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ORIGINAL ARTICLE

Biological and Chemical Evaluation of Some African Plants Belonging to *Kalanchoe* Species: Antitrypanosomal, Cytotoxic, Antitopoisomerase I Activities and Chemical Profiling using Ultra-Performance Liquid Chromatography/ Quadrupole-Time-of-Flight Mass Spectrometer

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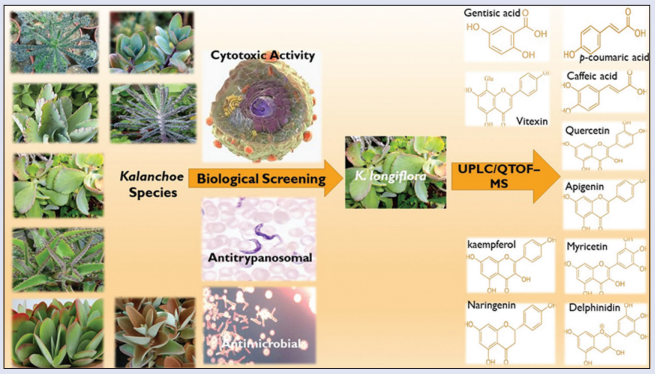
ABSTRACT

Background: Human African trypanosomiasis is one of the most serious neglected tropical diseases causing fatal symptoms and death. Natural products are a main source for anti-infective metabolites. **Objectives:** The objective of the study is to evaluate eight different plants belonging to the *Kalanchoe* species growing in Egypt for antitrypanosomal, antimalarial, antileishmanial, cytotoxic, and antimicrobial activities. **Materials and Methods:** The antitrypanosomal activity against *Trypanosoma brucei*; cytotoxic activities against human colon carcinoma, human hepatocyte carcinoma, and human breast adenocarcinoma cell lines; antileishmanial activity against *Leishmania donovani*; antimalarial activity against *Plasmodium falciparum*; and antimicrobial activities of all plant extracts have been examined. As well as the identification of the secondary metabolites for the most active extract was performed via ultra performance liquid chromatography coupled to high resolution quadrupole time of flight mass spectrometer operated in negative and positive ionization modes. **Results:** Among the examined plant extracts, *Kalanchoe longiflora* leaves extract showed promising activity against *T. brucei* with an inhibition concentration of sample at 50% fall in absorbance (IC_{50}) value of 17.6 μg/mL. *K. longiflora* with other extracts exhibited promising cytotoxic activities. Profiling of the polar secondary metabolites of *K. longiflora* revealed the presence of 47 metabolites including 31 flavonoids, 9 phenolic acids, 4 anthocyanidins, 2 chalcone glucoside, and 1 coumarin. To determine the mechanism of action of *K. longiflora* extract as a potent antitrypanosomal and cytotoxic agent, we investigate its ability to inhibit topoisomerase I enzyme. *K. longiflora* extract showed an excellent activity with an IC_{50} value of 0.148 μg/mL. **Conclusion:** These interesting results open the door for further research aiming at the development of a successful treatment for *Trypanosoma* from *K. longiflora*.

Key words: African trypanosomiasis, antitopoisomerase I, cytotoxic, *Kalanchoe*, ultra-performance liquid chromatography/ quadrupole-time-of-flight mass spectrometer

SUMMARY

- Biological investigation of *Kalanchoe* species growing in Egypt showed both antitrypanosomal and cytotoxic activities at which the responsible secondary metabolites for these activities were identified using advanced chromatographic analysis method, and quantification of the most valuable chemical classes was done for confirmation and explaining of these activities.



Abbreviations used: HCT-116: Human colon carcinoma; HEPG-2: Human hepatocyte carcinoma; MCF-7: Human breast adenocarcinoma; IC_{50} value: The inhibition concentration of sample at 50% fall in absorbance; Topo I: Topoisomerase I; Topo II: Topoisomerase II; HDAC: Histone deacetylase; WHO: World Health Organization; ESI: Electrospray ionization; RT: Retention time; UPLC/QTOF-MS/MS: Ultra-performance liquid chromatography/quadrupole-time-of-flight mass spectrometer; DMSO: Dimethyl sulfoxide; IMDM: Iscove's modified Dulbecco's medium; FBS: Fetal bovine serum; KEGG: Kyoto Encyclopedia of Genes and Genomes; a.m.u.: Atomic mass unit; Rham.: Rhamnose; Glu.: Glucose.

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INTRODUCTION

Human African trypanosomiasis or sleeping sickness is one of the most serious neglected tropical diseases.[1] The protozoan, *Trypanosoma brucei*, is the cause of human African trypanosomiasis through the bites of a tsetse fly (*Glossina* species).[2] According to the World Health Organization, *T. brucei* is endemic in 37 African countries causing fatal symptoms and death. These symptoms happen due to the ability of the parasite to multiply inside the human body, cross the blood–brain barrier, and attack the central nervous system directly.[3] The number of reported deaths in 2015 because of African trypanosomiasis was 3500.[4] In spite of these scary numbers, human African trypanosomiasis is still one of the neglected tropical diseases.[5]

Natural products reported as a source for anti-infective metabolites, either isolated from plants,[6] marine natural products,[7] or endophytic fungal sources.[8,9] *Kalanchoe* species belongs to family *Crassulaceae* (a family of 34 genera and 1410 species).[10] The genus *Kalanchoe* was established for the first time by Michel Adanson (1763), comprising 125 species.[11] *Kalanchoe* species were used extensively in different traditional medicines in many regions, especially Africa, China, India, and Brazil.[12] The antiprotozoal activity of different plants belonging to *Kalanchoe* species has been well documented.[13-15]

An important link had been emphasized between the antiprotozoal and the cytotoxic activities through different mechanisms, such as inhibition of histone deacetylase (HDAC) enzyme.[16] Antiprotozoal and cytotoxic activities were also exhibited by several synthetic compounds.[17-19] In addition, several natural products exhibited anticancer and antitrypanosomal activities, such as camptothecin and rebeccamycin, which were found to have the potential to inhibit the activity of topoisomerase I causing an arrest of the proliferation of cancer cells and *Trypanosoma cruzi*.[20] These findings prompted us to examine the cytotoxic activities for these plant extracts against human colon carcinoma (HCT-116), human hepatocyte carcinoma (HEPG-2), and human breast adenocarcinoma (MCF-7) cell lines.

