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Metabolic profiling of *Brachychiton rupestris* (T.Mitch. ex Lindl.) K. Schum. leaves using UPLC-ESI-MS and their antimicrobial potential

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ABSTRACT

Globally, the number of multi-drug resistant microbes increases critically and the search for novel antimicrobial agents from medicinal plants becomes necessary to overcome such serious problem. The antimicrobial activity of the methanol extract of *Brachychiton rupestris* leaves and its fractions was assessed against six pathogens through two *in-vitro* assays: Agar well diffusion and broth microdilution. UPLC-ESI-MS analysis of these samples was also performed. *Mucor circinelloides* was resistant to the tested samples. The inhibition zone of the samples ranged between 10 and 23 mm while the minimum inhibitory values ranged between 31.25 and 1000 μg/mL. The samples produced significant antimicrobial potential. Based on the UPLC-ESI-MS analysis, the phytochemical profile of both the methanol extract and the ethyl acetate fraction comprise a variety of phenolic compounds and hydroxy fatty acids while that of the *n*-butanol fraction composed of phenolic compounds mainly

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flavonoids. *B. rupestris* could be considered a promising source of natural antimicrobials.

Keywords: *Brachychiton rupestris*, antimicrobial, UPLC-ESI-MS, phenolics, microdilution assay

INTRODUCTION

Infectious diseases represent one of the major causes of morbidity worldwide. Despite the existence of a variety of potent antibiotics in the market, the death rate due to microbial infections increases recently. This may be due to the ability of the pathogenic microbes to develop resistance against the available antimicrobial drugs as a way to survive. The excessive and improper use of the available commercial antibiotics participated in the emergence of antimicrobial resistance (AMR). The increased number of multidrug resistance microbes side by side with the multiplicity of the undesirable side effects of the existing antimicrobials and the appearance of new maladies for which no treatment yet exists creates a crucial need to find out novel and potent antimicrobial agents[1,]

For centuries, man depends on medicinal plants, in the treatment, control and prevention of various illnesses. Nowadays, people in both developed and developing countries are still relying on medicinal plants in providing their primary health care needs by the reason of their affordability, availability and safety due to their lesser toxicity and lesser side effects in comparison with conventional synthetic drugs[3]. Presently, medicinal plants are considered a valuable source for searching for new drug leads based on their richness with bioactive secondary metabolites such as flavonoids and phenolic acids[4].

Brachychiton rupestris (T.Mitch. ex Lindl.) K. Schum. is one of the medicinal plants that is characterized by its bottle shaped trunk and belongs to the family Malvaceae. Too little studies have been conducted on *B. rupestris*. *B. rupestris* was reported to possess significant antischistosomal potential on *Schistosoma mansoni* as well as potent hypoglycemic and hepatoprotective activity on experimental animals in addition to antioxidant, antimicrobial and cytotoxic activities [5- 8]. Based on the previously published papers, the phytochemical profile of *B. rupestris* comprises flavonoids, phenolics, sterols and volatile compounds[9-13].

Ultra-high performance liquid chromatography (UHPLC) hyphenated with mass spectrometer (MS) is a newly applied separation and identification technique which is characterized by its remarkable high resolution, speed, sensi-

tivity and time and resources saving. It has been widely used in the separation and tentative identification of secondary metabolites existed in plant extracts and fractions[14].

This study aimed to investigate the antimicrobial activity of the methanol extract *B. rupestris* leaves as well as its ethyl acetate and *n*-butanol derived fractions against six microbial strains through two *in-vitro* methods. Also, separation and tentative identification of their phytochemical profile through the application of UPLC-ESI-MS in the negative ionization mode.

METHODOLOGY

Plant material

Leaves of *Brachychiton rupestris* were collected in September 2017 from Orman Garden, Giza, Egypt. Both Mrs. Treaze Labib (a consultant of plant taxonomy at the Agriculture Ministry and the ex-director of Orman Garden) and Mrs. Rehab Mohamed Eid (a botanist at Orman Garden Herbarium) performed the identification process of the plant specimen and a voucher sample (No. 278 BC) was deposited in Orman Garden Herbarium. The collected plant leaves were shade dried at room temperature and were then blended to a coarse powder using an electric blender.

Extraction and fractionation processes

The plant leaves fine powder (200 g) was successively extracted with 85% aqueous methanol at room temperature. The resultant extract was then filtered and evaporated under a reduced vacuum using a rotary evaporator (BUCHI, Germany) till complete dryness affording crude methanol extract of 33.58 grams (16.79%). The crude extract was then fractionated using petroleum ether, methylene chloride, ethyl acetate and *n*- butanol successively and evaporated under reduced pressure till complete dryness giving approximately 1 g of petroleum ether, 2.70 g of methylene chloride, 0.90 g of ethyl acetate, 4.50 g of *n*- butanol and 11.10 g of aqueous fractions.

