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Assessment of the Chemical Composition and *in vitro* Antimicrobial Potential of Extracts of the Liverwort *Scapania aspera*

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The chemical composition of *Scapania aspera* extracts was determined by solid phase micro extraction gas chromatography–mass spectrometry (SPME GC-MS) and 96 constituents were identified. The dominant compounds in the methanol extract were β-barbatene (25.1%), *o*-cymene (14.0%), α-barbatene (5.7%), allo-aromadendrene (4.9%) and β-bourbonene, while in the ethanol extract, o-cymene (17.8%), β-barbatene (17.6%), α-thujene (6.7%), octen-1-ol acetate (4.9%) and β-bazzanene (2.4%) were the major components. In the ethyl acetate extract, β-barbatene (14.3%), undecane (11.8%), 2-methyldecane (11.2%), decane (10.9%) and o-cymene (3.6%) were major components. The antimicrobial activity of the different extracts was evaluated against pathogenic and food spoilage microorganisms using disc diffusion and micro-broth dilution methods. The minimal inhibitory concentration (MIC) of extracts of *S. aspera* varied from 0.4 to 1.5 mg/mL and 1 to 3 mg/mL for yeast and bacterial strains, respectively. The zone of inhibition of the methanol extract for yeast strains was higher than that for bacterial strains. The results suggest that *S. aspera* extracts have potential as natural antimicrobial agents.

Keywords: *Scapania aspera*, Liverworts, SPME GC-MS, Chemical composition, Antimicrobial activity.

Some liverwort species have been used as medicinal plants in China [1]. So far, several hundred new compounds have been isolated from liverworts where more than 40 new carbon skeletal acetogenins, phenolic compounds and terpenoids have been found. Although liverworts are rich sources of new secondary metabolites which show interesting biological activity, only about 10% of liverwort species have been studied chemically [2]. Scapaniaceae is classified with 87 recent and one fossil species, [3] small to robust, often with red, purple or brown pigmentation [4]. The most common morphotype of *Scapania* is characterized by leaves with distinctly smaller dorsal lobes. To the best of our knowledge there are no reports on the SPME GC-MS analysis and antimicrobial properties of different extracts from the liverwort *S. aspera*. The main objectives of this study were to characterize the chemical composition of different extracts of this species and determine its antimicrobial properties against various pathogenic microbial strains.

More than 95 compounds of *S. aspera* were identified by the SPME GC-MS analysis of different extracts. The main components with their percentages and retention indices are listed in Table 1. In the methanol extract, 54 components were identified, which represented 54.6% sesquiterpenes, 15.1% monoterpenes, 7.1% non-terpene hydrocarbons, 7.5% aldehydes, 3.9% ketones, 3.3% alcohols, and 0.2% esters (about 98.9% of the total detected constituents). The major components were β-barbatene (25.9%), *o*-cymene (14%), α-barbatene (5.7%), allo-aromadendrene (4.9%) and β-bourbonene (4.4%). Fifty-seven components were identified from the ethanol extract, which represented 51.4% sesquiterpenes, 30.6% monoterpenes, 7.2% non-terpene hydrocarbons, 3.2% aldehydes and 2.4% ketones, which represented about 98.5% of the total composition; *o*-cymene (17.8%), β-barbatene (17.6%), α-thujene (6.7%), octen-1-ol acetate (4.9%) and β-bazzanene (2.4%) were the major components. Fifty-three components were identified from the ethyl acetate extract, which represented 52.2% non-terpene hydrocarbons, 31.7% sesquiterpenes, 7.9% monoterpenes, 1.5% alcohols and 1.4% esters; β-barbatene (14.3%), undecane (11.8%), 2-methyldecane (11.2%), decane (10.9%) and *o*-cymene (3.6%) were the main compounds.

Numerous terpenes appear to possess beneficial healthcare effects [5]. However, terpenes (*mono-*, *sesqui-* and *di-terpenes*) from different *Scapania* species have been reported in earlier works [6a-f]. *Scapania* spp. produce many kinds of sesquiterpenoid, which are ubiquitous in other liverworts. Two of the most common sesquiterpenes, anastreptene and aromadendrane, have been reported in eleven *Scapania* species [7]. A high percentage of sesquiterpenes and hydrocarbons in extracts of *S. aspera* could be responsible for considerable antimicrobial activity.