Topoisomerases are important nuclear enzymes playing a vital role in DNA replication, transcription, chromosome segregation, and recombination.[21] There are two types of topoisomerases: topoisomerase I (Topo I) and topoisomerase II (Topo II). Topo I is responsible for cleavage, relaxing, and releasing of one strand of the DNA duplex, while Topo II cleaves DNA helix simultaneously to remove DNA supercoiling.[22] Accordingly, topoisomerases are considered as important targets for cancer chemotherapy treatments.[23] Topoisomerase inhibitors block the ligation step of the cell cycle, generating single-stranded and double-stranded breaks that harm the integrity of the genome.[24] In addition, Topo I is considered a suitable target for antiprotozoal chemotherapy. Camptothecin has been examined and exerted great activity against trypanosomes and *Leishmania* through the inhibition of Topo I, leading to the promotion of protein–DNA adducts formation and inhibition of DNA synthesis.[25]

The profiling of plant's secondary metabolites using different mass spectrometric techniques has been progressively applied for medicinal plants analysis.[26] The ultra-high-performance liquid chromatography-quadrupole time-of-flight mass spectrometry (UHPLC/QTOF-MS) technique is a recent approach in the field of chromatography. It has the advantage of being fast, sensitive, and high-resolution separation technique.[27]

The initial impetus for the present study is to find out an effective treatment for a serious African disease using some African plants belonging to the *Kalanchoe* sp. In addition, exploring the mechanism of action and the chemical profile of the most active plant's extract are among our goals in this study.

MATERIALS AND METHODS

Plant material and extraction

Kalanchoe delagoensis, *Kalanchoe daigremontiana*, *Kalanchoe grandiflora*, *Kalanchoe longiflora*, *Kalanchoe marmorata*, *Kalanchoe orgyalis*, *Kalanchoe thyrsiflora*, and *Kalanchoe tubiflora* were collected and identified by Botanical team of Al-Orman Botanical Garden, Giza, Egypt, on January 2017 [Figure 1]. A voucher specimen (K-101 to K-108) has been deposited in the Pharmacognosy Department, Faculty of Pharmacy, Al-Azhar University, Cairo, Egypt. Samples of 10 g fresh leaves were prepared for extraction through cutting by a mixer. The cut plants exhaustively extracted with 70% ethanol and sonicated at 30 kHz for 30 min. Then, the samples were filtered; the marc was re-extracted 3 times as described before. The collected extracts were filtrated and dried under reduced pressure at 40°C.

Antitrypanosomal assay

A 2-day-old culture of *T. brucei* in the exponential phase was diluted to 5000 parasites/mL with Iscove's modified Dulbecco's medium (IMDM) according to the described protocol.[28] The maximum permissible limit of dimethyl sulfoxide (DMSO) in the assay was 0.5%. The assays were set up in clear 96-well microplates. For primary screening (single concentration of 20 μg/mL in duplicate), extract dilutions (1 mg/mL) were prepared from the stock extracts (20 mg/mL) in IMDM. Each well received 4 μL of diluted extract sample and 196 μL of the culture volume (total culture volume 200 μl). The plates were incubated at 37°C in 5% CO_2 for 48 h. Alamar blue (10 μl) (AbD Serotec, Catalog Number BUF012B) was added to each well, and the plates were incubated further for overnight. Standard fluorescence was measured on a Fluostar Galaxy fluorometer (BMG LabTechnologies) at 544 nm excitation and, 590 nm emission. Pentamidine and α-difluoro methyl ornithine (DFMO) were tested as standard. The extracts that have shown >90% inhibition of *T. brucei* growth in primary screening were subjected to secondary screening for dose-dependent–response analysis. Active extracts were screened at concentrations ranging from 10 to 0.4 μg/mL. The inhibition concentration of sample at 50% fall in absorbance (IC_{50}) and IC_{90} values were computed from dose-dependent–response growth inhibition curve by XLfit version 5.2.2.

Antileishmanial assay

The antileishmanial activity of the crude extracts, fractions, and isolated metabolites was tested *in vitro* against a culture of *Leishmania donovani* promastigotes using pentamidine and amphotericin B as positive controls.[29]

Figure 1: Figures of *Kalanchoe* species

Antimalarial assay

Crude extracts and fractions were tested on chloroquine-sensitive (D6, Sierra Leone) and chloroquine-resistant (W2, Indo-china) strains of *Plasmodium falciparum* based on plasmodial lactate dehydrogenase activity (LDH) activity using previously reported method; artemisinin and chloroquine have been used as positive controls.[30]

Antimicrobial assay

The antimicrobial activity of different extracts was screened for their ability to inhibit a panel of five bacteria and five fungi. Those bacteria and fungi are pathogenic to humans including *Staphylococcus aureus*, methicillin-resistant *S. aureus* (MRSA), *Escherichia coli*, *Pseudomonas aeruginosa*, *Mycobacterium intracellulare*, *Candida albicans*, *Candida glabrata*, *Candida krusei*, *Aspergillus fumigatus*, and *Cryptococcus neoformans*. The antimicrobial assay was carried out according to previously reported method using ciprofloxacin and amphotericin B as positive controls.[30,31]

