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Anogeissus latifolia leaf, flower and stem extracts: UPLC/ESI-qTOF-HRMS/MS profile with cytotoxicity and antiviral activity evaluation

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Abstract

Phytochemical and biological investigations were designed on *Anogeissus latifolia* extracts through LC/qTOF-MS/MS characterization alongside cytotoxicity and antiviral activity examination against four cancer cell lines and two viruses. Phytochemical profiles of leaves and flowers were determined by UPLC/HRESI-MS/MS. MTT assay was used for cytotoxicity evaluation against four human cancer cell lines. *In vitro* anti-HSV1 and HAV activity was evaluated with three different protocols to test protective, anti-replicative, and anti-infective antiviral activities, and three separate replications of each experiment were conducted using MTT colorimetric assay and IC_{50} with selectivity index (SI) using Vero cell line. By LC/ESI-MS, 66 and 27 metabolites were identified. *In-vitro*, all extracts inhibited HepG-2, Caco-2/ATB-37, MCF-7/HTB-22 and Panc-1 growth in concentration dependent manner. Leaves extract showed the moderate activity against Caco-2 (IC_{50}=81.78±0.43 µg/ml & SI= 11.83); stem, leaves and flowers demonstrated nearly weak cytotoxicity against HepG-2 (IC_{50}=203.3±5.33, 221.71±4.44, 329.35 ± 9.27 µg/ml). Flowers extract recorded weak activity against MCF-7 (IC_{50}=288±20.1) and promising protective activity against HSV1 with no significant difference with acyclovir (65.53±3.24 vs 68.44±7.62). Leaves extract demonstrated pronounced protective (82.99±1.56) and anti-infectivity (73.19±3.1) activities against HAV. Stem-extract exhibited least activity against HSV1 and no activity against HAV. Significant cytotoxicity and antiviral activity could be attributed mainly to the constitutive polyphenols.

Keywords: Anogeissus latifolia; LC/qTOF-MS/MS; Leaves; Flowers; Cytotoxicity; Antiviral activity

1. Introduction

For centuries, cultures around the world have learned how to use herbal medicine to improve healthcare regimen. The importance of plant-based products for disease treatment is growing exponentially due to the increased incidence of adverse drug reactions and the development of microbial resistance to the available antimicrobial drugs [1]. *A. latifolia* **(Roxb. ex DC.)** is a medium sized deciduous tree that belongs to Combretaceae family. This species is commonly known as axle-wood or button tree and widely distributed in tropical and sub-tropical countries as trees, shrubs, or small trees. In traditional medicine it has been used to treat broad spectrum of disorders such as diarrhea, colic, stomach disease, cough, and various skin diseases e.g. sores, boils, psoriasis, and itching [2]. It has been used also for the treatment of anemic conditions, UTI, cardiac disorder, and liver complaints [3]. The bark extract has been reported for the treatment of snake and scorpion bites [4]. Furthermore, the bark and the leaves extracts exhibited demulcent and astringent properties [2] alongside a wide range of biological activities, e.g. antiulcer, antimicrobial [5], antioxidant [6,7], anthelmintic [8], antiplasmodial [9], antidiabetic [10,11], anticonvulsant [12], hypolipid-emic [13], and hepato-protective activities [14]. The ethanolic extracts of the bark and leaves of *A. latifolia* were strongly active against human