The MIC and MBC/MFC of different *S. aspera* extracts were determined against various bacterial (*Salmonella enteritidis, Escherichia coli* and *Listeria monocytogenes*) and yeast (*Saccharomyces cerevisiae*, *Zygosaccharomyces bailii*, *Aerobasidium pullulans*, *Pichia membranaefaciens* and *Pichia anomala*) strains. These MIC and MBC/MFC values are shown in Tables 2 and 3. Different *S. aspera* extracts exhibited concentration-dependent growth inhibition. For the bacterial strains, the MIC varied from 1 to 3 mg/mL (Table 2). The MICs of methanol and ethanol extracts for Gram-positive bacteria (*L. monocytogenes*) was significantly ($p \leq 0.05$) lower than those for Gram-negative bacteria (*S. enteritidis*, *E. coli*), i.e. 1 mg/mL and 2 mg/mL, respectively. However, the MIC value of the ethyl acetate extract for *L. monocytogenes* was significantly ($p \leq 0.05$) higher than those for

Table 1: Chemical composition of *Scapania aspera* extracts.

Compounds %		Extracts [a]MeOH	[b]EtOH	[c]EtOAc
Monoterpene hydrocarbons	**RI**	**%**	**%**	**%**
α-Thujene	1038	0.0	6.7	0.0
Camphene	1077	0.0	1.4	0.2
β-Pinene	1136	0.0	0.3	0.0
Myrcene	1167	0.0	2.6	1.4
Limonene	1208	0.9	0.0	0.8
β-Phellandrene	1220	0.0	0.9	0.9
p-Cymene	1280	0.0	0.0	1.0
γ-Terpinene	1286	0.1	0.9	0.0
Terpinolene	1293	0.1	0.0	0.0
o-Cymene	1298	14.0	17.8	3.6
Total:		**15.1**	**30.6**	**7.9**
Sesquiterpene hydrocarbons				
α-Cubebene	1463	0.0	0.7	0.1
α-Ylangene	1494	0.0	0.2	0.6
Copaene	1503	0.6	0.7	0.1
β-Bourbonene	1528	4.4	4.2	3.8
β-Cubebene	1531	0.7	1.8	0.4
α-Isocomene	1550	0.0	0.0	0.1
α-Bergamotene	1562	0.0	0.4	0.2
β-Elemene	1571	1.2	2.5	0.0
β-Cedrene	1590	0.5	0.9	0.5
α-Barbatene	1592	5.7	4.1	0.0
β-Gurjunene	1598	0.3	0.0	0.2
α-Cedrene	1600	0.0	0.2	0.0
Isobazzanene	1605	0.7	0.8	0.8
β-Farnesene	1622	0.0	0.3	0.2
γ-Elemene	1633	0.0	0.8	0.4
trans-Cadina-1(6),4-diene	1630	0.0	1.0	0.2
Amorpha-4,11-diene	1636	0.6	1.1	0.8
allo-Aromadendrene	1642	4.9	3.5	2.8
β-Barbatene	1656	25.9	17.6	14.3
10-*epi*-β-Acoradiene	1662	0.1	0.0	0.0
α-Gurjunene	1665	0.3	1.1	0.6
Zingiberene	1684	0.1	0.4	0.0
Zonarene	1690	0.0	0.5	0.0
Didehydrocycloisolongifolene	1701	0.1	0.0	0.0
Germacrene D	1717	1.6	1.9	1.9
β-Selinene	1726	0.2	0.0	0.0
Bicyclogermacrene	1739	0.6	0.9	0.4
α-Bulnesene	1745	0.0	0.2	0.0
α-Chamigrene	1755	0.3	0.4	0.4
α-Curcumene	1776	0.4	0.8	0.5
β-Cadinene	1769	0.0	0.6	0.2
β-Bazzanene	1794	3.3	2.4	1.9
Calamenene	1855	0.8	1.2	0.3
Palustrol	1910	0.2	0.0	0.0
Calacorene	1934	0.0	0.1	0.0
Spathulenol	2129	0.4	0.1	0.0
trans-Sesquisabinene hydrate	2143	0.7	0.2	0.0
Total:		**54.6**	**51.6**	**31.7**
Non-terpene hydrocarbons				
2,6-Dimethyloctane	902	0.0	0.0	0.1
Decane	984	0.0	0.0	10.9
Pentadecane	961	0.0	0.0	0.5
3-Methylnonane	965	0.0	0.0	0.6
3,7-Dimethylnonane	1008	0.0	0.0	1.5
