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Bioactive properties and phenolic profile of *Momordica charantia* L. medicinal plant growing wild in Trinidad and Tobago

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ABSTRACT

A wild variety of bitter melon *Momordica charantia* L. (*Cucurbitaceae*) has been used in bush medicine of Trinidad and Tobago for treatment of diabetes, inflammations and cancer. Despite many studies regarding the cultivated bitter melon, the wild variety has been poorly investigated. This study evaluates the biological activities of the ethanol/water extract of aerial parts and correlates these activities with the presence of phenolic compounds. The extract exhibited antioxidant activity in the four assays (DPPH, reducing power, β-carotene bleaching and TBARS). The key role of oxidative stress in inflammation and tumorigenesis was supported by the results of anti-inflammatory (inhibition of nitric oxide production) and cytotoxicity (human tumor cell lines, namely HeLa, HepG2, MCF-7, and NCI-H460) assays. In contrast, no toxicity was observed in non-tumor cells. In the antibacterial screening, clinical resistant isolates were significantly affected (MIC_{50} = 10–0.625 μg/mL), being *Listeria monocytogenes* the most susceptible. Three phenolic acids and eleven flavonol glycosides derivatives were identified, quercetin-3-*O*-pentosylhexoside being the most abundant.

1. Introduction

With the growing incidence of both bacterial resistance and civilization diseases (*e.g.* cardiovascular disorders, cancer, diabetes, atherosclerosis, *etc.*) people started to pay more attention to the prevention of these problems and maintenance of their health *via* diet and supplementations (Paredes-Lopez et al., 2010). Moreover, the use of synthetically prepared food additives have been under the criticism for potential health risks lately and the food industry is constantly in search for novel nature-derived compounds and matrices with significant biological activities for both food safety and added health-promoting value (Carocho et al., 2014).

The plant kingdom remains the largest source of compounds with such qualities, especially tropical plants, which prove to be rich in the production of secondary metabolites due to the highly competitive environment and need for self-defense (Herms and Mattson, 1992). For example, 70% of plants with *in vitro* antitumor activity are of tropical origin (Pandey et al., 2015). Phenolic compounds, among others, have been associated with biological activities, such as antioxidant, antimicrobial, cytotoxic and anti-inflammatory (Heleno et al., 2015; Li et al., 2014; Martins et al., 2016; Roleira et al., 2015).

The ethnopharmacological approach with targeted screening based upon traditional medicinal use of plants is generally considered as the most effective way for discovering new bioactive agents (Gu et al., 2014). In Trinidad and Tobago, the bush medicine has a long tradition and many people still rely on remedies derived from local flora (Clement et al., 2015).

Momordica charantia L. is an annual climbing vine of the family *Cucurbitaceae*, locally known as bitter melon or karela in Trinidad

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and Tobago. This tropical plant occurs wildly in tropical and subtropical Africa, Asia, America and Caribbean and is also cultivated for edible fruits (Kubola and Siriamornpun, 2008). The fruits of the wild variety are of one-fifth comparing to the cultivated analogue. Karela has been used worldwide for a number of ailments, including diabetes, hypertension, obesity, cancer, as well as AIDS, and hundreds of studies have been published regarding these biological activities (Dandawate et al., 2016; Fang and Ng, 2011; Habicht et al., 2014; Tan et al., 2016; Upadhyay et al., 2015). The biological activity is usually attributed to the content of triterpenoids and proteins, for example momordicines, cucurbitacins and momorcharins (Grover and Yadav, 2004). However, despite the frequent use in natural medicine, the wild variety from the Caribbean is poorly investigated, especially with the possible relationship to its phenolic composition. As the wild varieties of tropical plants are often richer in bioactive phenolic compounds than its cultivated analogues (Braca et al., 2003), we decided to have a detailed look on the biological activity of this plant. Additionally, as World Health Organization (WHO) called for the scientific evaluation on the effectiveness of plant-derived drugs, the determination of claimed biological activities among widely used species is highly relevant (Bagozzi, 2003). Therefore, the aim of this work was to obtain reference data for the application of the crude extract of wild *Momordica charantia* L. as a natural agent with multiple biological activities for the production of new nutraceuticals and natural food additive development.

2. Materials and methods

2.1. Standards and reagents

Acetonitrile 99.9% of HPLC grade was from Fisher Scientific (Lisbon, Portugal). Trolox (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid), β-carotene and ellipticine were purchased from Sigma-Aldrich (St. Louis, MO, USA), as also acetic acid, phosphate buffered saline (PBS), sulforhodamine B (SRB), trypan blue, trichloroacetic acid (TCA), Tris-(hydroxymethyl)aminomethan (TRIS), and lipopolysaccharide (LPS). Phenolic compound standards were from Extrasynthèse (Genay, France). DPPH (2,2-diphenyl-1-picrylhydrazyl) was obtained from Alfa Aesar (Ward Hill, MA, USA). Dulbecco's Modified Eagle's Medium (DMEM) and RPMI-1640 medium, Fetal bovine serum (FBS), Hank's balanced salt solution (HBSS), L-glutamine, nonessential amino acids solution (2 mM), penicillin/streptomycin solution (100 U/mL and 100 mg/mL, respectively), and trypsin-EDTA (ethylenediaminetetraacetic acid) were from Hyclone (Logan, UT, USA). The Griess reagent system was purchased from Promega Corporation (Madison, WI, USA). The culture media Muller Hinton broth (MHB) and Tryptic Soy Broth (TSB) were obtained from Biomerieux (Marcy l'Etoile, France), as well as the blood agar with 7% sheep blood and Mac Conkey agar plates. The dye *p*-iodonitrotetrazolium chloride (INT) was purchased from Sigma-Aldrich (St. Louis, MO, USA) and was used as microbial growth indicator. All other chemicals were of analytical purity and obtained from common suppliers. Water was treated *via* the purification system *Milli-Q water* (TGI Pure Water Systems, Greenville, SC, USA).

