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Original Research Article

Antiglycation and Antioxidant Activities and HPTLC Analysis of *Boswellia sacra* Oleogum Resin: The Sacred Frankincense

Ahmed Al-Harrasi[1*], Liaqat Ali[1], Eliezer Ceniviva[2], Ahmed Al-Rawahi[1], Javid Hussain[1], Hidayat Hussain[1], Najeeb ur Rehman[1], Ghulam Abbas[1,3] and Rashid Al-Harrasi[1]

[1]Department of Biological Sciences and Chemistry, College of Arts and Sciences, University of Nizwa, Birkat Al-Mauz, Nizwa 616, Sultanate of Oman, [2] CAMAG Laboratory, Sonnenmattstrasse. 11, 4132 Muttenz, Switzerland, [3]COMSATS Institute of Information Technology, Abbotabad Campus, Abbotabad-22060, Pakistan.

For correspondence: **Email: aharrasi@unizwa.edu.om; **Tel:** +968-92828487; **Fax:** +968-25446289.*

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Abstract

Purpose: *To evaluate antiglycation and antioxidant activities as undertake HPTLC analysis of Boswellia sacra resin.*
Methods: *Sub-fractionation of the crude methanol extract of Hougari regular (HR) grade resin of Boswellia sacra was carried out by vacuum liquid chromatography. Free radical scavenging and anti-glycation activities of sub-fractions were characterized in order to assess their anti-aging properties. Furthermore, high performance thin layer chromatography (HPTLC) analysis of Boswellia sacra resins was also carried out.*
Results: *Polar fractions of the extract obtained exhibited the highest antiglycation activity while non-polar fractions showed more than 50 % inhibition in superoxide anion scavenging assay. Scavenging activity of reactive oxygen species results indicate that non-polar sub-fractions showed > 50 % inhibition, except Shabi frankincense (SF) oil which showed 33 % inhibition. Dichloromethane (CH_2Cl_2) fraction, 40 % dichloromethane (CH_2Cl_2)/n-hexane sub-fraction, and SF oil showed moderate activity in di(phenyl)-(2,4,6-trinitrophenyl)iminoazanium (DPPH) assay. Furthermore, HPTLC analysis indicates the presence of 11-keto-β-boswellic acid (KBA) and 3-O-acetyl-11-keto-β-boswellic acid (AKBA) along with some other terpenoids.*
Conclusion: *Various sub-fractions of Boswellia sacra exert effective antiglycation and antioxidant activities. The extracts should be studied further for possible formulation into pharmaceutical products.*

Keywords: *Frankincense, Boswellia sacra, Terpenoids, HPTLC, Antioxidant, Antiglycation*

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INTRODUCTION

Frankincense from *Boswellia* trees has been used for various therapeutic purposes since the very beginning of the human civilization [1]. It is still used from North Africa to China as a remedy in various formulations for the treatment of inflammation-related disorders, and a multitude of phytochemical and pharmacological properties of the gum resin have been documented [1]. Furthermore, the pharmacological effects of pentacyclic triterpenes, especially those reported from *Boswellia* species (boswellic acid derivatives) are numerous, and these include anti-inflammatory, hepatoprotective, anti-tumour, anti-HIV, anti-microbial, antifungal, anti-ulcer,

gastroprotective, hypoglycemic and antihyperlipidemic properties [1]. These boswellic acid derivatives have a similar molecular structure which makes their separation by simple thin-layer chromatography challenging.

High performance thin layer chromatography (HPTLC) is becoming a technique for the routine analysis for the identification of medicinal plants and derived products. The possibility of presenting chromatographic fingerprints as electronic images that can easily be stored, shared and compared for multiple samples in parallel is the principal advantage of the technique over Gas chromatography (GC), highy-performance liquid chromatography (HPLC), and other column chromatography [2,3]. While simplicity and cost efficiency are preserved, separation power and reproducibility as well as traceability of data are significantly improved over classical thin layer chromatography (TLC).