2-Methyldecane	1038	0.0	0.0	11.2
3-Methyldecane	1048	0.0	0.0	4.8
Undecane	1091	0.1	0.7	11.8
3,7-Dimethyldecane	1126	0.0	0.0	1.2
2,3-Dimethyldecane	1142	0.0	0.0	0.5
5-Undecene	1156	0.0	0.0	0.8
Dodecane	1190	0.0	0.5	3.7
2-Methylundecane	1224	0.0	0.0	2.0
1-Isopropoxy-2-propanol	1282	0.0	0.9	0.0
m-Diethylbenzene	1308	0.0	0.0	0.6
Dimethylformamide	1320	0.1	0.0	0.0
n-Butylbenzene	1322	0.0	0.0	0.2
1-Cyclododecene	1349	0.2	0.0	0.0
Octen-1-ol, acetate	1370	5.7	4.9	0.0
6-Butyl-1,4-cycloheptadiene	1372	0.0	0.0	0.6
2-Ethyl-6-methylpyrazine	1396	0.0	0.1	0.1
1-*sec*-Butyl-4-methylbenzene	1413	0.0	0.0	0.3
Ethyl octanoate	1416	0.0	0.1	0.0
1,3-Diethyl-4-methylbenzene	1438	0.0	0.0	0.8
Butyrolactone	1631	1.0	0.0	0.0
Total:		**7.1**	**7.2**	**52.2**
Alcohols				
2-Methyl-1-propanol	1079	0.0	0.3	0.3
1-Penten-3-ol	1193	2.4	0.2	0.0
2-Methyl-2-propen-1-ol	1260	0.1	0.0	0.0
cis-2-Penten-1-ol	1326	0.1	0.0	1.2
1-Octen-3-ol	1431	0.7	0.3	0.0
Total:		**3.3**	**0.8**	**1.5**
Aldehydes				
Pentanal	982	0.0	0.7	0.0
2-Butenal	1049	1.5	0.8	0.0
Hexanal	1097	4.9	1.4	0.0
(*E*)-2-Hexenal	1204	0.6	0.3	0.0
trans-2-Pentenal	1224	0.1	0.0	0.0
(*Z*)-2-Heptenal	1342	0.3	0.0	0.0
Hexa-2,4-dienal	1420	0.1	0.0	0.0
Total:		**7.5**	**3.2**	**0.0**
Ketones				
Pentanone	988	1.6	0.6	0.0
3-Penten-2-one	1123	1.3	0.0	0.0
2-Methyl-6-heptanone	1287	0.6	0.9	0.0
Cyclohexanone	1331	0.3	0.1	0.0
6-Methyl-6-hepten-2-one	1333	0.0	0.8	0.0
3-Octen-2-one	1416	0.1	0.0	0.0
Total:		**3.9**	**2.4**	**0.0**
Esters				
Benzene acetic acid, 6-ethyl-3-octyl ester	1232	0.0	0.0	1.4
Glycolic acid, methyl ester	1384	0.1	0.0	0.0
Propanoic acid, 3-hydroxy-, methyl ester	1545	0.1	0.0	0.0
Totals:		**0.2**	**0.0**	**1.4**
Others				
Acetic acid	1448	6.8	2.4	3.9
Propanoic acid	1519	0.2	0.0	0.0
Hexanoic acid	1861	0.2	0.7	0.0
Total:		**7.2**	**3.1**	**3.9**
Unidentified %		**1.1**	**1.5**	**1.5**
Total identified %		**98.9**	**98.5**	**98.5**

RI = Retention Index on CP WAX 52 CB capillary column; [a]MeOH: methanol extract; [b]EtOH: ethanol extract; [c]EtOAc: ethyl acetate extract.

the methanol and ethanol extracts. The MBC of the different extracts for bacterial strains varied from 2 to 3 mg/mL and showed a similar pattern, i.e. *S. enteritidis* (3 mg/mL) = *E. coli* (3 mg/mL) > *L. monocytogenes* (2 mg/mL). The MIC for streptomycin was 0.02–0.05 mg/mL and the MBC 0.10 mg/mL. Higher MIC/MBC values of Gram-negative bacteria could be due to the highly hydrophilic cell wall and presence of cell envelope (made up of lipopolysaccharide). The cell membrane of Gram-positive bacteria may facilitate the penetration of hydrophobic compounds [8]. The MIC of methanol, ethanol and ethyl acetate extracts for yeast strains varied from 0.4–1 mg/mL, 0.5–1.25 mg/mL and 1–1.5 mg/mL, respectively (Table 3).