2.2. Plant material

Upon consultation with local bush medicine practitioners, *Momordica charantia* L. was collected during May 2015 in Santa Cruz area, Trinidad. The plant was authenticated by Dr. Walcott at the National Herbarium, University of West Indies, St. Augustine Campus, Trinidad and Tobago, where the voucher specimen TRIN 40645 is deposited. Aerial parts from 20 specimens were mixed in order to obtain a homogenate sample.

2.3. Plant crude extract

A modified method of phenolic extraction previously described was used (Chandoura et al., 2015). Aerial parts were air dried (3 days, room temperature) and powdered in the laboratory scale mill (Grindomix, Retsch, Germany). Extraction of the fine powder (1.5 g) was done with two subsequent portions (30 mL) of ethanol/water (80:20, v/v). Each portion was stirred at 150 rpm for 1 h at room temperature. First portion was filtered and the solid residue was extracted again. Both filtered supernatants (Whatman No. 4 filter paper) yielded 60 mL of extract that was then evaporated under vacuum at 40 °C (Büchi R-210, Flawil, Switzerland). The remaining water residue was lyophilized (FreeZone 4.5 model 7750031, Labconco, KS, USA). The resulting fine powder (0.396 g, yield 26%) was homogenized and the crude extract was stored in the dark at room temperature until tested.

2.4. Analysis of phenolic compounds

A standard procedure used in our laboratory was applied (Barros et al., 2013). Dry lyophilized extract was dissolved in water/ethanol (80:20, v/v) in sonic bath, filtered through a 0.22 μm nylon filter for HPLC analysis.

The chromatographic system Dionex Ultimate 3000 UPLC with a diode array detector (DAD) was coupled to a Linear Ion Trap LTQ XL mass spectrometer (Thermo Scientific, San Jose, CA, USA). The UPLC consisted of a quaternary pump, degasser, auto-sampler (kept at 5 °C), and automated column compartment. Waters Spherisorb S3 ODS-2 C18 (3 μm, 4.6 × 150 mm, Waters, Milford, MA, USA) column thermostatted at 35 °C was used for chromatographic separation.

A previously described solvent system with gradient was applied (Barros et al., 2013) as follows: (A) 0.1% formic acid in water, (B) acetonitrile. An gradient elution was established: 15% B (0–5 min), 15% B to 20% B (5–10 min), 20–25% B (10–20 min), 25–35% B (20–30 min), 35–50% B (30–40 min), subsequent column re-equilibration with a flow rate of 0.5 mL/min. Double online detection was performed in the DAD (280 and 370 nm) and in a mass spectrometer (MS) connected *via* the DAD cell outlet.

MS detection with electrospray ionization was performed in negative mode under hereby mentioned conditions: sheath gas (N_2, 50 psi); source temperature 325 °C; spray voltage 5 kV; capillary voltage −20 V; tube lens offset voltage −66 V; collision energy 35 arbitrary units. The full scan data were collected from *m*/*z* 100 to 1500. Xcalibur® data system (ThermoFinnigan, San Jose, CA, USA) was used for data acquisition.

The identification of phenolic compounds was achieved by comparing retention times, UV–vis and mass spectra with available standard compounds. Otherwise, available data reported in the literature were applied to tentatively identify the compounds. Quantitative analysis was performed from calibration curves of each available phenolic standard constructed upon the UV signal. Identified phenolic compounds with unavailable commercial standard were quantified *via* the calibration curve of the most similar standard available. The analyses were carried out in triplicate and the results are expressed as mean values and standard deviations (SD), in mg/g of lyophilized extract.

2.5. Biological activity evaluation

2.5.1. Antioxidant activity

Two-fold serial dilutions were prepared from the stock solution (2.5 mg/mL in 80% ethanol) and further submitted to four *in vitro* assays previously described by the authors (Pinela et al.,

2012) in order to describe the antioxidant activity of each plant part reflecting different antioxidant mechanisms. The results were expressed as EC_{50}, the sample concentrations providing 50% of antioxidant activity (DPPH, β-carotene/linoleate and TBARS assays) or 0.5 of absorbance (reducing power assay) after calculation from the graphs. Trolox was used as standard positive control in all four assays carried out in triplicate.

2.5.1.1. DPPH radical scavenging activity. The reduction of the DPPH radical was determined by measuring the absorption at 515 nm with ELX800 Microplate Reader (Bio-Tek, Winooski, VT, USA). A portion of DPPH in methanol (270 μL; 60 μmol/L) was added to 30 μL of each concentration in the dilution row of the extract. The reaction mixture in 96-wells plates was left for 60 min in the dark before reading. The radical scavenging activity (RSA) was assessed as a percentage of DPPH discoloration, using the equation:

$$\%RSA = [(A_{DPPH} - A_{Sample})/A_{DPPH}] \times 100,$$

where A_{Sample} is the absorbance of the solution with extract and A_{DPPH} is the absorbance of the DPPH solution without extract.

2.5.1.2. Reducing power. Each prepared concentration of the extract (0.5 mL) was mixed with 0.5 mL of sodium phosphate buffer (200 mmol/L, pH 6.6) and 0.5 mL potassium ferricyanide (1% w/v). The mixture was incubated at 50 °C for 20 min. After, 0.5 mL of trichloroacetic acid (10% w/v) was added. A portion of 0.8 mL was placed into wells of a 48-wells plate, and mixed with deionized water (0.8 mL) and ferric chloride (0.1% w/v, 0.16 mL). The absorbance was measured at 690 nm using the microplate reader described above.