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cancer cell lines, including cancers of lung (A549), prostate (PC-3), breast (T47D), colon (HCT-16), and leukemia (THP-1, and HL-60) [4]. From the phytochemical standpoint, the plant was proved to be a rich source of tannins and having appreciable quantities of flavonoids, terpenes and saponins [2–4]. Several tannins have been isolated from the plant as 3,4,3'-tri-*O*-methylellagic acid, 3,4,3'-tri-*O*-methyl-flavellagic acid, 3,3'-di-*O*-methylellagic acid 4'-β-D-xyloside, 3,3'-di-*O*-methylellagic acid 4'-β-D-glucoside, [2] chebulic acid, [15] corilagin [16], and trigallic acid [4,15]. Quercetin, myricetin [17], luteolin 7-*O*-β-D-(galactopyranosyl)-6''-*O*-β-D-galac-topyranoside [18] and quercetin 3-*O*-β-D-(galac-topyranosy)-4''-rhamno-pyranoside [4] were the major flavonoids reported in the plant. Also 3-β-hydroxy-28-acetyltaraxaren and β-sitosterol were reported in the plant [3]. UPLC-MS/MS was optimized, among many of advanced hyphenation techniques, for a lot of fast and precise structural information to avoid the long-time consumption and tedious routine techniques of isolation and identification for known constituents, especially from the bioactive complex natural extracts [19]. The increasing of virus infections and cancer diseases has necessitated the ever-increasing demand of therapeutic natural product drugs due to lack of vaccines, the appearance of viral strains resistance to antiviral agents and the side effects of the cancer's chemotherapy. This has driven our interest to evaluate cytotoxic activity of *A. latifolia* against hepatocellular carcinoma (HepG-2), colorectal adenocarcinoma (Caco-2), breast cancer (MCF-7), and pancreas cancer (Panc-1) cell lines. In addition, the antiviral activity was estimated against some viral pathogens, including herpes simplex virus type 1 (HSV1), and hepatitis A virus (HAV) in regard to the acyclovir, a nucleoside analogue, as one of the approved drugs for treatment of HSV infections [20]. Doxorubicin is a chemotherapy medication used to treat cancer. It interacts with DNA by intercalation and inhibition of macromolecular biosynthesis. This inhibits the progression of topoisomerase II; an enzyme which relaxes super coils in DNA for transcription. Also, it stabilizes the topoisomerase II complex after it has broken the DNA chain for replication, preventing the DNA double helix from being released and thereby stopping the process of replication [21]. It is the first time to evaluate the antiviral activity of the different *A. latifolia* extracts using different protocols. Therefore, this work aimed at the tentative identification of the phytoconstituents in *A. latifolia* leaves and flowers extracts using LC/qTOF-MS/MS analytical tool, together with the evaluation of the cytotoxic and antiviral activities for leaves, flowers, and stem methanol extracts against four cancer cell lines and two viruses.

2. Materials and methods

2.1. Plant material

A. latifolia (Roxb. ex DC.) leaves, stem, and flowers were collected from Giza, Zoo Garden, Egypt, in April 2019. Mrs. Trease Labib identified the plant; senior botanist specialized of plant taxonomy, Orman Garden, Giza, Egypt, as well as by comparison with reference herbarium specimens. Voucher specimens (code AL-1619, AS-1619, AF1619) were deposited in the herbarium of the Department of Pharmacognosy, Faculty of Pharmacy (Girls), Al-Azhar University, Cairo, Egypt.

2.2. Preparation of the crude extracts

Air-dried powders of *A. latifolia* leaves (800 g), stems (235g) and flowers (60 g) were subjected, individually, to a continuous exhaustive defatting with petroleum ether (40–60°C) using Soxhlet apparatus. The left plant marcs were separately extracted with 70% aqueous MeOH (12 x 3 L, 12 x 2 L, and 10 x 500 ml, respectively, for 2 h each at 60°C), in a reflux system. Thereafter, the combined methanolic extracts were evaporated on a rotatory evaporator at low temperature (45°C) and reduced pressure to give dry extract samples of 395, 115 and 25 g, respectively. They were separately taken with hot MeOH under reflux, successively, producing dry MeOH-soluble portions of 190, 45 and 11g from leaves, stem, and flowers, respectively, after evaporation of MeOH. The major secondary metabolites of the leaves and stem samples were finally identified using UPLC/ESI-qTOF-HRMS/MS analysis through positive and negative modes of ionization