The present study employs HPTLC identification and biological evaluation of *Boswellia sacra* to explore the antiglycation and antioxidant potentials of the resin derived from *Boswellia* species.

EXPERIMENTAL

Chemicals

Chemicals such as 1,1-diphenyl-2-picrylhydrazyl radical (DPPH), naphthyl-ethylenediamine, sodium nitroprusside, sulfanilic acid, potassium hydrogen phosphate, dipotassium hydrogen phosphate, 3-(2-pyridyl)-5,6-di(*p*-sulfophenyl)-1,2,4-triazine, disodium salt (ferrozine), reduced *β*-nicotinamide adenine dinucleotide (NADH), 5-methylphenazium methyl sulfate (PMS), nitro blue tetrazolium salt (NBT), standard radical scavengers: propyl gallate, 3-*t*-butyl-4-hydroxyanisol, 7,8-dihydroxy flavone; and solvents: dimethylsulfoxide (DMSO), carbon tetrachloride, and ethanol were purchased from Sigma or Fluka and used without further purification. Water used for buffer preparation was deionized using Simplicity water purification system (Millipore).

Plant materials

The aerial parts of *B. sacra* and the various grades of resin were collected in April and May 2010 from different locations in Dhofar (southern part of Oman) and were supplied by a trusted Dhofari partner (Dr. Saleh Al-Amri). Super hougari green (SHG), hougari regular (HR), royal hougari white (RHW), and hougari yellow (HY) grade resins were collected from Wadi Hougar in Oman, while shabi frankincense (SF) was collected from Wadi Magsyl in Oman. Frankincense Oleogum resins were collected from *Boswellia sacra* tree by making careful incisions into the bark of the tree without harming the tree. A thick milky-white liquid oozed out and then solidified into pea-sized “tears”.

These samples were authenticated by Dr Mustafa Mansi (a taxonomist) of the Department of Biological Sciences and Chemistry, University of Nizwa, Sultanate of Oman and voucher specimen (no. BSHR-01/2012) of the plant was deposited in the herbarium of the Department of Biological Sciences and Chemistry.

Extraction and isolation

The air-dried ground material (500 g) of HR grade resin was exhaustively extracted with 100 % methanol at room temperature. The extract was evaporated to yield the residue (150 g). Sub-fractionation of the crude methanol extract (150 g) of HR grade resin was carried out by vacuum liquid chromatography. A silica gel column (1000 g, 70-230 mesh, Merck) was used for the fractionation with CH_2Cl_2/*n*-hexane (50:50) as a mobile phase. Various sub-fractions were obtained from liquid chromatography and compiled on the basis of the similarity observed on TLC plates. The essential oils of all available grades of the resin were extracted by hydro-distillation using 8 Quart Stove Still Home Distillation Unit with Clevenger’s apparatus until complete exhaustion.

HPTLC analysis

Chromatography was performed on pre-coated HPTLC silica gel glass plates 60 F254 (20 × 10 cm; E. Merck, Germany) for the development of characteristic fingerprinting profile of the selected samples of the resin from *B. sacra*. Each sample (0.5 g) was dissolved in HPLC grade methanol (5 mL) and sonicated for 10 min. The solution was then centrifuged at 3000 rpm for 10 min and the upper layer was used for HPTLC analysis after 1:1 dilution with methanol (the standard *Boswellia* extract was diluted 1:20). Thereafter, 0.5 and 2 µL of each sample were applied as bands of 8 mm using Automatic TLC sampler 4 (CAMAG, Switzerland) with a 25 µL syringe. Linear ascending development was carried out in Automatic Developing Chamber (ADC2, CAMAG, Switzerland) saturated with mobile phase for 20 min at room temperature (24 ºC) using a filter paper. For optimum conditions, relative humidity (RH) was controlled at 33 % in ADC2 using a saturated solution of $MgCl_2$.

Toluene: ethyl acetate: heptane: formic acid (80:20:10:3, v/v/v/v) was used as the mobile phase.