2.5.1.3. Inhibition of β-carotene bleaching. The reaction mixture of β-carotene and linoleic acid was prepared by transferring 2 mL of β-carotene in chloroform (0.2 mg/mL) into a round-bottom flask, evaporating the chloroform at 40 °C under vacuum, adding Tween 80 emulsifier (400 mg), linoleic acid (40 mg), and distilled water (100 mL) upon vigorous shaking. Aliquots (4.8 mL) of such emulsion were pipetted into test tubes containing 0.2 mL of the extract at different concentrations, vortexed and the zero time absorbance was measured at 470 nm as soon as possible. The tubes were incubated at 50 °C in a water bath for 2 h. β-carotene bleaching inhibition was calculated using the following equation:

$$(\text{absorbanceafter2 hofassay}/\text{initialabsorbance}) \times 100.$$

2.5.1.4. TBARS (thiobarbituric acid reactive substances) assay. The porcine brains (*Sus scrofa*) obtained from officially slaughtered animals were dissected and homogenized with a Polytron in ice cold Tris–HCl buffer (20 mM, pH 7.4). Such yielded 1:2 w/v brain tissue homogenate was centrifuged (3000*g*; 10 min) and 100 μL of the supernatant was incubated with 200 μL of the samples solutions together with ascorbic acid (0.1 mM; 100 μL) and $FeSO_4$ (10 mM; 100 μL) at 37 °C for 1 h. Afterwards, the reaction was stopped by the addition of trichloroacetic acid (28% w/v, 500 μL) and thiobarbituric acid (TBA, 2%, w/v, 380 μL). The mixture was heated at 80 °C for 20 min. Removal of the precipitated protein was performed by centrifugation (3000*g*; 10 min). In each supernatant, colour intensity of the malondialdehyde (MDA)-TBA complex was measured spectrophotometrically at 532 nm. The inhibition in percent was calculated from the formula:

$$\text{Inhibitionratio}(\%) = [(A_C - A_S)/A_C] \times 100\%,$$

where A_C was the absorbance of the control (no extract) and A_S was the absorbance of the sample solution.

2.5.2. Anti-inflammatory activity

In the anti-inflammatory activity assay, final dilutions ranging from 400 to 25 mg/mL were tested. Both extracts and LPS were dissolved in supplemented DMEM. Method previously described by Correa et al. (2015) was carried out.

2.5.2.1. Cell treatment. The mouse macrophage-like cell line RAW 264.7 was cultured at 37 °C under 5% CO_2, in humidified air, using a supplemented DMEM medium with glutamine, antibiotics and 10% heat-inactivated fetal bovine serum. Trypan blue dye exclusion test was performed to estimate the proportion of dead cells (less than 5%) after detaching the cells with a cell scraper. The cell density of 5×10^5 cells/mL was used for each experiment. Aliquots of 150,000 cells/well were seeded in 96-well plates and the cells were allowed to attach to the plate overnight. Further, attached cells were treated with prepared concentrations of the tested extract for 1 h. Subsequently, the cells were stimulated with LPS (1 μg/mL) for 18 h. Dexamethasone (50 μM) was used as a positive control for the experiment. In negative controls, LPS nor extracts were added.

2.5.2.2. Nitric oxide determination. Griess Reagent System kit (containing sulfanilamide, *N*-(1-napthyl)ethylenediamine hydrochloride (NED) and nitrite solutions) was used for the determination of nitric oxide. A reference curve of $NaNO_2$ (100 μM to 1.6 μM; $y = 0.0066x + 0.1349$; $R^2 = 0.9986$) was prepared in a 96-well plate. The cell culture supernatant (100 μL) was transferred to each plate well and mixed with sulfanilamide and NED solutions, 5–10 min each, at room temperature. The nitric oxide production was measured as absorbance at 540 nm (microplate reader ELX800 Biotek) and determined from the standard calibration curve. The assays were carried out in triplicate and the results are expressed as mean values and standard deviations (SD), EC_{50} values (μg/mL) corresponding to the sample concentration that provides 50%-inhibition of nitric oxide (NO) production.

2.5.3. Cytotoxicity evaluation

All the assays were carried out in triplicate and the results were expressed as GI_{50} values in μg per mL (sample concentration that inhibited 50% of the net cell growth) and ellipticine was used as positive control in the assays. Extract was tested at final concentration range from 400 to 1.5 μg/mL.

2.5.3.1. Cytotoxicity in human tumor cell lines. Four human tumor cell lines were used: HeLa (cervical carcinoma), HepG2 (hepatocellular carcinoma), MCF-7 (breast adenocarcinoma), and NCI-H460 (non-small cell lung cancer). RPMI-1640 medium containing 10% heat-inactivated FBS and 2 mM glutamine was used to routinely maintain the adherent cell cultures at 37 °C, in a humidified air incubator containing 5% CO_2. For the experiments, each cell line was placed at an appropriate density (1.0×10^4 cells/well) into 96-well plates. A procedure using the sulforhodamine B assay was performed (Barros et al., 2013).

2.5.3.2. Cytotoxicity in non-tumor liver cells primary culture. For hepatotoxicity assay, a cell culture was prepared from a freshly harvested porcine liver (obtained from a local slaughter house) and designated as PLP2 (Abreu et al., 2011). Briefly, the liver tissue was rinsed in Hank's balanced salt solution containing 100 U/mL penicillin + 100 μg/mL streptomycin, and was divided into $1 \times 1\ mm^3$ explants. Some of them were placed into 25 cm^2 tissue flasks containing DMEM medium (supplemented with 10% fetal bovine serum, 2 mM non-essential amino acids and 100 U/mL penicillin, 100 mg/mL streptomycin) and incubated at 37 °C under a humidified atmosphere with 5% CO_2. Phase contrast microscope was used for direct monitoring of the cell cultivation every 2 to 3 days. Before

reaching the confluence, cells were subcultured and plated in 96-well plates at a density of 1.0×10^4 cells/well, and cultivated in commercial DMEM medium supplemented with 10% FBS, 100 U/mL penicillin and 100 μg/mL streptomycin.

