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Antioxidant and Anti-adhesive Activity of Some Common Lichens

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ABSTRACT

Antioxidant, antimicrobial and anti-adhesive activity of six lichens in Central Texas region (*Ramalina celastri* (RA), *R. stenospora* (RS), *R. Americana* (RA), *T. chrysophthalmus* (TC), *Parmotrema austrosinense* (PA) and *P. perforatum* (PP)) was evaluated. Antioxidant activity was determined by Malondialdehyde (MDA) assay and ABTS radical quenching assay. Antimicrobial and anti-adhesive activity of sterile extracts against *P. aeruginosa* was determined by Kirby-Bauer disk diffusion assay and *ex-vivo* skin assay, respectively. The high molecular weight fraction (HMWF) had higher antioxidant activity and quenched 60-75% radicals compared to the low molecular weight fraction (LMWF) which only neutralized 20-40% of the radicals except PA where activity was 79%. All extracts were able to reduce MDA formation by 30-88% except LMWF of RA and PP. None of the extracts had antimicrobial activity in the Kirby-Bauer (KB) disc diffusion assay. All the HMWF were able to reduce adhesion of *P. aeruginosa* by 30-55% except PP. LMWF from RS, PP, PA and PP reduced bacterial adhesion by 41-54%. Present results suggest that lichens can be an important source for bioactive compounds with potential benefits against oxidative and infectious diseases and warrants further investigation.

Key words: Lichens, antibacterial activity, anti-adhesive activity, secondary metabolites, high molecular weight fraction, low molecular weight fraction

INTRODUCTION

Lichens are fungi (ascomycota and basidiomycota) that have an obligate symbiotic association with a photosynthetic partner usually algae or more rarely a cyanobacterium (Purvis, 2000; Grube and Berg, 2010; Bates *et al.*, 2011). More than 20,000 known species of lichens have been identified and inhabit diverse ecosystems ranging from arctic tundra to desert climates (Oboh and Ademosun, 2006). Lichens are ubiquitous on barks, stems, leaves and in soil but often grow in habitats less favorable for higher plants (Kershaw, 1985; Vrablikova *et al.*, 2006). Lichens have historically been used as food (edible lichens), dyes (for textile coloring) and in traditional medicine to treat topical and enteric ailments, including infections and wounds (Shukla *et al.*, 2010). Recently there has been a renewed interest in lichens as a potential source for bioactive compounds with therapeutic properties. Recent studies have revealed that these slow growing poikilohydric organisms produce a diverse array of secondary metabolites with antimicrobial, antiviral and anticancer activity (Johnson *et al.*, 2011). Lichen metabolites are from derived fungal (mycobiont) and algal/cyanobacterial (photobiont) metabolism organized into several distinct chemical classes such as polyketides, usnic acids, anthraquinones, chromones, depsides, dibenzofurans, etc.

(Johnson *et al.*, 2011; Oboh and Ademosun, 2006; Manojlovic *et al.*, 2012). In addition, lichens have also been identified as a source of biologically active enzymes, polysaccharides and fatty acids that may have pharmacological potential (Huneck and Yoshimura, 1996; Johnson *et al.*, 2011). Due to a relatively recent resurgence in lichen bioactivity, therapeutic potential of many classless of lichens and their metabolites in medicine has largely remained unexplored. Here, we report the antioxidant and anti-adhesive activity against *Pseudomonas aeruginosa* (*P. aeruginosa*) of six different lichens harvested from the Central Texas region along the banks of San Marcos and Blanco River.