Table 2: Antibacterial activity of *S. aspera* methanol, ethanol and ethyl acetate extracts (mg/mL).

	S. aspera extracts							
	[a]MeOH		[b]EtOH		[c]EtOAc		Strept.	
Bacterial strains	MIC	MBC	MIC	MBC	MIC	MBC	MIC	MBC
S. enteritidis 155	2.00	3.00	2.00	3.00	3.00	3.00	0.05	0.10
E. coli 555	2.00	3.00	2.00	3.00	2.00	3.00	0.05	0.10
L. monocytogenes 56 Ly	1.00	2.00	1.00	3.00	2.00	3.00	0.02	0.10

MIC: Minimal Inhibitory Concentration; MBC: Minimal Bactericidal Concentration; [a]MeOH: Methanol extract; [b]EtOH: Ethanol extract; [c]EtOAc: Ethyl acetate extract. Strept: Streptomycin.

Table 3: Anti-yeast activity of *Scapania aspera* methanol, ethanol and ethyl acetate extracts (mg/mL).

	Scapania aspera extracts						
	[a]MeOH		[b]EtOH		[c]EtOAc		Cycl.
	MIC	MFC	MIC	MFC	MIC	MFC	MIC
S. cerevisiae 635	0.40	1.00	0.50	1.00	1.00	2.00	<0.05
Z. bailii 45	1.00	2.00	1.00	2.00	1.50	3.00	<0.05
A. pullulans L6F	1.00	2.25	1.25	2.00	1.25	3.00	<0.05
P. membranaefaciens OC71	0.50	1.00	0.50	1.75	1.00	2.00	<0.05
P. membranifaciens OC70	1.00	1.75	1.00	2.00	1.25	3.00	<0.05
P. anomala CBS 5759	0.50	1.00	0.50	2.00	1.00	2.00	<0.05
P. anomala DBVPG 3003	0.50	1.00	1.00	2.00	1.25	3.00	<0.05

MIC: Minimal Inhibitory Concentration; MFC: Minimal Fungicidal Concentration; [a]MeOH: Methanol extract; [b]EtOH: Ethanolic extract; [c]EtOAc: Ethyl acetate extract, Cycl: cycloheximide.

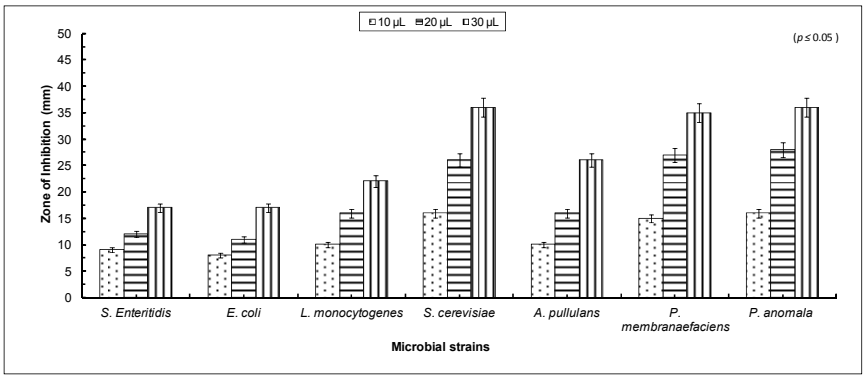

Figure 1: Antimicrobial potential of *S. aspera* extract using the disc diffusion method. Zone of inhibition due to the different concentrations (0.1, 0.2 and 0.3 mg/mL) of *S. aspera* extract against *S. enteritidis, E. coli*, *L. monocytogenes*, *S. cerevisiae*, *A. pullulans*, *P. membranaefaciens*, and *P. anomala.* (Column height represents the mean value of triplicate results and error bar represents the standard error).

The MIC values also varied with the yeast strain. *Z. bailii, A. pullulans,* and *P. anomala* showed higher MICs than *S. cerevisiae, P. membranaefaciens*, and *P. anomala* against different extracts. Similar pattern was found for the MFC of different extract against yeast strains, i.e. MFC of ethyl acetate extract > MFC of ethanol extract ≥ MFC of methanol extract. The commercial antibiotic cycloheximide used as a control, possessed much lower MICs (< 0.5 mg/mL) than the extracts against different yeast strains.