2.5.4. Antibacterial activity

2.5.4.1. Bacterial strains. The used bacterial strains were clinical isolates from patients hospitalized in the Local Health Unit of Bragança and Hospital Centre of Trás-os-Montes and Alto-Douro-Vila Real, Northeast of Portugal. Six Gram-negative bacteria (*Acinetobacter baumannii* and *Pseudomonas aeruginosa* isolated from expectoration, *Escherichia coli, Escherichia coli* spectrum extended producer of β-lactamases (ESBL), *Klebsiella pneumoniae* and *Klebsiella pneumoniae*, spectrum extended producer of β-lactamases (ESBL), isolated from urine); and four Gram-positive bacteria (*Enterococcus faecalis* isolated from urine, *Listeria monocytogenes* isolated from cerebrospinal fluid, MSSA: Methicillin-sensitive *Staphylococcus aureus* isolated from wound exudate, and MRSA: methicillin-resistant *S. aureus*, isolated from expectoration) were used to screen the antimicrobial activity of the extract.

2.5.4.2. Characterization of the antibiotic susceptibility of the bacterial strains. Microorganism's identification and susceptibility tests were performed using the MicroScan panels (MicroScan®; Siemens Medical Solutions Diagnostics, West Sacramento, CA, USA) by the microdilution method. The interpretation criteria were based on Interpretive Breakpoints as indicated in Clinical and Laboratory Standards Institute (CLSI, 2008) and in the European Committee on Antimicrobial Susceptibility Testing (EUCAST, 2013).

2.5.4.3. Microdilution method. Stock solution was prepared from hydroalcoholic extract by dissolving 110 mg in 1 mL of MHB or TSB media. The determination of the minimal inhibitory concentration (MIC) was performed by the microdilution method with rapid *p*-iodonitrotetrazolium chloride (INT) colorimetric assay according to Kuete et al. (2011) with some modifications (Pereira et al., 2015). Initially, 100 μL of each extract solution (110 mg/mL) were diluted in 400 μL of MHB or TSB media according to bacteria requirements (final concentration of 22 mg/mL), and then, 190 μL of this extract solution were added to each well (96-well microplate). Successive dilutions were carried out over the wells containing 90 μL of media and afterwards, 10 μL of inoculum (1.5×10^8 CFU/mL) were added to all wells containing the tested concentrations in the range of 20 to 0.156 mg/mL. Three negative controls were prepared (one with MHB/TSB, another one with the extract, and the third one with medium and antibiotic). For the Gram-negative bacteria, antibiotics, such as amikacin (*K. pneumoniae* ESBL and *P. aeruginosa*), tobramycin (*A. baumanii*), amoxicillin/clavulanic acid (*E. coli* and *K. pneumoniae*) and gentamicin (*E. coli* ESBL) were used. The concentration used was based on the MIC obtained (Supplementary material, **Table S1**). For the Gram-positive bacteria, ampicillin (*L. monocytogenes*) and vancomycin (MSSA, MRSA and *E. faecalis*) were selected (Supplementary material, **Table S2**).

2.5.4.4. One positive control was prepared with MHB and each inoculum. The plates were then incubated in an oven (Jouan, Berlin, Germany) at 37 °C for 24 h. The MICs of the samples were determined after addition of the INT (0.2 mg/mL, 40 μL) and after incubation at 37 °C for 30 min. The viable microorganisms reduced the yellow dye to pink. MIC was defined as the lowest extract concentration that prevented this change and exhibited the complete inhibition of bacterial growth. The assays were carried out in triplicate.

3. Results and discussion

3.1. Phenolic compounds profile

The phenolic profile of the crude extract (80% EtOH) of wild *Momordica charantia* L. was recorded at 280 and 370 nm, the latter chromatogram shown in Fig. 1. The compound characteristics, tentative identities and quantification are presented in Table 1. Fourteen compounds were identified, three of which were phenolic acid derivatives (hydroxycinnamic acid derivatives) and eleven flavonoids (flavonol glycoside derivatives). Compound 1 was tentatively identified as 4-*O*-caffeoylquinic acid derivative; it presented a pseudomolecular ion $[M-H]^-$ at *m/z* 435, releasing a fragment at *m/z* 353, that was easily distinguished as 4-*O*-caffeoylquinic acid derivative, based on the base peak at *m/z* 173 [quinic acid-H-H_2O]$^-$ and according to the fragmentation pattern described by Clifford et al. (2005, 2003). Compounds 2 and 4 were tentatively identified as 4- and 5-*O*-feruloylquinic acid taking into account its fragmentation pattern and relative intensities similar to those described by Clifford et al. (2005, 2003). Madala et al. (2014) described the presence of chlorogenic acids in samples of *M. charantia* leaves from South Africa, and one of the most abundant compound identified in hydromethanolic extracts was a *cis*-4-*O*-caffeoylquinic acid. Moreover, these authors also reported the presence of 4-*O*-feruloylquinic acid.