UPLC-ESI-MS profiling

Waters XEVO TQD UPLC-ESI-MS (MA01757, Milford, USA) equipped with ACQUITY UPLC- BEH C18 (1.7: 2.1 μm× 50 mm) column was used to separate and tentatively identify the major secondary metabolites in the *B. rupestris* methanol extract and its ethyl acetate and *n*-butanol derived fractions. A stock solution of the three test samples (100 μg/mL) was prepared through dissolution in HPLC analytical grade MeOH previously filtered with a 0.2 μm membrane disc filter. 10 μL of each sample was injected into the UPLC instru-

ment. The mobile phase elution was carried out at a flow rate of 0.2 mL/min using two eluents: eluent A is H_2O acidified with 0.1% formic acid and eluent B is MeOH acidified with 0.1% formic acid. The gradient elution was set as follows: 0–5 min, 10%–30% B; 5–15 min, 30%–70% B; 15–25 min, 70%–90% B, 25–32 min, 90%–100% B.

The negative ESI ionization ion mode was performed by applying the following parameters: source temperature 150 °C, cone voltage 60 eV, capillary voltage 3 kV, desolvation temperature 440 °C, cone gas flow 50 L/h, and desolvation gas flow 900 L/h. Mass spectra were detected in the ESI between *m/z* 100–1000. The peaks and spectra were processed using the Masslynx 4.1 software. The separated compounds were tentatively identified by comparing their retention time (R_t) and their mass spectra with the previously published literature.

Antimicrobial properties

Microbial strains and growth conditions

Four bacterial strains including two gram-positive bacteria; *Bacillus Subtilis* (ATCC 6633) and *Staphylococcus aureus* (ATCC 6538) and two gram-negative bacteria; *Escherichia coli* (ATCC 8739) and *Pseudomonas aeruginosa* (ATCC 90274) in addition to two pathogenic yeasts; *Candida albicans* (ATCC 10221) and *Mucor circinelloides* (AUMC 6696) were used to investigate the antimicrobial properties of the crude methanol extract of *B. rupestris* leaves and its ethyl acetate and *n*-butanol derived fractions. Non supplemented Mueller-Hinton agar or routine bacteriology laboratory Mueller-Hinton agar plates (pH, 7.2–7.4 after gelling) were used to culture the microbial strains. Gentamycin was used as a positive control.

Agar well diffusion assay

Briefly, Mueller-Hinton agar plates were inoculated with a suspension of the test microbes adjusted to 10^8 CFU/mL. Then, 6 mm diameter holes were made aseptically in the inoculated plates. In each hole, 100μL of the antimicrobial agent (gentamycin) or the plant test samples at a concentration of 10 mg/mL (methanol) were loaded. The agar plates were then incubated for 24 h at 37°C for bacteria or 48 h at 37°C for yeast. The antimicrobial agent as well as the test samples will diffuse in the agar medium and inhibit the growth of the microbial strains tested. After the incubation period, the zone of microbial growth inhibition was measured accurately in mm as an indication of antimicrobial activity[2]. The strength of the antimicrobial activity of the test samples against the test microorganisms was assigned based on the diameter of the growth inhibition zone as follows; potent antimicrobial potential when the growth inhibition

zone diameter is greater than 30 mm, strong when the growth inhibition zone diameter ranged between 30 and 21 mm, moderate when the growth inhibition zone diameter ranged between 20 and 16 mm, weak when the growth inhibition zone diameter ranged between 15 and 10 mm and little or no activity if the inhibition zone diameter is less than 10 mm[15].

Minimum inhibitory concentration (MIC)

The minimum inhibitory concentration is the lowest concentration of an antimicrobial agent that prevents the visible growth of a microorganism which can be determined via a broth microdilution susceptibility test. Briefly, pure cultures of the test microbes were grown overnight and diluted in Muller Hinton broth to a concentration between 10^5 CFU/mL to 10^6 CFU/mL. A stock solution of the test samples was then prepared by dissolving 10 mg of each sample in 10 mL of distilled water (1000 μg/mL). Furthermore, serial two fold dilutions of the test samples were prepared and distributed in 96-well microtiter plates ranging from 1000 to 0.06 μg/mL. Ten microliters of the prepared microbial suspension were added to the wells. Sterility and a growth control well were also included for every test microorganism. The microtiter plates were then incubated for 24 h at 37°C. The MICs were indicated via observing the turbidity which is the reflection of microbial growth. MIC is the lowest concentration where no visible growth is observed[1]fractions and isolated compounds from this plant. Bioassay guided fractionation was also undertaken to deeply evaluate the antibacterial activity of the water fraction of the leaves extract. This is to provide preliminary scientific evidence to the ethnopharmacology usage of this plant by investigating antibacterial properties of the plant and its isolated constituents. Methods: Bio-assay guided fractionation and subsequent isolation of compounds using open column chromatography. The antibacterial activity against gram positive and gram negative ATCC strain and resistant clinical strains were evaluated using microtiter broth dilution method to determine minimum inhibitory concentration (MIC.