MATERIALS AND METHODS

Lichen collection and extraction: Six different lichens (Table 1) were carefully harvested using a scalpel to collect the cut the thalli into individually marked plastic bags and stored at room temperature until extraction. For extraction (Huerta *et al.*, 2010; Everette and Islam, 2012), 1.5 g of sample was suspended in 30 mL of water in an Erlenmeyer flask at 80°C with stir bar and allowed to mix for 30 min at 250 rpm. The samples were then filtered under vacuum using a Buchner funnel equipped with Whatman No. 1 filter paper. The filtrate was called water extract and labeled as extract-1. The residue remaining on the filter paper after water extraction was scraped with a spatula into an Erlenmeyer flask containing a stirr bar. To this residue, 20 mL of 4N NaOH was added and the mixture was stirred and allowed to digest for 30 min at 250 rpm. The samples were then filtered under vacuum using a Buchner funnel and Whatman No. 1 filter paper. The pH of the filtrate was adjusted immediately to 7.0 and labeled as extract-2.

Total soluble phenolics assay: Total phenolics assay were assayed by modifying methods previously described (Mihalik *et al.*, 2007; Dhanasekaran and Ganapathy, 2011). Briefly, 50 μ L of extract was transferred into a 96 well microplate and mixed with of 50 μ L ethanol and 500 μ L of distilled water. To each sample 25 μ L of 50% (v/v) Folin-Ciocalteu reagent was added and mixed. After 5 min, 50 μ L of 5% Na₂CO₃ was added to the reaction mixture and allowed to stand in dark for 60 min at room temperature. Post-incubation the absorbance was read at 725 nm in a microplate reader (Biotek Instruments, VA). The absorbance values were converted to total phenolics and expressed in milligrams equivalents of gallic acid per gram Dry Weight (DW). Standard curves were established using various concentrations of gallic acid in 95% ethanol.

Antioxidant assays

2,2'-azinobis (3-ethyl-benzothiazoline-6-sulfonic acid) (ABTS) assay: The ABTS assay was conducted by modifying previously described methods (Annegowda *et al.*, 2010). Briefly, to 1 mL of 7 mM ABTS (in water, activated overnight with 140 mM potassium persulfate) was added 50 μ L

Table 1: List of lichens tested with their abbreviation, scientific name and common name

Scientific name	Common name	Abbreviation
<i>Parmotrema austrosinense</i>	Unwhiskered ruffle lichen	PA
<i>Parmotrema perforatum</i>	Perforated ruffle lichen	PP
<i>Ramalina americana</i>	Sinewed ramalina	RA
<i>Ramalina celastri</i>	Palmetto lichen	RC
<i>Ramalina stenospora</i>	Southern strap lichen	RS
<i>Teloschistes chrysophthalmus</i>	Gold-eye lichen	TC

of extracts 1 or 2 and the mixture was incubated for 2.5 min (RT). The absorbance was measured at 734 nm and compared with control containing ethanol in place of the extract. The percentage inhibition in ABTS radical due to the extract was calculated by:

$$\text{Inhibition(\%)} = \left[\frac{A_{734}^{\text{Control}} - A_{734}^{\text{Extract}}}{[A_{734}^{\text{Control}}]} \right] \times 100$$

Thiobarburic acid reactive substances (TBARS) assay: TBARS were measured by modifying methods previously described (Oboh and Ademosun, 2006). Briefly, an emulsion containing 1% linoleic acid and 1% Tween in 25 mL deionized water was sonicated for 3 min. Then 0.8 mL of emulsion was added to 0.2 mL of extracts to which 500 μL of 20% (w/v) trichloroacetic acid and 1 mL of 10 mM thiobarbutyric acid were added. Contents were vortexed and incubated for 30 min at 100°C. After incubation, tubes were centrifuged at 13,000 g for 10 min and the absorbance of the supernatant was measured at 532 nm. The concentration of malondialdehyde (MDA) was calculated from its molar extinction coefficient (ϵ) 156 $\mu\text{mol}^{-1} \text{cm}^{-1}$ and expressed as $\mu\text{mol g}^{-1} \text{FW}$. Inhibition of TBAR formation by the extracts was calculated by comparing with the control, which did not contain the extracts.

$$\text{Inhibition(\%)} = \left[\frac{A_{532}^{\text{Control}} - A_{532}^{\text{Extract}}}{[A_{532}^{\text{Control}}]} \right] \times 100$$

Bacterial strains: *Pseudomonas aeruginosa* (ATCC 15692) for routine antibacterial assays (was purchased from American Type Culture Collection (ATCC, Bethesda, MD) and were subcultured and maintained in LB at 37°C until the absorbance reached 0.6-0.7 units at 600 nm.