The plates were developed to a distance of 7 cm from the lower edge of the plate. Drying was carried out for 5 min in a stream of cold air. For derivatization, the plate was immersed in anisaldehyde-sulphuric acid reagent (170 mL of ice-cooled methanol mixed with 20 mL of acetic acid, 10 mL of sulfuric acid and 1 mL of anisaldehyde) followed by heating at 100 °C for 5 min. Images of the chromatograms were electronically documented with Visualizer (CAMAG Switzerland) under UV 254 nm before derivatization and under UV 366 nm and white light after derivatization. The identity of the zones in the sample corresponding to 3-O-acetyl-11-keto-β-boswellic acid (AKBA) was confirmed by comparing their densitometrically-obtained UV spectra with those of a AKBA reference standard (Phytolab, Vestenbergsgreut, Germany). HPTLC according to PhEur 7 Monograph for Indian Frankincense. Mobile phase*:* Anhydrous formic acid–heptane–ethyl acetate–toluene (3 + 10 + 20 + 80, v/v/v/v). Detection: UV light at 254 nm. Reference compound: KBA = 11-keto-β-boswellic acid.

Cytotoxicity and antiradical studies against the 1,1-diphenyl-2-picrylhydrazyl radicals were carried out at 37 °C, while the enzymatic reactions and superoxide scavenging studies were carried out at 28 °C. All studies were performed in 96-well microtitre plates using SpectraMax-340 and SpectraMax-384 spectrophotometers (Molecular Devices, CA, USA).

Antiglycation assay

Bovine serum albumin (BSA, 10 mg/mL) was dissolved in 67 mM phosphate buffer (20 µL) of pH 7.4 which incorporated 50 mg/mL anhydrous glucose (20 µL). Thereafter, a 3 mM sodium azide (20 µL) was added to inhibit bacterial growth. For assessment of antiglycation activity, each fraction (20 µL; 1 mg/1000 µL) was mixed and the mixture (60 µL in each well of 96-well plate) was incubated for a week at 37 °C. A blank sample containing only BSA dissolved in phosphate buffer and positive control sample containing both BSA and glucose, were prepared and incubated for a week at 37 °C. After incubation in 96- well plate for a week, samples were removed and cooled to room temperature, amd 6 µL of 100% trichloroacetic acid (TCA) was added to each well. The supernatant containing unbound glucose, inhibitor and test sample were removed after centrifugation at 14,000 rpm for 4 min, and 60 µL of PBS (pH 10) was added to dissolve the pellets left. Comparison of fluorescence intensity at 370 nm excitation and emission at 440 nm was obtained by spectrofluorimetry **(**RF-1500, Shimadzu, Kyoto, Japan) [4]. Rutin was used as standard inhibitor. Inhibition (%) was calculated as in Eq 1

$$\text{Inhibition} = 100 - \{(As/Ab) \times 100\} \quad \ldots\ldots\ldots\ldots (1)$$

where As and Ab are the absorbance of sample and blank, respectively.

Superoxide anion scavenging assay

Superoxide scavenging activities of the samples were determined by the method described by Gaulejac *et al* [5] with some modifications. The reaction was performed in triplicate in a 96-well plate and absorbance was measured on a multiplate reader (SpectraMax 340, Molecular Devices, CA, USA). The reaction mixture contained 40 µL of nicotinamide adenine dinucleotide (NADH), 40 µL of nitroblue tetrazolium (NBT), 90 µL of 0.1M phosphate buffer (pH 7.4) and 10 µL of the test compound pre-read at 560 nm. The reaction was initiated by the addition of 20 µL of phenazine methosulphate (PMS), and incubated at room temperature for 5 min. Formation of blue-colored formazan dye was measured at 560 nm. Control contained 10 µL of dimethyl sulfoxide (DMSO), instead of the test samples. The solutions of NBT, NADH and PMS were prepared in phosphate buffer, while the test samples were dissolved in DMSO.