Cytotoxic assay

Antiproliferative activity screening was carried out against three cancer cell lines (HCT-116, HEPG-2, and MCF-7 cell lines). The anticancer activity was measured quantitatively using the neutral red assay protocol as described by Borenfreund and Puerner.[32] Briefly, the cell lines were cultured in DMEM (Lonza group) supplemented with 200 mM of L-glutamine and 10% of fetal bovine serum (FBS). The test compounds were dissolved in a mixture of DMSO and DMEM with ratio 4:100 (v/v), respectively. An initial dose of (1 mg/mL) was tested on different cell lines and sub sequenced by seven more dilutions using two-fold dilution factor. Cells were seeded with a concentration of (6×10^4 cell/mL) for 24 h in the flat bottom 96-well plates and incubated at 37°C with 5% CO_2 until semi-confluent cell layer was obtained and then treated with 100 µL of each of serially diluted compounds. After 48 h, the anticancer activity of the compounds was measured quantitatively by an ELISA microplate reader at a wavelength of 540 nm using neutral red assay protocol.

Topoisomerase I assay

Kit components

Item specifications (48T/96T) storage

This kit was based on sandwich enzyme-linked immune-sorbent assay technology. Anti-TOP1 antibody was precoated onto 96-well plates and the biotin-conjugated anti-TOP1 antibody was used as detection antibodies. The standards, test samples, and biotin-conjugated detection antibody were added to the wells subsequently and washed with wash buffer. HRP streptavidin was added, and unbound conjugates were washed away with wash buffer. TMB substrates were used to visualize HRP enzymatic reaction. TMB was catalyzed by HRP to produce a blue color product that changed into yellow after adding acidic stop solution. The density of yellow is proportional to the TOP1 amount of sample captured in plate. Read the optical density absorbance at 450 nm in a microplate reader and then the concentration of TOP1 can be calculated.

Liquid chromatography-mass spectrometry/mass spectrometry

Chemicals

LC-MS grade acetonitrile and gradient solvents including isopropanol, methanol, dichloromethane, and ethyl acetate were provided by Thermo-Fisher (Thermo Fisher Scientific, USA). Formic acid 98%, ammonium hydroxide, ammonium formate, and ammonium acetate were purchased from Sigma-Aldrich (Sigma-Aldrich Co., Louis St., MO, USA).

Instruments and acquisition method

Separation of small molecules was carried out on an Axion AC system (Kyoto, Japan) connected with an autosampler system, an In-Line filter disks precolumn (0.5 µm × 3.0 mm, Phenomenex, USA), and an Xbridge C_{18} (3.5 µm × 2.1 mm × 50 mm) column (Waters Corporation, Milford, MA, USA) maintained at 40°C and a flow rate of 300 µL/min. The mobile phase consisted of solution (A) 5 mM ammonium formate in 1% methanol, adjusted to pH = 3.0 using formic acid and solution (B) acetonitrile 100% for the positive mode, while the negative mode solution (C) consisted of 5 mM ammonium formate in 1% methanol, adjusted to pH = 8.0 using ammonium hydroxide.

MS was performed using Triple TOF™ 5600+ system equipped with a Duo-Spray™ source operating in the electrospray ionization (ESI) mode (AB SCIEX, Concord, Canada). Subsequently, the top 15 intense ions were selected for acquiring MS/MS fragmentation spectra after each scan.[33]

Data processing

MS-DIAL 3.70 software (Yokohama, Kanagawa, Japan)[34] was used for non-targeting small molecule comprehensive analysis of the sample. According to the acquisition mode, ReSpect-positive (2737 records) or ReSpect-negative (1573 records) databases were used as reference databases. The identified compounds were retrieved for the pathway analysis using Kyoto Encyclopedia of Genes and Genomes (KEGG)[35] to investigate the integration of different molecule in the plant metabolic pathways.

Table 1: Cytotoxic activities of the examined plants using different cell lines

Plant species	HCT-116 IC_{50} (µg/ml)	HEPG-2 IC_{50} (µg/ml)	MCF-7 IC_{50} (µg/ml)
Kalanchoe tubiflora	42.71±1.3	45.51±1.2	43.26±0.78
Kalanchoe daigremontiana	10.55±0.87	9.17±1.1	11.48±0.79
Kalanchoe grandiflora	20.55±1.2	17.41±1.5	18.24±0.64
Kalanchoe marmorata	21.57±1.3	17.27±0.58	20.23±0.47
Kalanchoe orgyalis	34.52±0.94	31.45±0.76	30.17±1.21
Kalanchoe longiflora	22.45±1.45	23.14±1.2	19.22±2.15
Kalanchoe thyrsiflora	25.43±0.55	22.55±0.75	20.54±1.51
Kalanchoe delagoensis	41.57±0.75	40.81±1.21	39.59±0.89

HCT-116: Human colon carcinoma; HEPG-2: Human hepatocyte carcinoma; MCF-7: Human breast adenocarcinoma; IC_{50} value: The inhibition concentration of sample at 50% fall in absorbance

RESULTS AND DISCUSSION

Antiprotozoal assay

The eight *Kalanchoe* sp. extracts examined for antitrypanosomal, antileishmanial, and antimalarial activities. *K. longiflora* leaves extract only exhibited its activity against *T. brucei* with an IC_{50} value of 17.6 µg/mL.

