1.3	8
	ALP	48 ± 0.7	18 ± 2.4	30	38 ± 1.1	10
	SAS	49 ± 2.5	21 ± 1.6	28	32 ± 0.7	17
	NOS	57 ± 1.6	31 ± 3.2	26	47 ± 2.5	10
	α-GI	55 ± 2.3	32 ± 1.9	23	42 ± 0.9	13
	β-GI	52 ± 1.5	19 ± 1.3	33	43 ± 0.4	9
Sekikaic acid						
	DRS	65 ± 3.4	34 ± 5.6	31	56 ± 0.6	10
	ALP	57 ± 1.3	36 ± 6.2	21	45 ± 1.7	12
	SAS	69 ± 2.3	21 ± 2.1	48	60 ± 0.9	9
	NOS	72 ± 3.5	28 ± 3.4	44	63 ± 2.1	9
	α-GI	68 ± 1.3	32 ± 4.5	36	58 ± 1.3	10
	β-GI	72 ± 2.6	43 ± 2.5	29	60 ±1.9	12
Usnic acid						
	DRS	45 ± 3.1	26 ± 4.8	19	34 ± 2.1	11
	ALP	41 ± 2.6	19 ± 5.3	22	33 ± 1.7	8
	SAS	39 ± 3.4	14 ± 2.1	25	23 ± 1.8	16
	NOS	53 ± 1.6	23 ± 0.8	30	42 ± 0.5	11
	α-GI	56 ± 2.3	19 ± 1.6	37	42 ± 1.7	14
	β-GI	52 ± 2.4	22 ± 2.2	30	43 ± 2.8	9

DRS: DPPH radical-scavenging activity, ALP: Anti-linoleic acid peroxidation, SAS: Superoxide anion-scavenging activity, NOS: Nitric oxide-scavenging activity, α- GI: Alpha- glucosidase inhibitory activity, β- GI: Beta- glucosidase inhibitory activity.

potential of salazinic acid, sekikaic acid and usnic acid purified from the natural lichen species for anti-hyperglycemic effects. Before studies on the isolated and purified lichen metabolites, we have carried out the preliminary screening of the natural thallus of different lichen species using solvent extraction. The extracts of natural lichen thallus of *R. celastri, R. pacifica, R. nervulosa* showed moderate to high glucosidase inhibitory along with radical scavenging potential depending on the solvent used. Methanol extract showed highest activity in all the assays and found to be suitable for the extraction of glucosidase inhibitor and radical scavenging compounds from the studied lichen species. Crude extract always contains lichen substances along with accessory pigments etc. Probably these biological activities observed may be the synergistic action of those compounds. In order to know which phytochemical component/ active principle playing the vital role of the observed biological activities, we have only isolated and purified the lichen metabolite i.e. salazinic acid, sekikaic acid and usnic acid and then they were evaluated for their antioxidative potential, along with α-glucosidase and β-glucosidase inhibition activity.

These studies shown the IC_{50} value for α-glucosidase inhibition by the salazinic acid, sekikaic acid and usnic acid are 18.1, 14.6 and 17.7 μg/mL respectively. Where as the same acids have shown IC_{50} value for the β-glucosidase inhibition 13.8, 13.8

and 18.9 μg/mL respectively. On comparison with the standard α- glucosidase and β- glucosidase inhibitor, acarbose showed IC_{50} values for α- glucosidase inhibition as 14.7 μg/mL which is less than the α-glucosidase inhibition by salazinic acid and usnic acid. Sekikaic acid has shown α- glucosidase inhibition 14.6 μg/mL which is equivalent to the acarbose standard. Similarly β- glucosidase inhibition by the salazinic acid and sekikaic acid expressed as IC_{50} 13.8 μg/mL which was found to be lesser than the β-glucosidase standard castanospermine 16.6 μg/mL. As far as usnic acid on β- glucosidase inhibition is concerned the IC_{50} value was found little higher side 18.9 μg/mL than the standard castanospermine.

The antioxidative potential of lichen metabolites; salazinic, sekikaic and usnic acid are concerned, in general all the metabolites showed concentration dependant antioxidative potential. Salazinic acid showed 17.2 to 20.5, sekikaic acid 13.7 to 17.4, and usnic acid 18.8 to 25.5 as IC_{50}, μg/mL. Where as standard antioxidant showed the antioxidant activity as less or equivalent. Lankin et al. [44] reported that the severity of disturbance in carbohydrate metabolism in rats with alloxan-induced diabetes in the target organ (pancreas) prevented by the increase of antioxidant enzyme and inhibition of carbohydrate hydrolyzing enzyme activities. In this context, we believe that the compounds having potential in scavenging of radicals along with α- glucosidase and β- glucosidase inhibition may prevent severity of disturbance in the carbohydrate metabolism, and thereby reduce the development of degenerative diseases including diabetes, viral attachment and cancer.

Thadani et al. [43] reported the IC_{50} values for the lichen compounds against α-glucosidase enzyme as methylorsellinate 165 μM, methyl-β-orinolcarboxylate 140 μM and zeorin 100 μM.

However, in their report they have not described the kinetic mechanism of inhibition of the novel α- glucosidase inhibitors derived from the lichens. In the present study, we have determined the mode of inhibition of lichen metabolites for the α- and β- glucosidase activity. The lichen metabolite sekikaic acid and salazinic acid showed competitive inhibition, whereas usnic acid showed noncompetitive inhibition for α-glucosidase activity (Figure 2, 3, 4). Furthermore, the mode of inhibition of β- glucosidase activity by lichen metabolites; sekikaic acid and salazinic acid showed noncompetitive inhibition, and usnic acid showed uncompetitive inhibition (Figure 5, 6, 7).

Many natural plant derived compounds have been tested for anti-hyperglycemic agents as the synthetic anti-diabetic drugs have adverse side effects in humans. Phenolic phytochemicals from plants are reported to play an important role in modulating glucosidase activity [40,45,46]. Lichen produces secondary metabolites are mostly phenolic in nature, In our study, significant correlation was found between the total polyphenols in the extracts and glucosidase inhibitory and radical scavenging activity, hence our results are in agreement with those reported that phenolic compounds responsible for the glucosidase inhibition [11]. Marin et al. [47] reported that phytochemical content of the organism influenced by the genetics and growth conditions. This study investigated the anti-hyperglycemic potential of the three compounds, salazinic acid, sekikaic acid and usnic acid which produce under natural conditions by the lichen a symbiotic organism consists of fungus and algae. Therefore, we believe that the biological activities studied here are of the lichen compound or species specific.

Based on our in-vitro study, we recommend that these metabolites may have beneficial effects in managing the hyperglycemic effects. However, further preclinical and clinical studies should be pursued before its pharmaceutical applications. This investigation may also provide as additional information on anti-hyperglycemic properties of the lichen metabolites salazinic acid, sekikaic acid and usnic acid which are not reported earlier.

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