The flavonol derivatives identified were derived from quercetin (λ_{max} around 354 nm and an MS^2 fragment at *m/z* 301), kaempferol (λ_{max} around 348 nm, MS^2 fragment at *m/z* 285) and isorhamnetin (λ_{max} around 356 nm, MS^2 fragment at *m/z* 317) (Table 1). Quercetin-3-*O*-rutinoside (rutin, compound 6), quercetin-3-*O*-glucoside (isoquercitrin, compound 7), kaempferol-3-*O*-rutinoside (nicotiflorin, compound 10), kaempferol-3-*O*-glucoside (astragalin, compound 11) and isorhamnetin-3-*O*-rutinoside (compound 12) were positively identified according to their retention time, mass and UV–vis characteristics by comparison with commercial standards. Compound 3 ($[M-H]^-$ at *m/z* 625) and 5 ($[M-H]^-$ at *m/z* 595) corresponded to quercetin derivatives, releasing a MS^2 fragment at *m/z* 301 with the losses of two hexosyl moieties [M−H-162-162]$^-$ and a pentosyl and hexosyl moiety [M−H-132-162]$^-$, respectively, being tentatively assigned as quercetin-*O*-dihexoside and quercetin-*O*-pentosylhexoside. Compound 8 was identified as kaempferol-*O*-pentosylhexoside, taking into account the findings described above. Compound 9 presented a pseudomolecular ion $[M-H]^-$ at *m/z* 505 releasing an MS^2 fragment at *m/z* 301 (quercetin; [M−H-42-162]$^-$, loss of an acetylhexoside moiety), being assigned to a quercetin-*O*-acetylhexoside. Similar findings were performed for compounds 13 and 14, being identified as kaempferol and isorhamnetin *O*-acetylhexoside, respectively. Compounds 3, 5, 6, 10, 11, 12 were recently identified in *M. charantia* leaves and additionally, compound 13 was observed in *Momordica foetida* Schumach. leaves (Madala et al., 2016). From the flavonoids identified in this study, rutin (compound 6) was previously observed in *M. charantia* fresh leaves (Zhang et al., 2009) and fruits (Kenny et al., 2013). The presence of aglycon isorhamnetin was confirmed in *M. charantia* fruit by Lako et al. (2007). In contrary to our findings, several studies revealed the presence of flavan-3-ols in leaves of *M. charantia*, such as catechin, epicatechin and epigallocatechin (Choi et al., 2012; Kenny et al., 2013; Kubola and Siriamornpun, 2008). Hydromethanolic extracts of *M. charantia* leaf and stem from Korean farms showed high amounts of *p*-hydroxybenzoic acid, vanillic acid and quercetin, as well as small amounts of caffeic, ferulic and *p*-coumaric acids and kaempferol. Thus, in contrast, neither chlorogenic acid nor rutin were detected in any parts of the *M. charantia* sample (Choi et al., 2012). This discrepancy might be explained by different origin and extraction

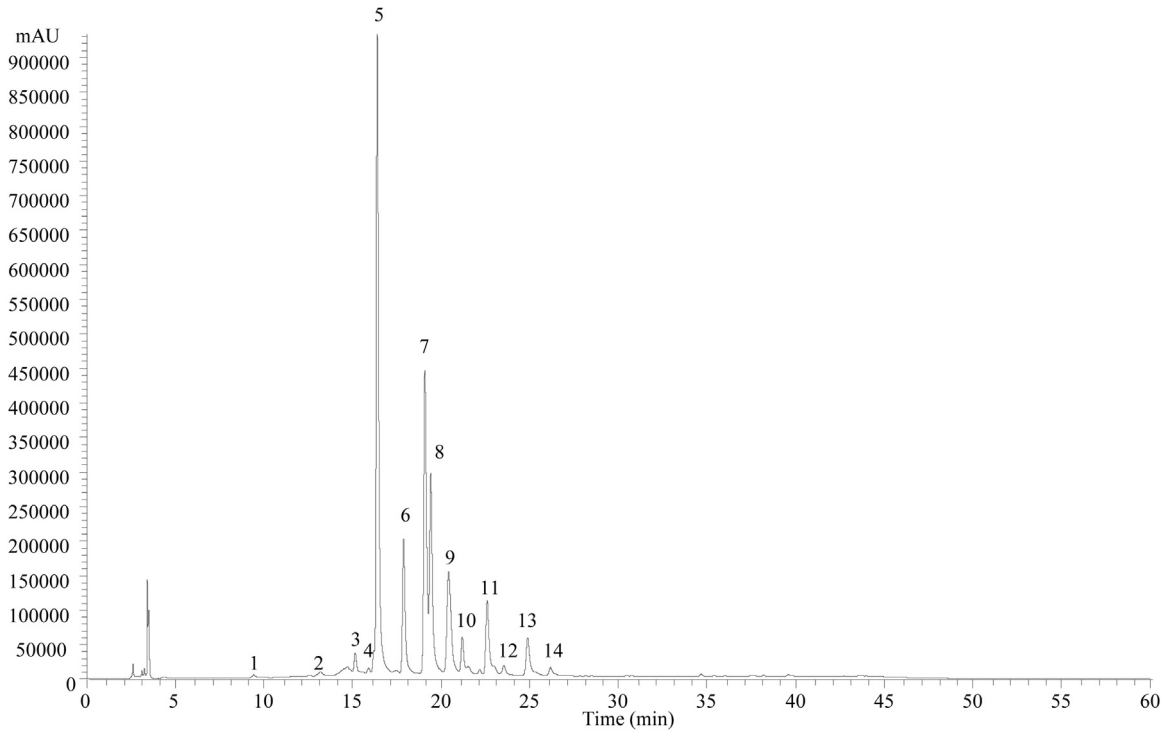

Fig. 1. Phenolic compounds profile of the wild *Momordica charantia* L. ethanol/water extract recorded at 370 nm.

Table 1
Retention time (R_t), wavelengths of maximum absorption in the visible region (λ_{max}), mass spectrometric data, and tentative identification of phenolic compounds in the wild *Momordica charantia* L. ethanol/water extract.

Compound	R_t (min)	λ_{max} (nm)	Pseudomolecular *(m/z)* ion $[M-H]^-$	MS^2 *(m/z)*	Tentative identification	Quantity (mg/g)
1	9.4	330	456	353(75),191(75),179(25),173(100),161(3),135(5)	4-*O*-Caffeoylquinic acid derivative	0.83 ± 0.06
2	14.7	332	367	193(34),191(46),173(100),155(20),134(33)	4-*O*-Feruoylquinic acid	0.48 ± 0.03
3	15.2	344	625	301(100)	Quercetin-*O*-dihexoside	0.057 ± 0.002
4	16.0	330	367	193(46),191(100),173(18),155(28),134(22)	5-*O*-Feruoylquinic acid	0.74 ± 0.04
5	16.4	355	595	301(100)	Quercetin-*O*-pentosylhexoside	7.78 ± 0.01
6	17.9	353	609	301(100)	Quercetin-3-*O*-rutinoside	1.59 ± 0.01
7	19.1	354	463	301(100)	Quercetin-3-*O*-glucoside	3.5 ± 0.1
8	19.4	348	579	285(100)	Kaempferol-*O*-pentosylhexoside	4.3 ± 0.1
9	20.4	354	505	463(30),301(80)	Quercetin-*O*-acetylhexoside	1.69 ± 0.03
10	21.2	346	593	285(100)	Kaempferol-3-*O*-rutinoside	0.795 ± 0.004
11	22.7	347	447	285(100)	Kaempferol-3-*O*-glucoside	1.66 ± 0.01
12	23.5	356	477	315(100)	Isorhamnetin-3-*O*-glucoside	0.291 ± 0.003
13	24.9	347	489	285(83)	Kaempferol-*O*-acetylhexoside	0.91 ± 0.01
14	26.2	358	519	315(100)	Isorhamnetin-*O*-acetylhexoside	0.26 ± 0.02
					Total phenolic acids	2.05 ± 0.01
					Total flavonoids	22.82 ± 0.01
					Total phenolic compounds	24.87 ± 0.01

procedures of the *M. charantia* samples, as well as the sensitivity of instrumentation.