RESULTS and DISCUSSION

Identification of the major chemical constituents of *B. rupestris* using (UPLC-ESI-(-ve)-MS)

The secondary metabolites existed in the crude methanol extract of *B. rupestris* leaves, the ethyl acetate and the *n*-butanol derived fractions were tentatively identified via UPLC-ESI-MS analysis. Their TICs were exhibited in Figure 1.

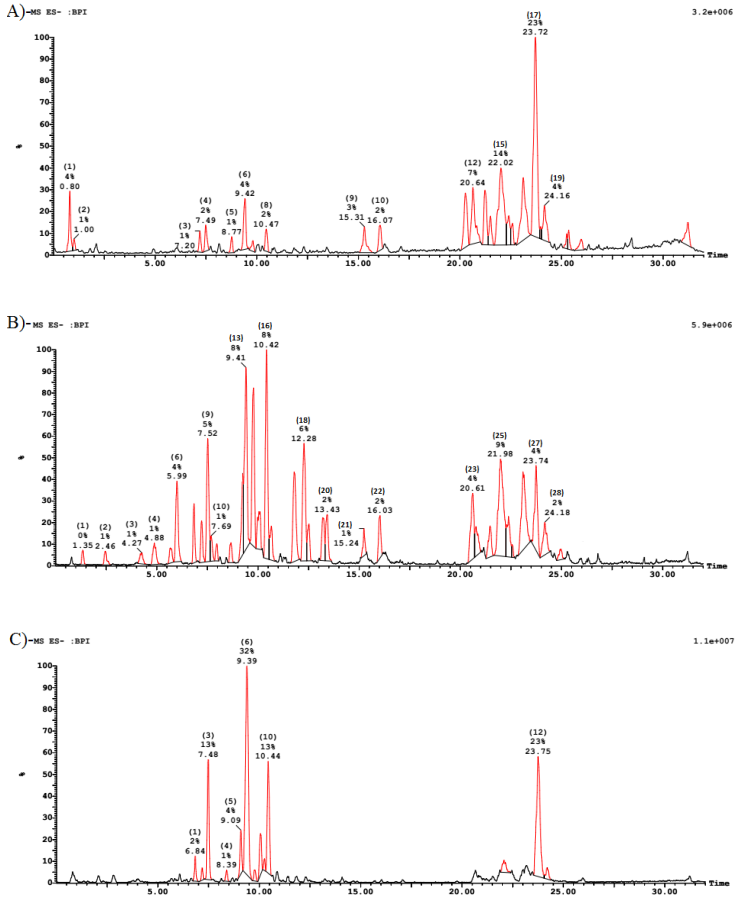

Figure 1. Total ion chromatograms (TIC) of A)-85% MeOH extract, B)- EtOAC fraction and C)-n-BuOH fraction of *B. rupestris* leaves, Numbering of peaks indicates compounds tentatively identified in the Tables (1-3).

Compounds tentatively identified in the methanol extract of *B. rupestris* leaves

Ten phenolics and five hydroxy fatty acids were identified in the methanol extract of *B. rupestris* leaves. The retention time, the molecular weight and the fragmentation data of these compounds were presented in **Table 1**. Peak 1, R_t 0.79 min, exhibited $[M-H]^-$ ion at m/*z* 341 and product ions at m/*z* 179 $[M-H-162]^-$ and 135 which are the characteristic fragments of caffeic acid produced due to loss of hexosyl residue (162 Da). So, this compound was identified as caffeoyl hexoside[16]. Peak 2, R_t 1.00 min, showed a molecular ion peak at m/*z* 191 with a fragment ion at m/*z* 127 $[M-H-CO-2H_2O]^-$ so that it was identified