In general, different *S. aspera* extracts showed significant antimicrobial activity where yeast strains were more sensitive than bacterial strains (Tables 2 and 3). The difference in antimicrobial activity of different extract for similar bacterial and yeast strains was also associated with the presence of the sesquiterpenes β-barbatene, α-barbatene, allo-aromadendrene and β-bourbonene, which were present in higher amounts in the methanol (54.6%) and ethanol (51.4%) extracts than in the ethyl acetate extract (31.6%). Recently, it was reported that sesquiterpenes show strong antimicrobial activity against selected pathogens [9]. High content of sesquiterpene components may account for the higher antimicrobial activity of methanol and ethanol extracts than the ethyl acetate extract of this liverwort [10]. Some volatile sesquiterpene hydrocarbons, such as β-selinene, α-guaiene, α-bisabolene, α-cedrene, caryophyllene, α-amorphene, α-chamigrene, bulnesene and valencene, acted synergistically to kill a broad range of plant- and human-pathogenic microorganisms. Our results are in agreement with previous data [11] in regard to the variation in antimicrobial activity of different *S. aspera* extracts. Antimicrobial activities of various sesquiterpenoids, methanol and diethyl ether extracts from the Tahitian liverwort *Mastigophora diclados* were evaluated against *Staphylococcus aureus* NBRC 15035 and *Bacillus subtilis* NBRC 3134. The MICs of methanol and diethyl ether extracts of *M. diclados* were 64 and 16 mg/mL, respectively [12]. These MIC values are higher than our results. The antimicrobial activity of the methanol extract of the liverwort, *Ptilidium pulcherrimum* was evaluated against five bacterial and six fungal species and the MIC for bacterial and fungal strains varied from 10–20 mg/mL to 0.5–2.5 mg/mL, respectively [13]. These results are similar and supported by our results.

The antimicrobial potential of the methanolic *S. aspera* extracts was also observed in terms of zone of inhibition generated by the diffusion of the *S. aspera* components into microorganism inoculated agar plates. The zone of inhibition increased with increasing concentration (i.e. 0.1 mg/mL, 0.2 mg/mL, and 0.3 mg/mL) of *S. aspera* extracts (Figure 1). The trend observed here with respect to the antimicrobial activity was similar to that observed in MIC determinations i.e. the zone of inhibition in the case of Gram-negative bacteria was less than that for the Gram-positive bacteria. The inhibition zone due to 0.3 mg/mL *S. aspera* extracts was *S. enteritidis* ≈ *E. coli* (17 mm) < *L. monocytogenes* (22 mm) < *A. pullulans* (26 mm) < *P. membranaefaciens* (35 mm) < *P. anomala* ≈ *S. cerevisiae* (36 mm).

S. aspera extracts represent a source of natural substances that exhibit antimicrobial potential which could be used to prevent the growth of food spoiling and pathogenic microorganisms. Additional investigations based on isolated components of *S. aspera* extract and experiments on real food systems could be useful for the food industry for improving shelf life and food safety (as natural preservative in food).

Experimental

Plant materials: *Scapania aspera* M. & H. Bern. was collected in April 2009 from a locality close to the river *Djetinja*, Uzice (Serbia). A voucher specimen (No. 16613) has been deposited in the herbarium at the Institute of Botany and Botanical Garden 'Jevremovac', University of Belgrade. Material was dried at room temperature for further use.

Extract preparation: Dried plants were pulverized into fine powder using an electric blender. Powdered material (10 g) was extracted with 100 mL methanol, ethanol and ethyl acetate for 24 h at room temperature. The mixture was then filtered through Whatman filter paper No 1. The solvents were evaporated in a rotary vacuum evaporator (Laborota 4001, Heidolph) at 40ºC. The yields of were 1.34%, 1.12% and 1.08% for methanol, ethanol and ethyl acetate extracts, respectively. The obtained extracts were stored at 4 ºC for further observations.