Flavonoids were the most represented group of phenolic compounds, being quercetin-*O*-pentosylhexoside (compound 5) the most abundant molecule. To our best knowledge, this is the first quantitative report of flavonoid glycosides (except for rutin) in aerial parts of wild *Momordica charantia* as the available studies on these compounds (Madala et al., 2016, 2014) described qualitative, but not quantitative, occurrence in cultivated *M. charantia*. More specifically, compounds 4, 7, 8, 9, 13 and 14 were determined for the first time in *Momordica charantia* species.

3.2. Biological activity screening

3.2.1. Antibacterial activity

The growing demand for new agents with strong antimicrobial activity comes hand in hand with the uprising resistance of bacterial strains to common antibiotics (Normanno et al., 2007). Especially in hospital environment, the occurrence of multiresistant microorganisms is alarming. Plant-derived secondary metabolites proved to be an unlimited source for strong antimicrobials with generally lower toxic effects (Bajpai et al., 2012).

Therefore, *M. charantia* extract was screened for antibacterial properties which might be useful for the development of new nutraceuticals in prevention of serious microbial infec-

Table 2
Antibacterial activity of the wild *Momordica charantia* L. ethanol/water extract against selected hospital isolates.

Microorganism	MIC (mg/mL)	Microorganism	MIC (mg/mL)
Gram-positive bacteria		Gram-negative bacteria	
Enterococcus faecalis	1.25	*Acinetobacter baumannii*	10
Listeria monocytogenes	0.625	*Escherichia coli*	5
MSSA	5	*Escherichia coli* ESBL	5
MRSA	5	*Klebsiella pneumoniae*	5
		Klebsiella pneumoniae ESBL	5
		Pseudomonas aeruginosa	10

MRSA = methicillin-resistant *Staphylococcus aureus*.
MSSA = methicillin-sensitive *Staphylococcus aureus*.
ESBL = spectrum extended producer of β-lactamases.
MIC = minimal inhibition concentration.

tions. Eight clinical isolates representing both Gram-positive and Gram-negative bacteria were used in broth microdilution method with INT colorimetric evaluation. *Acinetobacter baumannii*, *Klebsiella pneumoniae*, *Pseudomonas aeruginosa* and *Staphylococcus aureus* belong to the ESKAPE pathogens (***E**nterococcus faecium*, ***S**taphylococcus aureus*, ***K**lebsiella pneumoniae*, ***A**cinetobacter baumannii*, ***P**seudomonas aeruginosa*, and ***E**nterobacter* species) that exhibit a high rate of resistance (Pendleton et al., 2013). Table 2 summarizes the minimal inhibitory concentrations (MICs) of the extracts against these clinical isolates. As it can be seen, all bacteria were inhibited making it possible to obtain the MIC values. In general, Gram-positive bacteria were more sensitive presenting lower MIC concentrations ranging from 0.625 to 5 mg/mL, whereas MICs for Gram-negative bacteria ranged from 5 to 10 mg/mL. *Listeria monocytogenes* was the most susceptible microorganism, with a MIC of 0.625 mg/mL, while *A. baumannii* and *P. aeruginosa* gave the highest MICs of 10 mg/mL. These results suggest that the extracts present phytochemicals with a broad-spectral activity against both Gram+ and Gram- bacteria. Several studies confirmed antibacterial activity of *M. charantia* leaves to *S. aureus*, *E. coli* and *P. aeruginosa* and *K. pneumoniae* (Braca et al., 2008; Leelaprakash et al., 2011; Mada et al., 2013). In a previous study, *Momordica charantia* leaf extract (EtOH) inhibited clinical isolates of *S. aureus*, *K. pneumoniae*, *Shigella dysenteriae* and *Salmonella typhi* at concentrations of 20 mg/mL (Olufunke, 2011). In another study, clinical isolates of *P. aeruginosa, E. coli* and *K. pneumoniae* were not inhibited by leaf methanolic extract of *M. charantia*, however the fruit methanolic extract inhibited these strains at 1 mg/mL, nevertheless *S. aureus* was affected by both extracts (Mwambete, 2009). Furthermore, Rakholiya et al. (2014) found aerial extract of *M. charantia* in 75% methanol to be effective against *L. monocytogenes*, *S. aureus* and *P. aeruginosa* in agar assay. Leaves from *M. charantia* collected in Mauritius exhibited antibacterial potential on *E. coli* (MIC = 1 mg/mL) and *P. aeruginosa* (MIC = 7 mg/mL), however neither MSSA nor MRSA were inhibited (Mahomoodally et al., 2010). Costa et al. (2010) described much lower MICs in comparison to our results for MSSA, MRSA, *E. coli* and resistant *E. coli* (256, 256, 27 and 32 μg/mL, respectively). In agreement to our study, the MSSA and MRSA were inhibited equally giving the same MICs, suggesting that the antibacterial activity of *M. charantia* does not involve resistance-specific mechanisms. *M. charantia* vine (stem and leaves) extract (250 μg/mL) was reported to inhibit *A. baumannii* (Phatthalung et al., 2012). Lu et al. (2011) did not observe any inhibition for *S. aureus*, *E. coli* or *P. auruginosa* at 10 mg/mL of extracts from deseeded fruits of wild *M. charantia*. On the other hand, Jagessar et al. (2008) found ethanol extract of wild *M. charantia* from Guyana (3 mg/mL) to give wide inhibition zones for *E. coli*, *S aureus* and even *Candida albicans* yeast (22 mm, 18 mm and 20 mm, respectively). The discrepancy in the results can be attributed to different methods used (agar plate *vs.* broth microdilution) and various origin of the bacterial strains, especially in the case of clinical isolates as well as different plant origin and plant parts used in the assays. It has to be noted, that all authors noticed higher antimicrobial activity of extracts in less polar solvents (MeOH, EtOH, EtOAc) in comparison to water. Flavonoids, such as quercetin, kaempferol, quercitrin and rutin have been previously reported to exhibit antimicrobial activity (Hodek, 2012). In the studied sample, kaempferol and quercetin derivatives, including rutin, were found and therefore this activity could be related to the presence of these compounds in the extract.