Disk-diffusion method for antibacterial assessment: 100 μL aliquots of this culture were uniformly spread on LB plates; they were left to dry for 15 min under a laminar flow hood. Sterile paper discs (diameter 10 mm) of high grade cellulose were coated with different amounts (50, 100 and 150 and 200 μL) of LMWF and HMWF. Once disks were laid onto LB plates, the plates were incubated at 37°C for 24 h and the diameters of inhibitory zones (no growth) were measured. All tests were done in triplicates and mean values of growth inhibition for each treatment are reported (Oyetayo, 2007; Dwarakanath *et al.*, 2007).

Ex vivo anti-adhesion activity on porcine skin: Pig ears from a freshly slaughtered animal were obtained from a local slaughter house. The animals were slaughtered for food and not for research. The ears were washed to remove dust and debris and immediately used or stored at -80°C. Using a hair clipper the hair from pig ears was removed. The skin was cut into strips (3×2 cm^2) using a scalpel. The strips were transferred into sterile petri plates and washed with 20 mL diluted disinfectant for one minute, aspirated, washed thoroughly with sterile DH_2O and transferred to another sterile petri dish 100 μL bacteria+100 μL , 10 mM sterile PBS for control (or sample) was applied using a glass spreader and incubate at 30°C for one hour. The skin samples were then thoroughly washed twice with 20 mL with sterile DH_2O to remove non-adherent bacteria and aspirated. They were then transferred to a stomacher bag with 20 mL sterile 10 mM PBS and Homogenized for 120 sec on normal setting. From the homogenized solution, the supernatant was spread plated and incubated to count the total number of adherent bacteria (Farrag, 2001; Maisch *et al.*, 2007).

RESULTS

Total phenolic content: PA (1.6 mg g^{-1}) and PP (1.1 mg g^{-1}) had the highest phenolic content in the LMWF (Fig. 1). This was followed by RA (0.34 mg g^{-1}), RC (0.215 mg g^{-1}) and RS (0.217 mg g^{-1}). The LMWF of TC had 0.116 mg g^{-1} of total phenolics which was lowest among all the lichens. The total phenolic content of the HMWF was higher than the LMWF for all lichens tested. PA (3.4 mg g^{-1}), PP (3.3 mg g^{-1}) and RA (1.6 mg g^{-1}) had the highest phenolic content (Fig. 1). In HMWF of RC, RS and TC, the phenolic content was determined to be 0.345 , 0.335 and 0.393 mg g^{-1} , respectively.

ABTS radical quenching activity: The free radical quenching antioxidant activity of HMWF was higher than the LMWF, except in PA. The LMWF and HMWF of PA decreased the ABTS radical by 80 and 75%, respectively. The LMWF's of PP, RS and RC decreased ABTS radicals by 43, 32 and 29%, respectively (Fig. 2). The free radical quenching activity of LMWF's of RA and TC reduced the ABTS radicals by 22 and 13%, respectively (Fig. 2). Similar to PA, The HMWF of PP decreased ABTS radicals by 73%. The HMWF's of RA, RC and TC decreased ABTS radicals by 62% followed by HMWF of RS where a 59% reduction in ABTS radicals was observed (Fig. 2).