Antimicrobial assay

The plant extracts were evaluated for their antibacterial and antifungal activities against *C. albicans*, *C. glabrata*, *C. krusei*, *A. fumigatus*, *Cryptococcus neformans*, *S. aureus*, MRSA, *E. coli*, and *P. aeruginosa.* Unfortunately, neither one of the tested plant extracts showed any promising activity.

Cytotoxic assay

The well-proven relation between antiprotozoal and cytotoxic activities prompted us to examine the cytotoxic activities of the plant extracts against HCT-116, HEPG-2, and MCF-7 cell lines. Several plant extracts exhibited good activities, against the tested cell lines [Table 1].

Topoisomerase I inhibitory activity

The most active antitrypanosomal extract (*K. longiflora*) was evaluated for its inhibitory activity against Topo I enzyme. Staurosporine was used as a positive control in this procedure. The results were recorded as an IC_{50} calculated from the concentration–inhibition response curve. Topo I was efficiently inhibited by *K. longiflora* ethanolic extract which displayed excellent inhibitory activity with an IC_{50} value of 0.148 µg/mL. The inhibitory activity of staurosporine was very near to *K. longiflora* with an IC_{50} value of 0.135 µg/mL [Figure 2]. This result was consistent with that of *in vitro* antitrypanosomal and cytotoxic activities of *K. longiflora* ethanolic extract. This result indicates that the expected mechanism of action of *K. longiflora* ethanolic extract as an antitrypanosomal and cytotoxic agent is due to its ability to inhibit Topo I enzyme.

Profiling of *Kalanchoe longiflora* secondary metabolites via ultra-performance liquid chromatography–quadrupole-time-of-flight mass spectrometer

The valuable biological effects of *K. longiflora* ethanolic extract prompted us to identify its phytochemical profile through non-targeted profiling method using ultra-performance liquid chromatography (UPLC) coupled with a high-resolution quadrupole-time-of-flight mass spectrometer (QTOF-MS) operated in the negative and positive ionization modes [Figures 3 and 4]. The extract was analyzed in both positive and negative-ion ESI MS modes to avoid any change in competitive ionization and suppression effects due to the changes in ESI polarity can often circumvent or significantly alter, revealing otherwise suppressed metabolite signals.

In total, 30 peaks from *K. longiflora* ethanolic extract were identified based on their negative-ionization mass spectral data versus 17 in the positive-ion mode [Table 2 and Figure 5]. A total of 47 secondary metabolites were detected and identified. Metabolites belonged to several natural product classes including 31 flavonoids, nine phenolic acids, four anthocyanidins, one coumarin, one chalcone glycoside, and one dihydrochalcone glucoside.

Identification of flavonoids

The flavonoid glycosides resort to generate $[M–H]^-$ ions more than $[M+H]^+$ ions. In their MS/MS spectra, losses of glycosyl moieties in both negative- and positive-ion mode could be observed as well as their major characteristic fragment ions due to retro-Diels-Alder fragmentation pathway [Figure 5]. In case of O-flavone glycosides, the common losses of 132, 146, 162, and 176 a.m.u indicated the losses of pentose (arabinose or xylose), rhamnose, hexose (glucose or galactose), and hexuronic acid, respectively. Furthermore, flavonoids tended to lose 28 a.m.u. (CO), 18 a.m.u (H_2O), and 15 a.m.u (CH_3), suggesting

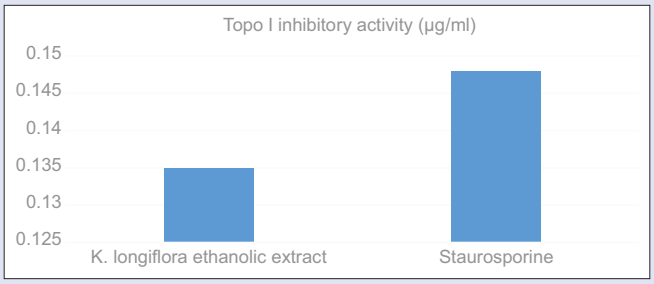

Figure 2: Topoisomerase I inhibitory activity of *K. longiflora* ethanolic extract against staurosporine

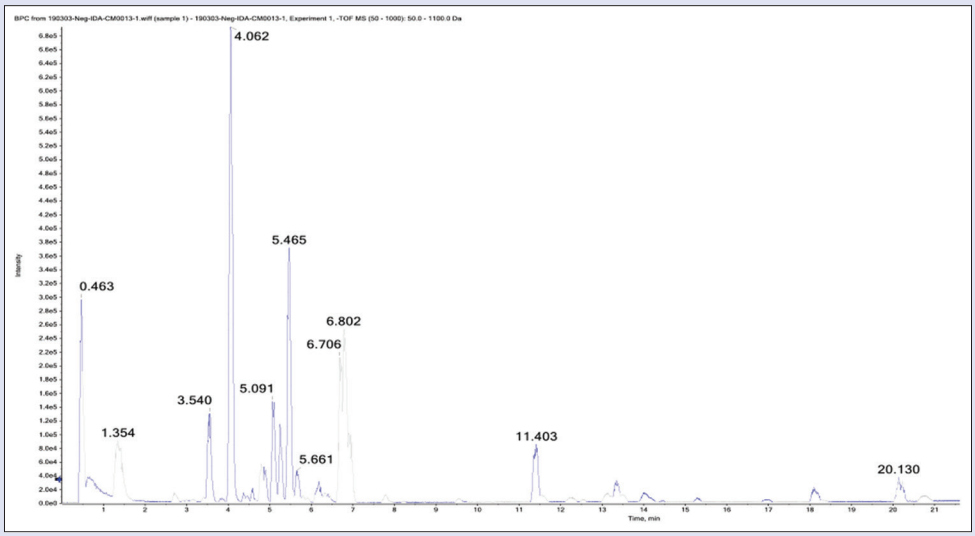

Figure 3: Base peak chromatogram (BPC) of *Kalanchoe longiflora* ethanolic extract in negative electrospray ionization mode