DPPH free radical scavenging assay

Free radical scavenging activity of the test samples were determined by measuring the change in absorbance of DPPH (l,l-Diphenyl-2-picrylhydrazyl radical) spectrophotometrically at 515 nm [6]. The reaction mixture comprised of 95 µL of ethanol solution of $DPPH^{\cdot}$ and 5 µL of the test sample dissolved in DMSO. Total reaction volume was 100 µL, with final concentrations of 300 µM and 1000 µM of DPPH and test compound, respectively. The reaction mixture was then incubated at 37 °C for 30 min. After incubation, decrease in absorption was measured at 515 nm spectrophotometrically (Molecular Devices, CA, USA). The control contained 5 µL of DMSO instead of the test sample and the reactions were performed in triplicates. To avoid solvent evaporation during incubation, the 96-well plate was covered with parafilm immediately after the addition of DPPH solution and the reaction mixtures were thoroughly mixed by shaking the plate for 1 min. The absorbance of the yellow-colored reduced form of DPPH produced after incubation was measured at 562 nm using a multiplate reader (SpectraMax-

340), and radical scavenging activity (RSA, %) was determined according to Eq 2.

% RSA = 100 – {At/Ac) x 100} …….…… (2)

where, At is the absorbance of radicals and formazan dye in the presence of the test sample and Ac is the absorbance of control, formazan dye without test sample.

Statistical analysis

The data obtained were analyzed statistically using Statistic Analysis System (SAS, version 9.1). Each experiment was repeated three times and values expressed are means ± standard error. Differences were considered significant at $p < 0.05$.

RESULTS

HPTLC fingerprinting profile

Boswellic acids are pharmacologically active compounds isolated from the resins (frankincense) of various species of *Boswellia*. HPTLC allows rapid investigation, identification and comparison of the quality of different kinds of frankincense (*Olibanum*). In the present study, the HPTLC profile of the crude resin (Figure 1; tracks 3 and 4), the crystalline medium polarity fraction (tracks 5 and 6), and the hydrodistillate essential oil (tracks 7 and 8) were analyzed at two quantitative levels (0.5 and 2.0 µL). *Boswellia serrata* extract (track 1) and the gum resin (track 2), and compared with the standard sample, 3-*O*-acetyl-11-keto-*β*-boswellic acid (AKBA, track 9). All samples showed the presence of 11-keto-*β*-boswellic acid (KBA, lower arrow, track 8) and 3-*O*-acetyl-11-keto-*β*-boswellic acid (AKBA, upper arrow, track 8) with different concentration (intensity) in all the three test samples (Figure 1). The same compounds were identified in approximately the same concentration for the standard *B. serrata* extract and the gum resin (KBA and AKBA, black arrows, tracks 1 and 2). These results were further confirmed by matching the UV spectra of the corresponding bands in the test samples with those of the standard AKBA.

These results and comparison with the literature [7] confirm the identity of the investigated samples as *B. sacra* based on various concentrations of KBA and AKBA. The concentrations of the same compounds in *B. serrata* were approximately equal. These observations were further confirmed through derivatization of the HPTLC plate with the anisaldehyde reagent (Figure 2).

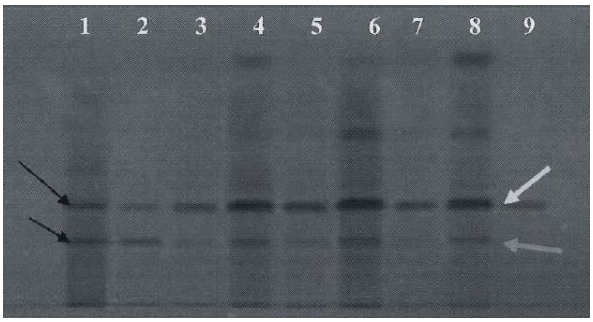

Figure 1: Detection and comparison of boswellic acids in different samples of Boswellia sacra. ***Track 1 =*** *Boswellia serrata* extract (CAMAG); ***Track 2 =*** *Boswellia serrata* gum (CAMAG); ***Track 3 & 4 =*** *Boswellia sacra* resin (test sample, 0.5 and 2.0 µL, respectively); ***Track 5 & 6*** *Boswellia sacra* crystalline fraction (test sample, 0.5 and 2.0 µL respectively); ***Track 7 & 8 =*** *Boswellia sacra* resin (test sample, 0.5 and 2.0 µL respectively); ***Track 9 =*** standard AKBA.