as quinic acid[17]. Peak 3, R_t 7.20, had a $[M-H]^-$ ion at m/*z* 401 in addition to two base peaks at m/*z* 269 formed due to loss of pentosyl residue (132 Da) and 161 formed as a result of loss of both pentosyl and hexosyl residues (132 Da+162 Da). This compound was identified as benzyl alcohol hexose pentose[18]. Peak 4, R_t 7.50 min, exhibited the deprotonated molecule ion at m/*z* 593 and fragment ion at m/*z* 431 due to loss of hexose sugar in addition to two more fragment ions at m/*z* 163 and 119 which are indicative of coumaroyl residue thus this compound was identified as Kaempferol-3-*O*-coumaroylhexoside[19,20]. Peak 5, R_t 8.77 min, displayed a parent ion peak at m/*z* 739 and two main fragments at m/*z* 430 $[M-2H-146-162]^-$ (loss of hexose and deoxyhexose sugars) and 284 formed due to loss of deoxyhexosyl residue from the previous peak. This MS data suggested that this compound could be kaempferol deoxyhexoside hexoside deoxyhexoside [21]. Peak 6, R_t 9.41 min, had a parent ion at m/*z* 609 in addition to two major fragment ions at m/*z* 463 and 300 such MS data indicated that this compound could be rutin[22]. Peak 7, R_t 9.81 min, showed the $[M-H]^-$ ion at m/*z* 433 and two fragments ions at m/*z* 300 and 152 which are indicative of quercetin aglycone that was formed as a result of loss of pentose sugar (132 Da) so that this compound was identified as quercetin pentoside[23]. Peak 8, R_t 10.48 min, exhibited a molecule ion at m/*z* 593 and two fragments at m/*z* 447 (loss of rhamnosyl moiety) and 285 (loss of rutinosyl moiety). This MS data indicated that this compound could be kaempferol rutinoside[24]. Peak 9, R_t 15.31 min, exhibited a molecular ion peak at m/*z* 327 and fragment ions at m/*z* 239, 229, 211 and 171. This compound was identified as oxo-dihydroxy-octadecenoic acid isomer based on data published by[25]. Peak 10, R_t 16.08 min, showed a molecular ion peak at m/*z* 329 in addition to base peaks at m/*z* 229, 221 and 171. This compound was identified as trihydroxy-octadecenoic acid based on similar MS data reported by[25]. Peak 11, R_t 20.25 min, showed a molecule ion at m/*z* 293 in addition to a fragment ion at m/*z* 275. These MS fragments were consistent with MS data of hydroxy-octadecatrienoic acid that was reported by[26]. Peak 13, R_t 21.24 min, displayed a molecule ion at m/*z* 295 with three fragment ions at m/*z* 277, 195 and 171. Also, peak 14, R_t 21.15 min, displayed a molecule ion at m/*z* 309 with two fragment ions at m/*z* 295 and 180. These compounds were identified as hydroxy-octadecadienoic acid and dihydroxy-octadecadienoic acid, respectively depending on MS fragmentation patterns reported by[26]. Peak 15, R_t 22.02 min, exhibited a deprotonated molecule ion at m/*z* 577 and fragment ions at 397, 353 [M–H–104–120]; the loss of 104 Da indicated the partial fragmentation of *C*- deoxyhexosyl moiety ($^{0,2}X_o$)$^-$ ion while the loss of 120 Da is characteristic for *C*- hexosyl flavone and 311. According to the mentioned MS data, this compound was identified as Api-

genin 8-*C*-deoxyhexoside-6-*C*-hexoside[27]. Peak 16, R_t 23.13 min, exhibited a molecular ion peak at m/*z* 325 and a base peak at m/*z* 293 formed due to loss of O_2 molecule. This compound was identified as *p*-coumaroyl hexoside[20].

Table 1. Compounds tentatively identified in methanolic extract of *B. rupestris* leaves by UPLC-ESI-(-ve)-MS

Peak no.	RT (min)	MW	$[M-H]^-$ (m/z)	Fragments (m/z)	Tentative Identification
	0.79	342	341	179, 135	Caffeoyl-*O*-hexoside
	1.00	192	191	127	Quinic acid
	7.20	402	401	269, 161	Benzyl alcohol hexose pentose
	7.50	594	593	431, 163, 119	Kaempferol-3-O-coumaroylhexoside
	8.77	740	739	430, 284	Kaempferol deoxyhexoside hexoside deoxyhexoside
	9.41	610	609	463, 300	Rutin
	9.81	434	433	300, 152	Quercetin pentoside
	10.48	594	593	447, 285	Kaempferol rutinoside
	15.31	328	327	239, 229, 211, 171	Oxo-dihydroxy-octadecenoic acid isomer
	16.08	330	329	229, 211, 171	Trihydroxy-octadecenoic acid
	20.25	294	293	275	Hydroxy-octadecatrienoic acid
	20.64	298	297	265	Unknown
	21.24	296	295	277, 195, 171	Hydroxy-octadecadienoic acid
	21.51	310	309	295, 180	Dihydroxy-octadecadienoic acid
	22.02	578	577	397, 353, 311	Apigenin 8-*C*-deoxyhexoside-6-*C*-hexoside
	23.13	326	325	293	*p*-coumaroyl hexoside
	23.71	556	555	337	Unknown
	23.97	572	571	540, 483, 447, 339	Unknown
	24.17	582	581	483, 455, 339, 163	Unknown