Table 2: Peak annotations of metabolites in *Kalanchoe longiflora* ethanol extract using ultra-performance liquid chromatography-quadrupole time-of-flight mass spectrometry in negative and positive ionization modes

Peak	RT (min)	MS [-] MS/MS *m/z*	MS [+] MS/MS *m/z*	Chemical formula	Error (ppm)	Tentative assignment	Compound type	References
1	0.46	191 [M-H-Caffe]$^-$, 173 [M-H-Caffe-H_2O]$^-$	-	$C_{16}H_{18}O_9$	5.4	Chlorogenic acid	Phenolic ester	[36]
2	0.48	173.0095 [M-H]$^-$, 154 [M-H-H_2O]$^-$, 129 [M-H-CO_2]$^-$	-	$C_7H_{10}O_5$	0.2	(-)-Shikimic acid	Phenolic acid	[37]
3	0.54	-	319.0345 [M+H]$^+$, 291 [M+H-CO]$^-$, 273 [M+H-H_2O-CO]$^-$	$C_{15}H_{10}O_8$	1	Myricetin	Flavonol	[38]
4	0.62	-	303.083 [M]$^+$	$C_{15}H_{11}O_7^+$	2.6	Delphinidin	Anthocyanidin	[39]
5	0.65	153.0305 [M-H]$^-$, 125 [M-H-CO]$^-$, 109 [M-H-CO_2]$^-$	-	$C_7H_6O_4$	10.2	Gentisic acid	Phenolic acid	[40]
6	0.69	163.0402 [M-H]$^-$, 119 [M-H-CO_2]$^-$	-	$C_9H_8O_3$	0.4	*p*-coumaric acid	Phenolic acid	[38]
7	0.73	181.1218 [M-H]$^-$, 135 [M-H-CO_2]$^-$	-	$C_9H_8O_4$	0.1	Caffeic acid	Phenolic acid	[41]
8	1.06	301.0011 [M-H]$^-$, 257 [M-H-H_2O-CO]$^+$	-	$C_{15}H_{10}O_7$	−2.6	Quercetin	Flavonol	[42]
9	1.10	449.1088 [M-H]$^-$, 287 [M-H-glu]$^-$	-	$C_{21}H_{22}O_{11}$	0.8	Isookanin-7-glucoside (Flavanomarein)	Flavone glycoside	[43]
10	1.14	289.0739 [M-H]$^-$, 271 [M-H-H_2O]$^+$,123 [1,2B]$^+$	-	$C_{15}H_{14}O_6$	−3.5	Cianidanol (catechin)	Flavanol	[36]
11	1.19	137.0244 [M-H]$^-$, 109 [M-H-CO]$^-$, 94 [M-H- H_2O-CO]$^+$	-	$C_7H_6O_3$	1	Salicylic acid	Phenolic acid	[40]
12	1.61	609.1513 [M-H]$^-$, 447 [M-H-Glu]$^-$, 285 [M-H-2Glu]$^-$	-	$C_{27}H_{30}O_{16}$	−5.8	Luteolin-3',7-di-O-glucoside	Flavone glycoside	[44]
13	2.53	593.1527 [M-H]$^-$, 447 [M-H-p-coumaroyl]$^-$, 285 [M-H-3-O-(6-p-coumaroyl)-Glu]$^-$	-	$C_{30}H_{26}O_{13}$	−0.9	Tiliroside (kaempferol-3-O-(6""-p-coumaroyl)-glucoside)	Flavonol glycoside	[45]
14	2.75	285 [M-H-H_2O]$^-$, 257 [M-H-H_2O-CO]$^-$, 193 [M-H-Bring]$^-$, 179 [1,4B], 153 [1,3A], 149 [0,2A]	-	$C_{15}H_{12}O_7$	−2	(±)-Taxifolin	Flavanonol	[42]
15	2.76	285.0402 [M-H]$^-$	-	$C_{15}H_{10}O_6$	0.5	Kaempferol	Flavonol	[46]
16	2.76	433.1152 [M-H]$^-$, 271 [M-H-Glu]$^-$	-	$C_{21}H_{22}O_{10}$	−2.1	Prunin (naringenin-7-O-glucoside)	Flavanone glycoside	[47]
17	2.89	-	611.1614 [M]$^+$, 465 [M-Rham]$^+$, 303 [M-Rham-Glu]$^+$	$C_{27}H_{31}O_1^+$	0.1	Tulipanin	Anthocyanidin glycoside	[48]
18	2.89	385.1862 [M-H]$^-$, 223 [M-H-Glu]$^-$, 205 [Sinapic acid-H-H_2O]$^-$	-	$C_{17}H_{22}O_{10}$	1.6	1-O-sinapoyl-beta-d-glucose (1-o-b-d-glucopyranosyl sinapate)	Phenolic glycosides	[49]
19	2.89	359.1272 [M-H]$^-$	-	$C_{18}H_{16}O_8$	−3.7	Rosmarinic acid	Phenolic ester	[36]
20	3.14	177.0201 [M-H]$^-$	-	$C_9H_6O_4$	−2.4	Esculetin (6,7-dihydroxycoumarin)	Coumarin	[50]

Contd...

Table 2: Contd...