2.3. UPLC/ESI-qTOF-HRMS/MS analysis

The LC/MS analysis was carried out in proteomics and metabolomics unit in children cancer hospital (CCHE 57357), Cairo, Egypt. It was performed on an UPLC standard interface (ExionLC, Sciex) combined with a quadruple time-of-flight (QTOF, with HR-TOF scan) mass spectrometer (Triple TOF 5600+, Sciex) that is used for MS/MS selective fragmentation analysis and collection of the structural information. The analytical parameters were set using both negative

and positive modes of ionization. Chromatographic separation was performed on Xbridge reversed phase C18 column (2.1x50 mm, 3.5 µm; Waters, USA) and maintained at 40°C with an in-line filter disks pre-column (3.0mm x 0.5µm; Phenomenex). The mobile phase working solution (1 ml; deionized [DI] H_2O, methanol, and acetonitrile) was separately added to a 50 mg amount from the methanol extract of leaves, or flowers to prepare the stock solutions. Each of the three solutions was vortexed for 2 min. followed by ultra-sonication for 10 min., then centrifugation for 10 min. at 10000 rpm. After that 20 µl stocks (50 mg/1000 µl) was diluted with 1000 µl reconstitution solvent so that the final injected concentration was 1 µg/µl. The eluents were (**A**) DI-H_2O containing 0.1% formic acid for the positive mode, **(B)** 5 mM ammonium formate buffer (*p*H 8.0) containing 1% methanol for the negative mode, and **(C)** 100% acetonitrile for the positive/negative modes. They were applied with multi-step linear gradient as follow; mobile phase A or B with gradient increase from (10-90) of mobile phase C over 28 min. at a flow rate of 0.3 ml/min to allow comprehensive elution of the different analytes. MasterView was used for feature (peaks) extraction from the total ion chromatogram (TIC) based on features should have Signal-to-Noise greater than 5 (non-targeted analysis) and intensities of the sample-to-blank should be greater than 5. PeakView (Sciex) software was used for features annotation and removing isotopic peaks. It was used again to identify peaks based on their fragments using Build-in database and online database (MoNA-MassBank of North America).

2.4. Preparation of stock solution of different plant extracts

Stock solutions of the tested plant were prepared by solubilized 1 g of each extract in 10 ml of Eagle's minimal essential medium (MEM), sterilized the solutions, and stored it in stock concentrations of 100 mg/ml. Further dilutions were made in cell culture medium MEM for use in the *in vitro* experiments.

2.5. In vitro cytotoxicity assay (viability assay)

2.5.1. Cancer cell lines and culture

Four human cancer cell lines were obtained from the Lab of Virology, Microbiology Department, Faculty of Medicine (for Girls), Al-Azhar University, Cairo, Egypt. The cell lines were derived from different cancers including liver (HepG-2), colon (Caco-2, ATB-37), breast (MCF-7, HTB-22) and pancreas (Panc-1). All cell lines were cultured in RPMI- 1640 growth medium except MCF-7 cell line that was cultured in DMEM medium. Both growth media (pH 7.2) were supplemented with 10% FCS, 1% penicillin (100 U/ml) and streptomycin (100µg/ml), in tissue culture flask in an incubator at 37°C with 95% relative humidity and 5% CO_2 gas environment.

2.5.2. Determination of cytotoxicity by MTT assay (Viability assay)

Four human cancer cell lines were employed to test the cytotoxicity of the tested extracts using the MTT assay. This assay assesses concentrations of doxorubicin as a positive control and tested extracts that cause toxicity to 50 % of cells (IC_{50}). The cytotoxicity evaluation was performed by MTT [3-(4,5-dimethylthiazole-2-yl)-2,5-diphenyl tetrazollium bromide] method [22]. Briefly, cells were cultivated in the 96 well tissue culture plate with $1x10^5$ cells/well (100µl/well) and incubated at 37°C for 24 h to develop a complete monolayer sheet. Afterwards, the growth medium was decanted from 96 well micro titer plates and 0.1ml of each dilution (two-fold dilutions of tested samples was made in RPMI medium with 2% serum maintenance medium) was tested in different wells leaving 3 wells as control, receiving only maintenance medium. The plates were incubated at 37°C with 95% relative humidity and 5% CO_2 gas environment. After 2 days, 20 µl of MTT solution (5 mg/ml in PBS, BIO BASIC CANADA INC) prepared in cell culture medium, were added to each well. Thereafter, the wells are placed on a shaking table for 5 min. to mix thoroughly the MTT into the media and the plates were incubated for 4 h to allow the MTT to be metabolized. After the MTT solution was removed without disturbing the cells, 200 µl of DMSO was added to each well to dissolve the formazan crystals (MTT metabolic product). After gently shaking the plates (150 rpm/5 min), the crystals were completely dissolved. The optical density (OD) of each well was recorded using a microplate reader at a wavelength of 560 nm.