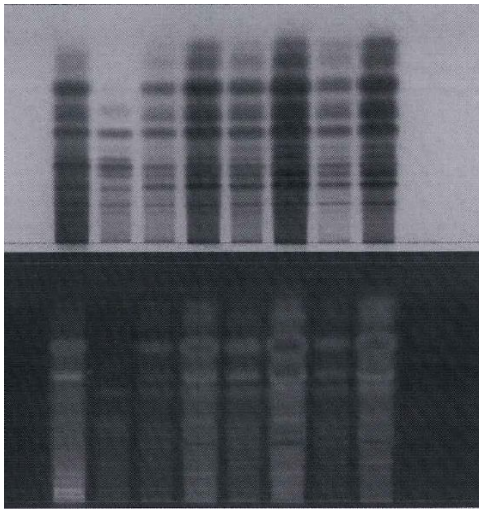

Figure 2. Detection and comparison of boswellic acids in different samples of Boswellia sacra. ***Track 1 =*** *Boswellia serrata* extract (CAMAG); ***Track 2 =*** *Boswellia serrata* gum (CAMAG); ***Track 3 & 4 =*** *Boswellia sacra* resin (test sample, 0.5 and 2.0 µL, respectively); ***Track 5 & 6*** *Boswellia sacra* crystalline fraction (test sample, 0.5 and 2.0 µL respectively); ***Track 7 & 8 =*** *Boswellia sacra* resin (test sample, 0.5 and 2.0 µL respectively); ***Track 9 =*** standard AKBA.

Antiglycation activity

All the tested samples showed varied inhibitory potential *in vitro* at a concentration of 1 mg/1000 µL (Table 1). The sub-fraction obtained with 36 % CH_2Cl_2/*n*-hexane showed the highest inhibitory activity (69.5 %), followed by 2 % methanol/ CH_2Cl_2 (66.9 %) and Royal lower (RL) oil (54.3 %). The sub-fraction obtained with 40 % methanol/CH_2Cl_2 and Shabi frankincense (SF) oil showed moderate activity in antiglycation assay

Table 1: Antiglycation and antioxidant activities (mean ± standard error) of various fractions of *Boswellia serrata*

Sample	Inhibitory activity (%)		
	Antiglycation	***DPPH***	***Superoxide***
HR oil[a]	9.20±0.02	16.30±0.04	56.40±0.01
RL oil	54.30± 0.01	5.60±0.02	50.50±0.02
RU oil	5.30±0.06	2.80±0.01	52.80±0.05
SF oil	37.20±0.01	33.40±0.04	33.10±0.08
Crude extract	10.90±0.03	14.30±0.05	49.00±0.02
Pure n-hexane	6.80±0.02	6.60±0.03	50.80±0.14
36% CH_2Cl_2/*n*-hexane	69.50±0.06	11.90±0.12	13.40±0.03
40% CH_2Cl_2/*n*-hexane	14.40±0.01	31.30±0.03	16.90±0.05
60% CH_2Cl_2/*n*-hexane	7.80±0.01	15.60±0.08	23.40±0.01
80% CH_2Cl_2/*n*-hexane	10.30±0.03	13.30±0.05	33.50±0.03
Pure CH_2Cl_2	12.40±0.07	31.80±0.14	49.70±0.04
2% MeOH/CH_2Cl_2	66.90±0.02	10.90±0.08	6.20±0.01
8% MeOH/CH_2Cl_2	9.90±0.03	12.10±0.07	11.80±0.02
40% MeOH/CH_2Cl_2	41.20±0.04	10.00±0.2	50.80±0.04
Standard	Rutin (82.50 %)	Propyl gallate (90.30 %)	Propyl gallate (92.50 %)

[a]*HR = Hougari regular; RL = Royal lower; RU = Royal upper; SF = Shabi frankincense*

whereas the other samples were weakly active or completely inactive (Table 1).