Peak	RT (min)	MS [-] MS/MS *m/z*	MS [+] MS/MS *m/z*	Chemical formula	Error (ppm)	Tentative assignment	Compound type	References
21	3.34	-	433.0999 $[M+H]^+$, 415 $[M+H-H_2O]^+$, 397 $[M+H-2H_2O]^+$, 361 $[M+H-4H_2O]^+$, 295 $[M+H-120-H_2O]^+$,	$C_{21}H_{20}O_{10}$	−1	Vitexin	Flavone C- glycoside	[51]
22	3.41	463.0895 $[M-H]^-$, 301 $[M-H-Glu]^-$	-	$C_{21}H_{20}O_{12}$	−0.6	Quercetin-4'-glucoside (Spiraeoside)	Flavonol glycoside	[44]
23	3.52	593.1537 $[M-H]^-$, 447 $[M-H-rham]^-$, 285$[M-H-rutinose]^-$	-	$C_{27}H_{30}O_{15}$	−1.7	Datiscin (Datiscetin-3-O-rutinoside)	Flavonol glycoside	[52]
24	3.53	-	595.1642$[M+H]^+$, 449 $[M+H-Rham]^+$, 287 $[M+H-$ rutinose$]^+$	$C_{27}H_{30}O_{15}$	−0.5	Nicotiflorin (kaempferol-3-O-rutinoside)	Flavonol glycoside	[53]
25	3.53	-	449.108 $[M+H]^+$, 303 $[M+H-Rham]^+$	$C_{21}H_{20}O_{11}$	−0.6	Vincetoxicoside b (quercetin-7-O-rhamnoside)	Flavonol glycoside	[53]
26	3.69	-	285.1099 $[M+H]^+$	$C_{16}H_{12}O_5$	−0.6	Acacetin	Flavone	[53]
27	3.97	591.136 $[M-H]^-$	-	$C_{28}H_{32}O_{14}$	−1	Acacetin-7-O-rutinoside	Flavone glycoside	[54]
28	4.04	-	287.0545 $[M+H]^+$, 269 $[M+H-H_2O]^+$, 241 $[M+H-H_2O$ $-CO]^-$	$C_{15}H_{10}O_6$	−1	Luteolin	Flavone	[42]
29	4.06	577.1539 $[M-H]^-$, 431 [M-H-Rham]−, 285 $[M-H-2Rham]^-$	-	$C_{27}H_{30}O_{14}$	3.9	Kaempferol-3,7-O-bis-alpha-L-rhamnoside	Flavonol glycoside	[49]
30	4.41	-	741.2239 $[M+H]^+$, 595 $[M+H-Rham]^+$, 433 [M+H-robionse-Rham]$^+$, 287 $[M+H-$ robionse$]^+$	$C_{33}H_{40}O_{19}$	−0.2	Robinin (kaempferol-3-O-robinoside-7-O-rhamnoside)	Flavonol glycoside	[55]
31	4.70	-	209.1358 $[M+H]^+$, 181 $[M+H-CO]^+$	$C_{11}H_{12}O_4$	0.8	Sinapyl aldehyde	Phenolics	[53]
32	4.80	431.099 $[M-H]^-$, 285 $[M-H-2Rham]^-$	-	$C_{21}H_{20}O_{10}$	−0.2	Kaempferin (Kaempferol-3-O-alpha-L rhamnoside)	Flavonol glycoside	[38]
33	4.84	435.129 $[M-H]^-$, 273 $[M-H-Glu]^-$	-	$C_{21}H_{24}O_{10}$	1.3	Phlorizin	Dihydro chalcone glycoside	[39]
34	5.20	287.0586 $[M-H]^-$, 287 $[M-H]^-$, 151 $[1,3A]^-$	-	$C_{15}H_{12}O_6$	−3.8	Eriodictiol (3' 4' 5 7-tetrahydroxyflavanone)	Flavanone	[56]
35	5.26	447.0944 $[M-H]^-$, 301 $[M-H-Rham]^-$	-	$C_{21}H_{20}O_{11}$	−0.9	Quercitrin	Flavonol glycoside	[53]
36	5.54	-	597.254 $[M]^+$	$C_{26}H_{29}O_{16}^+$	2.3	Delphinidin-3-O-sambubioside	Anthocyanidin glycoside	[48]
37	5.71	-	433.1832 $[M+H]^+$	$C_{21}H_{20}O_{10}$	1.1	Kaempferol-7-O-rhamnoside	Flavonol glycoside	[38]
38	6.28	271 $[M-H]^-$, 151 $[1,3A]^-$, 177 [M-H-B-ring]$^-$.	-	$C_{15}H_{12}O_5$	−0.3	Naringenin	Flavanone	[42]
39	6.52	269.0459 $[M-H]^-$, 151 $[M-H-C_8H_6O]^-$, 117 $[M-H-C_8H_6O]^-$, 107 $[M-H-C_6H_3O_2]^-$	-	$C_{15}H_{10}O_5$	0	Apigenin	Flavone	[42]

Contd...

Table 2: Contd...