3.2.2. Antioxidant activity

Four methods have been chosen to cover several mechanisms of oxidative damage prevention by antioxidants. DPPH is a rapid assay reflecting the free radical scavenging potential of compounds with ability to donate either hydrogen or electron. In reducing power assay, Fe^{3+} ions are used as oxidizing agents and Fe^{2+} detection is evaluated to study antioxidants that are able to reduce these ions *via* single electron transfer. In β-carotene bleaching assay, the linoleic radical formed from peroxidation of linoleic acid at 50 °C attacks the double bonds of β-carotene, which can be prevented by antioxidants possessing HAT (hydrogen atom transfer) mechanism. TBARS uses the lipid peroxidation biomarkers thiobarbituric acid (TBA) and malonylaldehyde (MDA) to evaluate the potential of antioxidants to prevent formation of TBA-MDA chromogen (Carocho and Ferreira, 2013).

The results obtained in each assay are shown in Table 3. The *M. charantia* extract showed antioxidant activity in all four assays with EC_{50} ranging from 25 ± 2 to 151 ± 1 μg/mL. The β-carotene bleaching and reducing power assay gave promising results (25 ± 2 and 58 ± 1 μg/mL, respectively) comparable to those of trolox, a hydrophilic analogue of vitamin E (18 ± 1 and 41.7 ± 0.3 μg/mL, respectively). Due to the exhibited reducing power, it can be concluded that wild *M. charantia* extract contains compounds with the ability to maintain redox status in cells or tissues. Additionally, a relationship between reducing power and the degree of hydroxylation together with extent of conjugation in phenolic compounds has been previously stated (Pulido et al., 2000). In their study, quercetin and rutin were found to have higher antioxidant activity in FRAP (Ferric Reducing Antioxidant Power) assay comparing to trolox. Isoquercitrin (compound 7), rutin (compound 6) and quercetin glycosides are probably responsible for the strong antioxidant activity in our *M. charantia* extract, as they previously exhibited better or comparable antioxidant activity than that of BHT (butylhydroxytoluen) or α-tocopherol (von Gadow et al., 1997). Isoquercitrin, as the third most abundant compound in our study, as well as nicotiflorin (compound 10) and astragalin (compound 11) displayed strong radical scavenging activity (Yang et al., 2013).

The antioxidant activity of *M. charantia* has been confirmed in many studies (Grover and Yadav, 2004; Choi et al., 2012; Kubola and Siriamornpun, 2008). Regarding the wild varieties of *M. cha-*

Table 3
Antioxidant, anti-inflammatory and cytotoxic activities of the wild *Momordica charantia* L. ethanol/water extract.

Antioxidant activity (EC_{50} values, μg/mL)		
	Extract	Trolox
DPPH scavenging activity	151 ± 1	41 ± 1
Reducing power	58 ± 1	41.7 ± 0.3
β-carotene bleaching inhibition	25 ± 2	18 ± 1
TBARS inhibition	123 ± 11	23 ± 1
Anti-inflammatory activity (EC_{50} values, μg/mL)		
	Extract	Dexamethasone
Nitric oxide (NO) production	146 ± 8	16 ± 1
	Extract	Ellipticine
Cytotoxicity to tumor cell lines (GI_{50} values, μg/mL)		
MCF-7 (breast carcinoma)	112 ± 6	0.91 ± 0.04
NCI-H460 (non-small cell lung cancer)	210 ± 17	1.0 ± 0.1
HeLa (cervical carcinoma)	145 ± 14	1.91 ± 0.06
HepG2 (hepatocellular carcinoma)	193 ± 13	1.1 ± 0.2
Cytotoxicity to non-tumor cell lines (GI_{50} values, μg/mL)		
PLP2	>400	3.2 ± 0.7

The antioxidant activity was expressed as EC_{50} values, which means that higher values correspond to lower reducing power or antioxidant potential. EC_{50}: extract concentration corresponding to 50% of antioxidant activity or 0.5 of absorbance in the reducing power assay. Cytotoxicity results are expressed in GI_{50} values corresponding to the sample concentration achieving 50% of growth inhibition in human tumor cell lines or in liver primary culture PLP2.
Anti-inflammatory activity is expressed as EC_{50} values corresponding to 50% of inhibition of the NO production in comparison with the negative control (100% of NO production). Trolox, dexamethasone and ellipticine, respectively, were used as positive controls in the assays.
All values are means ± SD (n = 9).

rantia, Wu and Ng, (2008) obtained similar results for DPPH assay of wild *M. charantia* fruits extracts in EtOH (IC_{50} = 157 μg/mL) exceeding vitamin E activity (IC_{50} = 172 μg/mL). Nevertheless, it was weak in TBARS assay. Lu et al. (2012) studied 16 cultivars of wild *M. charantia* from Taiwan and the cultivar with the highest antioxidant potential extracted with methanol gave similar results to our DPPH assay (IC_{50} = 181 μg/mL), when compared to the water extract (IC_{50} = 246 μg/mL). Nagarani et al. (2014) proved that wild *Momordica* species extract contain more effective antioxidants and anti-inflammatory drugs when compared to the commercial varieties. However, detailed determination of active phenolic compounds remains scarce, especially regarding flavonoid glycosides. Therefore it is evident, that the present study brings more information regarding these compounds in the wild *Momordica charantia*.