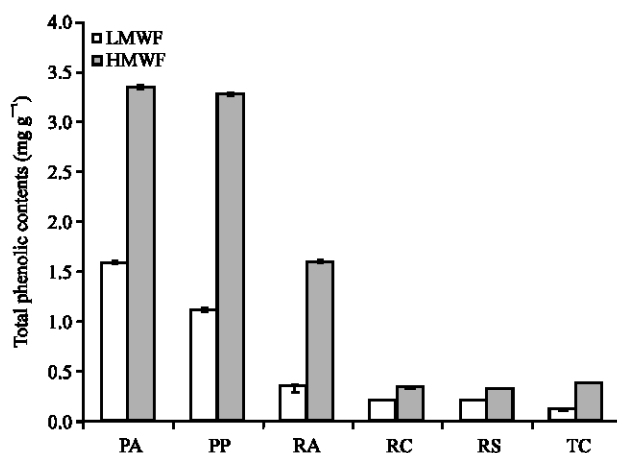


Fig. 1: Total Phenolic content of LMWF and HMWF of Lichens

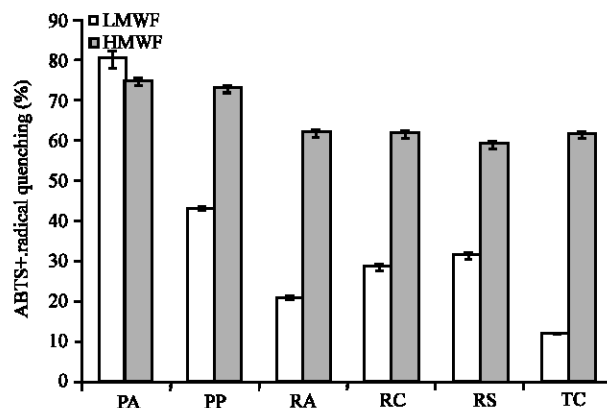


Fig. 2: ABTS radical quenching activity of LMWF and HMWF of Lichens

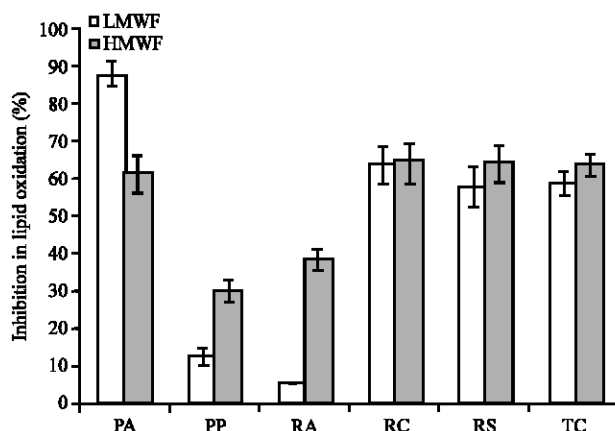


Fig. 3: Inhibition of lipid oxidation by LMWF and HMWF of Lichens

Inhibition of lipid oxidation: Among all the lichens tested, the highest inhibition of lipid oxidation was observed with the LMWF of PA, where 88% reduction in TBAR formation was noted. This was followed by LMWF's of RC, TC and RS, where a 63.7, 58.6 and 57.8% reduction in lipid oxidation was observed, respectively. LMWF's of PP and RA decreased lipid oxidation only by 12.5 and 5.2%, respectively. HMWF's of RC, RS and TC decreased lipid oxidation by 64% and was followed by HMWF of PA, where a 61% reduction in lipid oxidation was noted. At 38.5 and 30% reduction in lipid oxidation, the HMWF's of RA and PP had the lowest antioxidant activity (Fig. 3).

Antibacterial and Anti-adhesive activity: In the disc diffusion assay, none of the lichens tested had any antimicrobial activity against a common pathogenic bacteria *P. aeruginosa* (Ansari and Sitaram, 2011). In the *ex-vivo* skin adhesion assay, the LMWF of PP had the highest anti-adhesive activity and reduced the adhesion of *P. aeruginosa* by 52.8%. This was followed by the LMWF of RA, where a 48.9% reduction in bacterial adhesion was noted. The LMWF of RC decreased adhesion of bacteria by only 10.4%. The LMWF's of PA, RS and TC did not decrease the adhesion of *P. aeruginosa* to porcine skin.