Peak	RT (min)	MS [-] MS/MS *m/z*	MS [+] MS/MS *m/z*	Chemical formula	Error (ppm)	Tentative assignment	Compound type	References
40	6.77	299.0595 $[M-H]^-$, 284 $[M-H-CH_3]^-$, 256 $[M-H-CH_3-CO]^-$	-	$C_{16}H_{12}O_6$	0.4	Kaempferide (3 5 7-trihydroxy-4'-methoxyflavone)	Flavonol	[42]
41	7.03	315.0518 $[M-H]^-$, 300 $[M-H-CH_3]$	-	$C_{16}H_{12}O_7$	−0.9	Isorhamnetin (3'-methoxy-4',5,7-trihydroxyflavonol)	Flavonol	[42]
42	10.84	-	435.1984 $[M+H]^+$	$C_{20}H_{18}O_{11}$	1.4	Guaijaverin (quercetin-3-arabinoside)	Flavonol glycoside	[53]
43	12.67	-	449.1561 $[M+H]^+$, 431 $[M+H-H_2O]^+$, 413 $[M+H-2H_2O]^+$,395 $[M+H-3H_2O]^+$,	$C_{21}H_{20}O_{11}$	2.7	Orientin (luteolin-8-C-glucoside)	Flavone glycoside	[51]
44	17.73	-	449.1252 $[M]^+$, 287 [M-glucose]$^+$	$C_{21}H_{21}O_{11}$	3.9	Kuromanin (cyanidin-3-glucoside)	Anthocyanin glycoside	[48]
45	2.36	449.1082 $[M-H]^-$, 169, 150	-	$C_{21}H_{22}O_{11}$	2.2	Marein (Okanin-4'-O-glucoside)	Chalcone glycoside	[43]
46	7.79	-	287.1501 $[M+H]^+$, 269 $[M+H-H_2O]^+$	$C_{16}H_{14}O_5$	−2.1	Sakuranetin (4',5-dihydroxy-7 methoxyflavone)	Flavone	[42]
47	3.53	-	417.1524 $[M+H]^+$	$C_{21}H_{20}O_9$	0.2	Puerarin (daidzein-8-C-glucoside)	Isoflavone	[57]

MS: Mass spectrometry; RT: Retention time

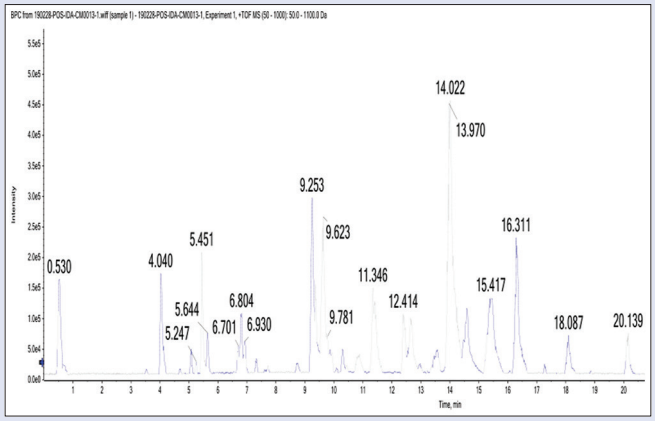

Figure 4: Base peak chromatogram (BPC) of *Kalanchoe longiflora* ethanolic extract in positive electrospray ionization mode

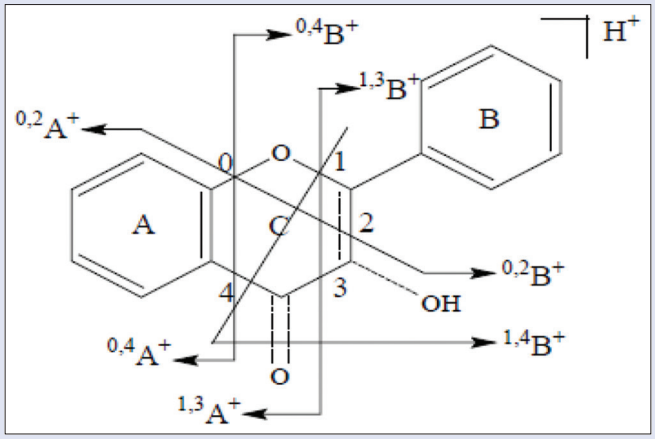

Figure 5: Fragmentation pattern of flavonol

the existence of phenolic hydroxyl and methyl groups, thus allowing the characterization of the flavonoid subgroups.[46,58]

Among 47 different peaks, flavonoids represented the most abundant class in *K. longiflora*, with 31 peaks. Seventeen of them were tentatively assigned to flavonol subclass. The presence of seven characteristic parent ion peaks in positive- and negative-ion mode at 741.2239 $[M+H]^+$, 595.1642 $[M+H]^+$, 593.1527 $[M-H]^-$, 577.1539 $[M-H]^-$, 433.1832 $[M+H]^+$, 431.099 $[M-H]^-$, 299.0595 $[M-H]^-$, and 285.0402 $[M-H]^-$, corresponding to kaempferol and its glycosides [Table 2]. However, quercetin and its glycosides assigned to four characteristic parent ion peaks at 463.0895 $[M-H]^-$, 449.108 $[M+H]^+$, 435.1984 $[M+H]^+$, and 301.0011 $[M-H]^-$ [Table 2]. In addition, parent ion peaks for myricetin, quercetin, and isorhamnetin have been observed. Furthermore, nine flavones ion peaks such as apigenin at 269.0459 $[M-H]^-$, vitexin at 431.0999 $[M-H]^-$, acacetin at 285.1099 $[M+H]^+$, acacetin-7-*O*-rutinoside at 591.136 $[M-H]^-$, luteolin and its glycosides at 609.1513 $[M-H]^-$, 449.1561 $[M+H]^+$, and 287.0545 $[M+H]^+$ have been recorded. Further, other subclasses, three metabolites belonging to flavanone (prunin, eriodyctiol, and naringenin), 1 isoflavone (puerarin), and 1 flavononol (taxifolin), have been detected.