Solid phase, micro extraction, gas chromatographic-mass spectrometric (SPME GC-MS) analysis: A Divinyl benzene/Carboxen/Poly dimethyl siloxane (DVB/CAR/PDMS) coated stable flex fiber (65 µm) and a manual SPME holder (Supelco Inc., Bellefonte, PA) were used after preconditioning according to the manufacturer's instruction manual. Before each headspace sampling, the fiber was exposed to the GC inlet for 5 min for thermal desorption at 250°C. Samples (5 mg) were put into sealed vials (20 mL) and then equilibrated for 10 min at 40°C. The SPME fiber was exposed to each sample for 10 min by manually penetrating the septum and the fiber was inserted into the injection port of the GC for 10 min for desorption. GC–MS analyses were carried out on an Agilent 6890 gas chromatograph (Agilent Technologies, Palo Alto, CA) coupled to an Agilent 5970 mass selective detector operating in electron impact mode (ionization

voltage, 70 eV). A Chrompack CP Wax 52 CB capillary column (50 m length, 0.32 mm i.d., 1.2 μm df) was used (Chrompack, Middelburg, Netherlands). The temperature program was 50°C for 0 min, then programmed at 5°C min^{-1} to 230°C for 10 min. Injector, interface, and ion source temperatures were 250, 250, and 230°C, respectively. Injections were performed with a split ratio of 1:50 and helium (1 mL min^{-1}) as the carrier gas. Identification of compounds was carried out by comparison of their mass spectra with those available on the databases of NIST05 and WILEY8 libraries, and those of pure standards.

Chemicals and microbial strains: Analytical grade chemical reagents (methanol, ethanol, acetone) used for livewort extraction were purchased from Sigma-Aldrich GmbH (Germany). For antimicrobial activity, the different dry extracts were dissolved in dimethyl sulfoxide (DMSO) (Sigma–Aldrich GmbH, Germany). For yeast and bacterial cultivation, Yeast extract Peptone Dextrose (YPD) and Tryptic Soy Broth (TSB) (Merck KgaA Darmstad, Germany) were used, respectively. Bacterial and yeast strains were isolated from food. Different bacterial (*Salmonella enteritidis* 155, *Escherichia coli* 555 and *Listeria monocytogenes* 56Ly) and yeast strains (*Saccharomyces cerevisiae* 635*, Zygosaccharomyces bailii* 45*, Aerobasidium pullulans* L6F*, Pichia membranaefaciens* OC71, *Pichia membranaefaciens* OC70, *Pichia anomala* CBS 5759 and *Pichia anomala* DBVPG 3003) were obtained from the strain collection of the Department of Agricultural and Food Sciences of Bologna University, Italy. The strains of yeasts and bacteria used in this study were grown in Yeast extract Peptone Dextrose (YPD) at 28ºC for 48 h and Tryptic Soy Broth (TSB) at 32ºC for 24 h, respectively. Further microbial cells were suspended in sterile physiological water and used immediately.

Antimicrobial activity of different extracts of S. aspera: In order to investigate the antimicrobial activity of the extracts, the modified microdilution technique was used [14]. MIC determination was performed by the micro-broth dilution technique using 96-well microtiter plates. The extracts were dissolved in 0.5% dimethylsulfoxide (DMSO) and added to TSB and YPD broth with bacterial (10^6 cfu mL^{-1}) and yeast (10^5 cfu mL^{-1}) inoculum, respectively. The microplates were incubated for 24 h at 32ºC for bacteria and 48 h at 28ºC for yeasts, respectively. The lowest concentration without visible growth was defined as MIC. Minimal bactericidal concentration (MBC)/minimal fungicidal concentration (MFC) of the extracts were evaluated. MBC/MFC values were defined as the minimal concentrations of the tested molecule not allowing microbial growth on agar medium after incubation at optimal temperature. DMSO was used as negative control.

Disc diffusion method: The agar disc diffusion method was employed for the determination of antimicrobial activities of the methanolic extract of *S. aspera* [14]. Briefly, a suspension of the tested microorganism (100 μL of $1x10^6$ cfu/mL) was spread on the TS/YPD agar media plates. Filter paper discs, 6 mm in diameter (Schleicher & Schuell, Germany), were soaked with 10 μL of *S. aspera* extract and placed on the inoculated plates, which were incubated at 32ºC for 24 h and 28°C for 48 h for bacterial and yeast strains, respectively. The volume of *S. aspera* extract tested was varied (0.1, 0.2 or 0.3 mg/mL) by using an appropriate number of sterile discs. The diameters of the inhibition zones were measured in mm.

Statistical analyses: All the experiments were conducted in triplicate and repeatability was established. Significance of differences among treatments ($P \leq 0.05$) was analyzed using one way ANOVA (SPSS, 10.0 version).

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