Compounds tentatively identified in the ethyl acetate fraction derived from the methanol extract of *B. rupestris* leaves

Twenty-two phenolics and three hydroxy fatty acids were tentatively identified in the ethyl acetate fraction derived from the methanol extract of *B. rupestris*. The retention time, the molecular weight and the MS data of these compounds were presented in **Table 2**. Peak 1, R_t 1.35 min, exhibited a parent ion at m/*z* 169 and a daughter ion at m/*z* 125 which are indicative to gallic acid[28]. Peak 2, R_t 2.46 min, showed a precursor ion at m/*z* 153 that was fragmented by losing CO_2 molecule producing ion at m/*z* 109 and hence this compound was identified as protocatechuic acid[29]. Peak 3, R_t 4.27 min, showed a molecule ion at m/*z* 153 and a fragment ion at m/*z* 108 which was formed due to loss of carboxylic group (– COOH, 45 Da) so that it was identified as dihydroxybenzoic acid[30]. Peak 4, R_t 4.88 min, displayed $[M-H]^-$ ion at m/*z* 183 and a fragment ion at m/*z* 124 which are indicative of methyl ester of gallic acid[31]. Peak 5, R_t 5.67 min, showed a molecule ion at m/*z* 177 that was further fragmented by losing CO_2 molecule producing ion at m/*z* 133. So, this compound was identified as 6, 7-dihydroxycoumarin (esculetin)[32]. Peak 6, R_t 5.99 min, showed a parent peak at m/*z* 179 and a base peak at m/*z* 135 formed due to loss of 44 Da (CO_2) which suggested that this compound could be caffeic acid[25]. Peak 7, R_t 6.84, has a molecule ion at m/*z* 633 and three fragment ions at m/*z* 463, 301 and 166. This compound was identified as corilagin as its MS data was consistent with the reported MS data of corilagin[33]. Peak 8, R_t 7.21, showed a parent ion at m/*z* 327 $[2M-H]^-$ and fragment ions at 283 $[2M-H-44]^-$ formed due to loss of CO_2, 237 $[2M-H-2\times45]^-$ formed due to loss of two carboxylic groups (COOH) and 163 (*p*-coumaric acid). So, this compound was identified as a dimer of coumaric acid. Peak 9, R_t 7.52 min, exhibited a molecular ion peak at m/*z* 593 with fragment ion at 431 formed due to loss of hexose sugar in addition to two more fragments at m/*z* 163 and 119 which are indicative to coumaric acid thus this compound was identified as Kaempferol-3-*O*-coumaroylhexoside [34]. Peak 10, R_t 7.69 min, showed a molecule ion at m/*z* 305 and fragment ions at 273 and 247. This compound was identified as methyl brevifolin carboxylate[24]. Peak 11, R_t 7.97 min, presented a molecule ion at m/*z* 303 and fragments at m/*z* 285, 151, 125 and 119. Based on this MS data, this compound was identified as taxifolin[35]. Peak 12, R_t 8.67 min, showed a precursor ion at m/*z* 615 and daughter ions at 463 and 301. This fragmentation pattern was consistent with quercetin galloyl hexoside[28]. Peak 15, 16 and 19 possessed the same MS pattern; $[M-H]^-$ ion at m/*z* 593 and fragment ions at m/*z* 447 and 285 and so identified as kaempferol rutinoside isomers that was also identified in MeOH ext. of *B. rupestris*. Peak 13, 14, 21, 22, 24, 25 and 26 were identified as rutin,

quercetin hexoside, oxo-dihydroxyoctadecenoic acid, trihydroxyoctadecenoic acid, dihydroxy-octadecadienoic acid, apigenin 8-C-deoxyhexoside-6-C-hexoside and *p*-coumaroyl hexoside, respectively as they had already identified in the MeOH extract of *B. rupestris* based on the MS data of the same compounds reported in the literature. Peak 17, R_t 11.79 min, exhibited a molecular ion at m/*z* 301 and fragment ions at m/*z* 289, 179 and 151 and so was identified as quercetin[35]. Furthermore, peak 18, R_t 12.28 min, possessed a molecule ion at m/*z* 285 and a fragment at m/*z* 151 and hence was identified as luteolin or kaempferol[35]. Also, peak 20, R_t 13.43 min, had a molecule ion at m/*z* 269 and a fragment ion at m/*z* 151 and was identified as apigenin[36].