With HMWF's of PA, RA and PP where a 31.2, 23 and 8.26% reduction in bacterial adhesion was observed, HMWF's from RC, RS and TC did not decrease the adhesion of *P. aeruginosa* to porcine skin (Fig. 4).

DISCUSSION

A preliminary bioactivity analysis of six lichens from Central Texas region indicated a presence of free radical quenching antioxidant activity. Recent studies with lichens of different species have also reported antioxidant activity (Manojlovic *et al.*, 2012). All lichens were also effective in decreasing accelerated oxidation of lipids at high temperatures. The phenolic content of the HMWF was more than the LMWF, which suggests that most phenolic secondary metabolites made by the lichens were not readily water soluble (Huerta *et al.*, 2010). As noticed with other natural products (Afolayan *et al.*, 2008; Olajuyigbe and Afolayan, 2011), phenolic content also correlated with antioxidant activity of the fractions. In all lichens that we tested, the ABTS radical quenching activity was higher in the HMWF than LMWF. HMWF fraction typically contains dimeric and multimeric phenolic metabolites that tend to have a higher radical quenching capacity as a result

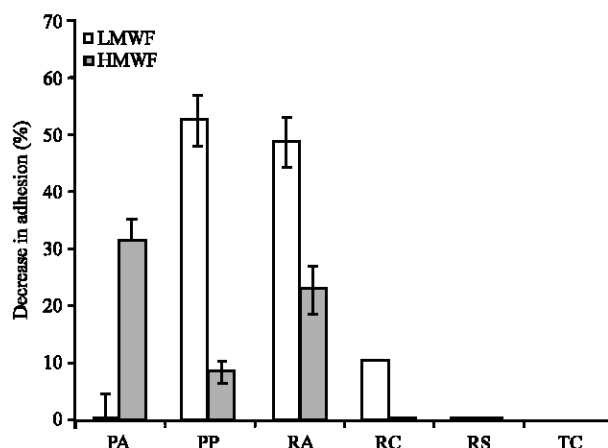


Fig. 4: Anti-adhesive properties of LMWF and HMWF of Lichens against *P. aeruginosa* in *ex-vivo* porcine skin assay

of increased resonance delocalization of the free radicals (Huerta *et al.*, 2010; Kwon *et al.*, 2006). The HMWF were also more effective in reducing lipid-peroxidation, which probably is due to the ability of the larger partially hydrophobic polyphenols to more effectively disperse at the lipid-water interface (Huerta *et al.*, 2010; Lin *et al.*, 2005). A strong anti-adhesive activity against *P. aeruginosa* was also noted in four lichens at sub-lethal levels. The LMWF especially were more effective in decreasing bacterial adhesion. We have previously reported that LMWF contain weakly acidic metabolites that can dissociate at the bacterial plasma membrane resulting in the hyper-acidification (Lin *et al.*, 2005; Chun *et al.*, 2005). Additionally, molecules such as ellagic acid can possibly stack or embed itself in the bacterial membrane. Together, these may impair the plasticity of the membrane and decrease the interaction of bacterial exopolysaccharide monomers with glycoprotein receptors, resulting in lower adhesion (Townsend *et al.*, 2010; Vamanu, 2011). As we did not see a direct correlation between phenolic content and anti-adhesive activity, it is possible that similar to other nutraceuticals and prebiotics described in the literature (Ogueke *et al.*, 2010; Singh and Sachan, 2011), lichen metabolites may also interfere with the fimbriae-adhesion interactions and decrease bacterial adhesion. Inhibition of bacterial adhesion and eventual reduction in pathogenicity of *P. aeruginosa* suggests that the lichens may have novel antimicrobial compounds that act on novel bacterial targets and that are less susceptible to resistance determinants already active in the opportunistic pathogen *P. aeruginosa* (Mihalik *et al.*, 2008).

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