3.2.3. Anti-inflammatory activity

The oxidative stress and subsequent damage to cell structures like lipid membrane components, DNA or proteins are involved in the mechanisms of neurodegeneration, inflammatory diseases and oncogenesis (Pisoschi and Pop, 2015). Therefore, according to the promising results from antioxidant screening, we decided to investigate the *in vitro* anti-inflammatory and anti-tumor activity of the extract as well. Nitric oxide (NO) is a pleiotropic mediator produced at inflammatory sites and is used to determine anti-inflammatory activity in LPS-stimulated RAW 264.7 macrophages (Chen et al., 2001).

In our study, we observed a dose-dependent inhibition of NO production with EC_{50} = 146 ± 8 μg/mL for the hydroethanolic extract of wild *M. charantia* (Table 3). Similar activity was obtained for *M. charantia* fruit EtOAc extract by Hsu et al. (2013); in the same study, these authors also described the inhibition of IL-6, PGE2, TNF-α, and MCP-1 production.

Cucurbitane triterpenods previously isolated from leaves of wild *M. charantia* potently suppressed *Porphyromonas gingivalis*-induced IL-8, IL-6, and IL-1β levels (Tsai et al., 2016). Methanolic extract of *M. charantia* leaves inhibited the carrageenan-induced edema *in vivo* with results comparable to indomethacin (Ganesan et al., 2008).

Hsu et al. (2012) described the potential of wild *M. charantia* extract in suppressing the cytokine and matrix metalloproteinase (MMP)-9 levels in *Propionibacterium acnes*-induced inflammation of THP-1 cells and attributed the anti-inflammatory potential to phenolic compounds present in the extract. In a study on LPS-stimulated RAW 264.7 macrophages, the wild *M. charantia* fruit extract gave better results than cultivated *M. charantia*, as it showed 64% reduction of NO at concentration lower than 50 μg/mL (Lii et al., 2009).

Phenolic compounds have been generally recognized as important contributors to anti-inflammatory properties in plant extracts (Liang and Kitts, 2016). From the flavonoid glycosides determined in the current study, astragalin (Lee et al., 2011), isoquercitrin (Rogerio et al., 2007), nicotiflorin (Soberon et al., 2010) and rutin (Lee et al., 2015), as well as chlorogenic acids (Liang and Kitts, 2016) have been previously stated as demonstrating anti-inflammatory activity.

3.2.4. Cytotoxic properties

Results regarding the anti-proliferative effect of the wild *Momordica charantia* hydroethanolic extract in different human tumor cell lines (HeLa, Hep2G, NCI-H460, and MCF-7) are presented in Table 3. All cell lines were inhibited in a dose-dependent manner. The lowest GI_{50} (112 ± 6 μg/mL) was obtained for MCF-7, proving the extract to be most effective in this line. The lowest growth inhibition was observed in NCI-H460 (210 ± 17 μg/mL). Despite only exhibiting half of the activity in human tumor cell lines comparing to ellipticine standard, the extract did not show hepatotoxicity to primary PLP2 culture within the tested concentrations (up to 400 μg/mL). Various *in vivo* and *in vitro* studies with *M. charantia* crude extracts have shown antitumor activity against breast cancer, lung adenocarcinoma, skin tumor, leukemia, choriocarcinoma, prostatic cancer, *etc.* (Grover and Yadav, 2004). Cytotoxic activities toward human fibrosarcoma HT 1080 cells comparable to doxorubicin were observed in water and methanolic extracts of fruits from wild *M. charantia* cultivars from Taiwan (Lu et al., 2011).

Previously, Bai et al. (2016) isolated a cucurbitane-type triterpene from wild *M. charantia* that inhibited MCF-7 and MDA-MB-231 cell lines. Despite the anti-proliferative effect is generally attributed to the triterpenoids from *M. charantia* (Grover and Yadav, 2004), the phenolic compounds have been also reported as potent antitumor agents. For example, isoquercitrin was found to be more effective than rutin in cell proliferation assay with MTT tetrazolium dye (Razavi et al., 2009). Quercetin and its glycosides (for example quercetin-3-*O*-β-D-glucopyranoside) showed potent anti-proliferative activities against MCF-7 and HepG2 lines (He and Liu, 2008).

The anti-proliferative activity has been positively correlated with the antioxidant potential in many studies and generally, the oxidative mechanisms are involved in carcinogenesis (Roleira et al., 2015). Our results indicate the possible relationship between antioxidants, inflammation and cytotoxicity in tumor cell lines as the *M. charantia* extracts were active in all assays.

4. Conclusions

Numerous studies have been conducted on the bioactivity related to triterpenoids and proteins found in *Momordica charantia*, however little attention has been paid to the phenolic

compounds, despite being often associated with strong antioxidant, anti-inflammatory, cytotoxic and antibacterial potential. In the presented study, the wild *Momordica charantia* extract of aerial parts showed to be a good source of natural antioxidants that can play an important role in inflammation and cancer treatment and prevention. Moreover, the extract revealed strong antimicrobial potential in inhibiting multi-resistant clinical bacterial isolates. Three phenolic acid derivatives and eleven flavonoid glycosides were identified in wild *M. charantia*, of which six were identified for the first time. Significant biological activities were already attributed to some of these compounds and, therefore, we suggest they may contribute to the biological prospect of this plant. *Momordica charantia* wildly growing in Trinidad and Tobago could be considered as a promising source for new nutraceuticals for its anti-inflammatory and antitumor activity, as well as natural food preservatives development for its strong antioxidant and antimicrobial activity.

Conflict of interest

No conflict of interest.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.indcrop.2016.10.046.

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