Table 2. Compounds tentatively identified in EtOAc fraction derived from methanolic extract of *B. rupestris* by UPLC-ESI-(-ve)-MS

Peak no.	RT (min)	MW	$[M-H]^-$ (m/z)	Fragments (m/z)	Tentative Identification
	1.35	170	169	125	Gallic acid
	2.46	154	153	109	Protocatechuic acid
	4.27	154	153	108	Dihydroxybenzoic acid
	4.88	184	183	124	Methyl gallate
	5.67	178	177	133	6,7-dihydroxycoumarin (Esculetin)
	5.99	180	179	135	Caffeic acid
	6.84	634	633	463, 301, 166	Corilagin
	7.21	328	327	283, 237, 163	Dimer of coumaric acid
	7.52	594	593	431, 163, 119	Kaempferol-3-*O*-coumaroylhexoside
	7.69	306	305	273, 247	Methyl brevifolin carboxylate
	7.97	304	303	285, 151, 125, 119	Taxifolin
	8.67	616	615	463, 301	Quercetin galloyl hexoside
	9.41	610	609	463, 301	Rutin
	9.77	434	433	301	Quercetin pentoside
	10.05	594	593	447, 285	Kaempferol rutinoside isomer
	10.42	594	593	447, 285	Kaempferol rutinoside isomer
	11.79	302	301	289, 179, 151	Quercetin

	12.28	286	285	151	Luteolin or Kaempferol
	12.52	594	593	447, 285, 227	Kaempferol rutinoside isomer
	13.43	270	269	151	Apigenin
	15.24	328	327	239, 229, 211, 171	Oxo-dihydroxyoctadecenoic acid
	16.03	330	329	229, 211, 171	Trihydroxyoctadecenoic acid
	20.61	298	297	265	Unknown
	21.47	310	309	295, 180	dihydroxy-octadecadienoic acid
	21.98	578	577	397, 353, 311	Apigenin 8-*C*-deoxyhexoside-6-*C*-hexoside
	23.10	326	325	293, 277	*p*-coumaroyl hexoside
	23.74	556	555	337	Unknown
	24.18	582	581	483, 455, 339, 279	Unknown

Compounds tentatively identified in the *n*-butanol fraction derived from the methanol extract of *B. rupestris* leaves

Eleven phenolic compounds were tentatively identified in the *n*-butanol fraction derived from the methanol extract of *B. rupestris*. The retention time, the molecular weight and the MS fragmentation data of these compounds were presented in **Table 3**. Peak 1, R_t 6.84 min, exhibited a molecular ion at m/*z* 633 with a fragment ion at m/*z* 301. This compound was identified as corilagin which was also identified in the ethyl acetate fraction of *B. rupestris*. Peak 2, R_t 7.19 min, showed a parent ion at m/*z* 401 and a fragment ion at m/*z* 269 resulting from loss of pentose sugar. So, this compound was identified as benzyl alcohol hexosyl pentoside[18]. Peak 3, 6 and 7 were identified as kaempferol-3-*O*-coumaroylhexoside, rutin and quercetin pentoside based on their MS fragmentation data that is consistent with previous reports. These compounds were also identified in both the *B. rupestris* MeOH ext. and its ethyl acetate derived fraction. Peak 8 and 10 had the same $[M-H]^-$ ion at m/*z* 593 as well as the fragment ions at m/*z* 447 and 285 and so they were identified as kaempferol rutinoside isomers that were also identified in the MeOH ext. and its EtOAc derived fraction. Peak 4, R_t 8.39 min, exhibited $[M-H]^-$ ion at m/*z* 755 and fragment ions at m/*z* 593 $[M-H-162]^-$, 445 $[M-2H-162-146]^-$ and 289. So, this compound was identified as Kaempferol hexosyl deoxyhexosyl hexoside[37]. Peak 5, R_t 9.09 min, showed a molecular ion peak at m/*z* 739 and fragment

ions at m/*z* 431 [M–H–308] $^-$ produced due to loss of rutinosyl moiety and 285[M–H–308–146] $^-$ formed as a result of losing of one more deoxyhexosyl moiety. This compound was identified as Kaempferol hexosyl di-deoxyhexoside[38]. Peak 9, R_t 10.26 min, showed [M–H] $^-$ ion at m/*z* 591 with two major base peaks at m/*z* 445 [M–H–146] $^-$ resulting from loss of deoxyhexosyl sugar and 269 [M–H–146–176] $^-$ formed as a result of breaking down of glucuronide moiety from the previous peak leading to formation of apigenin aglycone. So, this compound was identified as apigenin glucuronide deoxyhexoside[39]. Peak 11, R_t 21.94 min, showed a precursor ion at m/*z* 593 and fragment ions at m/*z* 577, 353 and 311. This compound was identified as vicenin-2[29].