Several scientific reports indicated the antiprotozoal activity of kaempferol,[59] which was one of the most three active flavonoids as antiamoebic and antigiardial agent among 18 natural flavonoids.[59] Furthermore, kaempferol and its glycosides were reported before to have antileishmanial activity.[60,61] Quercetin was described as a potent antileishmanial flavonoid[61] and proved to induce apoptosis of *T. brucei* previously.[62] In fact, different flavonoids exhibited antiprotozoal activities, for instance, fisetin, 3-hydroxyflavone, luteolin, and quercetin showed promising activities against *T. cruzi* with IC_{50} values of 0.6, 0.7, 0.8 and 1.0 μg/mL, respectively.[63] Sakuranetin, which is a flavonoid isolated from the leaves of *Baccharis retusa* (*Asteraceae*), exhibited good activities against different species of *Leishmania* with IC_{50} values in the range of 20–52 μg/mL.[64] As well as some flavonoids isolated from the aerial parts of *Dodonaea viscosa* (*Sapindaceae*)

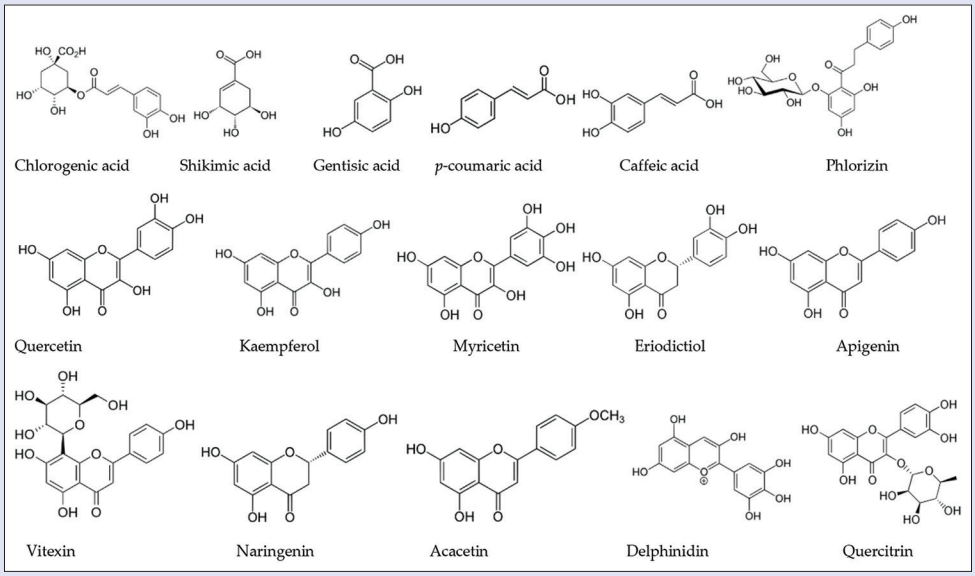

Figure 6: Some compounds tentatively identified in *K. longiflora* ethanol extract using Ultra-performance liquid chromatography/quadrupole-time-of-flight mass spectrometer

exhibited antileishmanial activities with IC_{50} value ranging from 16.6 to 19.06 μg/ml.[65]

The well-documented antiprotozoal activities of flavonoids, besides the detection of a high content of flavonoids (especially, these which reported for antiprotozoal effects) in *K. longiflora* ethanolic extract, suggested that the promising antitrypanosomal activity of *K. longiflora* may be linked to its flavonoid content.

Identification of phenolic acids

Phenolics are a group of secondary metabolites processing different types of promising biological activities.[66] Phenolic acids are commonly reported metabolites in most of the profiling studies of medicinal plants. Phenolic acids produced generally precursor ion $[M-H]^-$ corresponding to deprotonated molecule and fragment ion $[M-H-44]^-$ corresponding to loss of CO_2 from the carboxylic acid group.[65] In this study, nine phenolic acids were identified including 2 esterified (chlorogenic acid and rosmarinic acid), 1 phenolic glycoside (1-O-sinapoyl-β-D-glucose), and 6 free (shikimic acid, gentisic acid, *p*-coumaric acid, caffeic acid, salicylic acid, and sinapyl aldehyde).

The antitrypanosomal activity of phenolic acids was discussed before, for example, gallic acid (the famous plant phenolic) exhibited good antitrypanosomal activity against *T. brucei* with an LD_{50} value of 46.9 μM.[67]

The presence of phenolic acids in *K. longiflora* extract in high concentrations may contribute its antitrypanosomal activity.

Other metabolites

The chemical profiling of *K. longiflora* extract revealed the presence of four anthocyanidins including delphinidin (aglycon) and 3 other glycosides (tulipanin, kuromanin, and delphinidin-3-O-sambubioside), 1 dihydroxycoumarin (esculetin), 1 dihydrochalcone glucoside (phlorizin), and 1 chalcone glycoside (marein) as shown in Figure 6.

CONCLUSION

The ethanolic extract of *K. longiflora* leaves exhibited promising antitrypanosomal activity against *T. brucei* with an IC_{50} value of 17.6 ug/ml. In addition, it showed promising cytotoxic activities against HCT-116, HEPG-2, and MCF-7 cell lines. Chemical profiling of the polar secondary metabolites in *K. longiflora* via UPLC coupled to high-resolution QTOF-MS operated in negative and positive ionization modes resulted in tentative identification of 47 metabolites including 31 flavonoids, 9 phenolic acids, 4 anthocyanidins, 1 coumarin, 1 chalcone, and 1 dihydrochalcone glucoside. The proposed mechanism of action of *K. longiflora* extract as antitrypanosomal and cytotoxic agent may be through its ability to inhibit Topo I enzyme (IC_{50} value of 0.148 μg/ml).

These interesting results open the door for further research aiming at the development of a successful treatment for *Trypanosoma* from *K. longiflora*.

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Conflicts of interest

There are no conflicts of interest.

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