Superoxide anion scavenging activity

The results obtained indicate that the non-polar sub-fractions exhibited > 50 % inhibition, except n-hexane fraction, CH_2Cl_2 fraction and 40 % methanol/ CH_2Cl_2 fraction all of which exhibited approximately the level same inhibition which was close to 50 %. Other test samples showed < 50 % inhibition was close to 50 %. Other test samples showed < 50 % inhibition.

DPPH free radical scavenging activity

CH_2Cl_2 fraction and the sub-fraction of 40 % CH_2Cl_2/*n*-hexane fraction, as well as SF oil showed moderate activity (Table 1), while the other samples were largely inactive. Standard rutin showed 82.5 % inhibition of glycation at 3 mM concentration with IC_{50} of 98.01 ± 2.03 μM.

DISCUSSION

Triterpenic acids are an important group of natural compounds with confirmed pharmacological activity [1]. They occur in many medicinal herbs and plants. The similarity of their chemical structures makes their TLC separation very difficult. There are some chromatographic systems described in the literature [8-11], but they do not offer better separation, and the result is poor overall yield of the pure natural products. In this regard, our approach using HPTLC offers more effective separation and higher yield.

Oxidation process plays an important role in glycation end-products (AGEs) formation and is essential to many living organisms for the production of energy to fuel the biological processes. On the other hand, reactive oxygen species (ROS) can damage DNA and thus cause mutation and chromosomal damage [12-14]. Furthermore, production of excessive free radicals stimulates oxidative damage which is responsible for more than one hundred disorders in humans including atherosclerosis, coronary heart disease, neurodegenerative disorders, cancer, and aging process. Therefore, agents with antioxidative or metal-chelating properties may retard the process of AGEs formation by preventing metal-catalyzed glucose oxidation [12-14].

Free radicals, such as hydroxyl radical, are generated from sequential reduction of oxygen during the normal course of aerobic metabolism. Over-abundant radicals cause oxidative stress which can lead to cell injury and tissue damage [15]. *B. sacra* extract is a potential source of natural antioxidants, and incorporation of these extract into foods could enhance their nutritional and antioxidant potentials.

DPPH radicals are widely used to investigate the scavenging activity of natural compounds. These free radicals are stable in ethanol and show maximum absorbance at 517 nm. When DPPH radicals encounter a proton-donating substance such as an antioxidant, the radicals are scavenged and their absorbance reduced [15]. *B. sacra* extracts and essential oils showed

scavenging activities against DPPH radicals. This is not surprising since they contain a large variety of terpenes [16], which could be electron donors, and hence can react with free radicals to convert them to more stable products and terminate radical chain reaction.

The results gleaned from antioxidant and antiglycation assays indicated that the fractions obtained by the use of a *n*-hexane or CH_2Cl_2 solvent were comparatively more active in almost all the assays. Furthermore the crystalline sub-fractions and the more polar fractions (40 % methanol/CH_2Cl_2) also showed comparatively higher activity than the other tested samples. These observations indicate that overall activity increases the purer the sample and the higher the polarity of the fraction. The polar sub-fractions obtained when methanol solvent system was added contain chemical compounds that are believed to be responsible for their higher antiglycation activity. Our assumption is supported by a study on *Plantago asiatica* which showed higher antiglycation activity (75 %) for polar fractions [17].

CONCLUSION

An HPTLC method has been applied successfully for the simultaneous fingerprint identification of boswellic acid derivatives in *B. sacra.*

The method was found to be simple and specific and suitable for further qualitative analysis of the plant material. The tested materials possessed high antioxidant and antiglycation activities. Thus the plant may offer additional sources of ingredients that can be formulated into products for health promotion.

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