Table 3. Compounds tentatively identified in n-BuOH fraction derived from methanolic extract of *B. rupestris* by UPLC-ESI-(-ve)-MS

Peak no.	RT (min)	MW	$[M-H]^-$ (m/z)	Fragments (m/z)	Tentative Identification
	6.84	634	633	301	Corilagin
	7.19	402	401	269	Benzyl alcohol hexosyl pentoside
	7.48	594	593	431, 163, 119	Kaempferol-3-*O*-coumaroylhexoside
	8.39	756	755	593, 445, 289	Kaempferol hexosyl deoxyhexosyl hexoside
	9.09	740	739	431, 285	Kaempferol hexosyl dideoxyhexoside
	9.39	610	609	463, 301	Rutin
	9.76	434	433	300	Quercetin pentoside
	10.06	594	593	447, 285	Kaempferol rutinoside isomer
	10.26	592	591	445, 269	Apigenin glucuronide deoxyhexoside
	10.44	594	593	447, 285	Kaempferol rutinoside isomer
	21.94	594	593	577, 353, 311	Vicenin -2 (Apigenin-6,8-di-C-hexoside)
	23.75	556	555	---	Unknown

Antimicrobial properties

Agar well diffusion assay

The antimicrobial potential of the crude methanol extract of *B. rupestris* leaves as well as its ethyl acetate and *n*-butanol derived fractions was assessed against six pathogenic microorganisms using agar well diffusion assay **(Table 4, Figure 2)**. In particular, the three test samples exerted no antimicrobial activity against the pathogenic yeast *Mucor circinelloides* (AUMC 6696). They also showed moderate antibacterial activity against *B. subtilis*. The *n*-butanol fraction exhibited strong antibacterial activity against *S. aureus* with an inhibition zone of 21 mm in diameter while the crude extract and the ethyl acetate fraction showed weak antibacterial activity with an inhibition zone of 15 mm and 13 mm, respectively. Also, the methanol extract exhibited strong antibacterial activity against *E. coli* with an inhibition zone of 23 mm diameter while the *n*-butanol fraction exhibited moderate antibacterial activity against *E. coli* with an inhibition zone of 18 mm diameter. The anti- *E. coli* response of the methanol extract and the *n*-butanol derived fraction was greater than the response produced by the used positive control (gentamycin) at the same concentration and conditions where its inhibition zone was 17 mm diameter. Both the ethyl acetate and the *n*-butanol fractions exerted moderate antimicrobial activity against *P. aeruginosa* and *C. albicans*, respectively with an inhibition zone of 16 mm in diameter. Moreover, the methanol extract and the *n*-butanol fraction had weak anti-*P. aeruginosa* with inhibition zone of 15 mm and 12 mm diameter, respectively. The same response was also observed for the crude extract and the ethyl acetate fraction against *C. albicans* whose inhibition zone diameter was 14 mm and 12 mm, respectively.

Table 4. Antimicrobial activity via well diffusion assay of the methanol extract, the ethyl acetate and the *n*-butanol derived fractions of *B. rupestris* leaves

Results are expressed as zone of growth inhibition (mm), NA: no growth inhibition

Sample / Pathogenic microorganism	Crude MeOH extract	EtOAc derived fraction	*n*-BuOH derived fraction	Gentamycin
Zone of growth inhibition (mm)				
Gram-positive bacteria				
Bacillus subtilis (ATCC 6633)	16	17	19	25
Staphylococcus aureus (ATCC 6538)	15	13	21	19
Gram-negative bacteria				
Escherichia coli (ATCC 8739)	23	10	18	17
Pseudomonas aeruginosa (ATCC 90274)	15	16	12	22
Fungal strains				
Candida albicans (ATCC 10221)	14	12	16	21
Mucor circinelloides (AUMC 6696)	NA	NA	NA	16

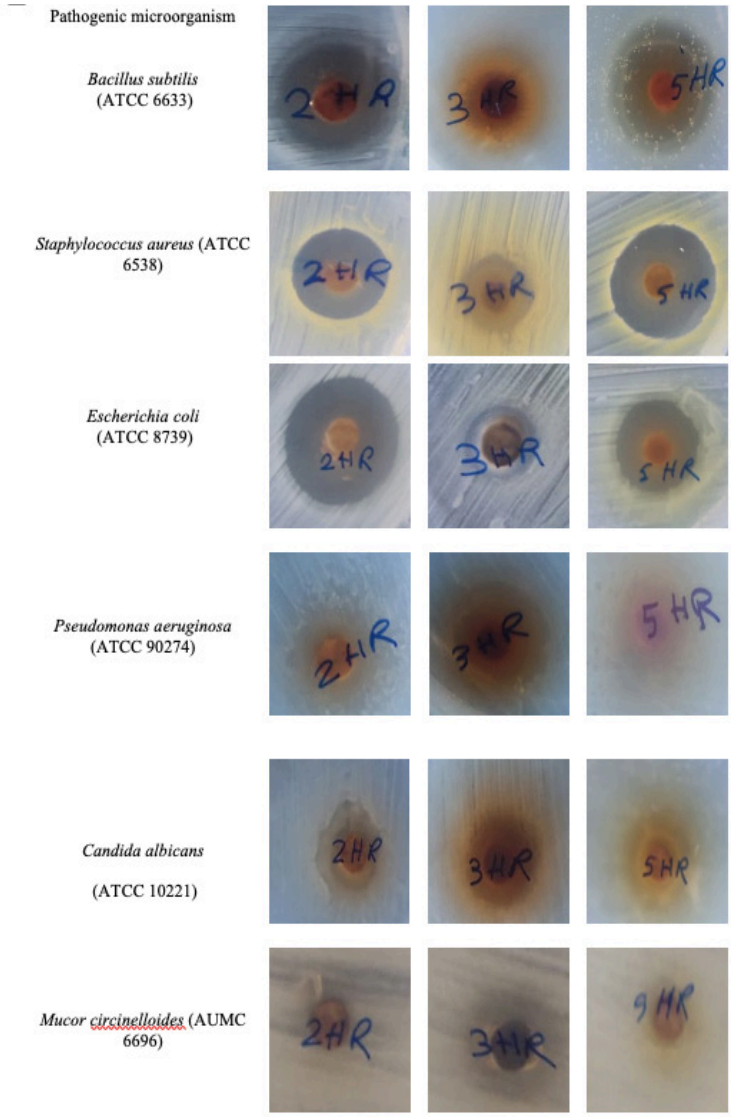

Figure 2. Antimicrobial activity via well diffusion assay of 2HR: 85% MeOH extract, 3HR: EtOAC derived fraction and 5HR: n-BuOH derived fraction of B. rupestris leaves (10 mg/mL) against tested microorganisms in MHA medium after incubation for 24 h at 37° for bacteria or 48 h at 37° for yeast.

Minimum inhibitory concentration (MIC)

The MIC values of the three samples under investigation against the five susceptible pathogens were demonstrated in **Table 5**. The test sample is considered a strong antimicrobial agent if its MIC value equals or lower than 500 μg/mL[1]. Accordingly, the methanol extract of *B. rupestris* leaves and its ethyl acetate fraction could be considered strong microbial inhibitors against the test samples where their MIC values ranged between 31.25 to 250 μg/mL and 100 to 500 μg/mL, respectively. Also, the *n*-butanol fraction exhibited strong antimicrobial activity against all the test microbes except for *P. aeruginosa* where its MIC values ranged between 62.5 and 1000 μg/mL. The most potent antimicrobial activities of the methanol extract, the ethyl acetate fraction and the *n*-butanol fraction were observed against E. coli, E. coli and B. subtilis, respectively where their MIC values were 31.25, 100 and 62.5 µg/mL, respectively.

Table 5. Minimal inhibitory concentrations of the methanol extract, the ethyl acetate and the n-butanol derived fractions of *B. rupestris* leaves against tested microorganisms

Samples / Pathogenic microorganisms	Crude MeOH extract	EtOAc derived fraction	*n*-BuOH derived fraction
Conc. (µg/mL)			
Bacillus subtilis (ATCC 6633)	125	125	62.5
Staphylococcus aureus (ATCC 6538)	125	500	250
Escherichia coli (ATCC 8739)	31.25	100	250
Pseudomonas aeruginosa (ATCC 90274)	250	125	1000
Candida albicans (ATCC 10221)	125	500	250

STATEMENTS OF ETHICS

Not applicable

CONFLICT OF INTEREST STATEMENT

The authors declare no conflict of interest.

AUTHOR CONTRIBUTIONS

EAE, MME and ESA conceived and supervised the study. HRM and MS performed experimental work. HRM wrote the first draft of the manuscript. All authors contributed to data analysis and interpretation, revising the article,

gave final approval of the version to be published, and agreed to be accountable for all aspects of the work.

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