% cytotoxicity = $[(A_1–A_2)/A_1]\times 100$

A_1= Absorbance of cells without treatment; A_2= Absorbance of cells with treatment

2.6. Antiviral assays

2.6.1. Cell culture and viruses

African green monkey kidney-derived Vero cells (CCL-81; American Type Culture Collection, USA) maintained in MEM supplemented with fetal calf serum (FCS; 10% v/v), L-glutamine (2 nM), penicillin (100 U/ ml), and streptomycin (100 µg/ml) were used for the antiviral assays. Propagation of cells for up to 4 weeks, then the cells incubated in a 5% CO_2 humidified atmosphere at 37°C as cell line stock. HAV and HSV1 viruses were provided by the Laboratory of Virology, Microbiology Department, Faculty of Medicine (for Girls), Al-Azhar University, Cairo, Egypt.

2.6.2. Preparation of virus stock

Each virus was propagated in Vero cells by infecting a confluent Vero cell monolayer in 75 cm^2 culture flasks and allowed to be adsorbed for 1 h. The non-adherent particles were washed off using 2% MEM (maintenance medium), and the infected cells were overlaid with 20 ml of 2% MEM and incubated until a full cytopathogenic effect (CPE) was observed daily for up to 4–6 days. This step was repeated twice, then the challenge dose of each virus was determined by using plaque formation assay [23,24] and each virus harvest was stored at – 20°C until use.

2.6.3. Cytotoxicity assay

The maximum nontoxic concentration (MNTC) of each extract was determined by double-fold dilutions of the different plant extracts in MEM with FCS. Then, 0.1 ml of each dilution was used to treat confluent Vero cells in four wells in a 96-well plate (Falcon; Corning, USA), and two rows were left as the control. Acyclovir (Sigma-Aldrich, St. Louis, MO, USA) was dissolved in distilled water and its MNTC was also evaluated. The plates were incubated at 37°C. The cells were examined daily using an inverted microscope to determine the minimum concentration required to produce alterations in cell morphology. The treated Vero cells did not show any morphological differences at the MNTC compared with the control Vero cells, even after 7 days. The MTT colorimetric assay (Bio Basic Inc., Canada) was performed as described previously [22].

2.6.4. Mode of action of virus inhibition

The possible mechanism of the antiviral activity of the plant extracts was examined at three different stages of the virus propagation cycle; the challenge dose of the virus (CDV) was inoculated into Vero cell cultures that were treated with the MNTC of each extract and its serial dilution according to three main protocols [25–27]. For consistency, each protocol was repeated three times independently of each other and the mean value of the three experiments of each protocol was presented.

2.6.5. Protocol A: virus pretreatment (anti-infective activity)

This protocol was used to test the virucidal activity of the extracts [25,27]. The challenge virus was exposed to the nonlethal dilution (MNTC) of extracts (1:1 v/v) for 1 h at 37°C. Then, the confluent monolayers of Vero cells in 96-well flat-bottomed microtiter plates were overlaid with 0.5 ml of medium containing the extract and an equal volume of the CDV virus suspension of each virus. After 60 min. of virus adsorption, the plates were incubated at 37°C. In control wells, Vero cells were incubated with the virus but not treated with the extract as (negative control), whereas acyclovir was used as a positive control. Finally, the virus-induced CPE was scored for 72 h under an inverted microscope using the MTT colorimetric assay.

2.6.6. Protocol B: post-infection treatment (anti-replicative activity)

This protocol tested the effect of the extract on virus replication [26,27]. A confluent layer of Vero cells in 96-well plates was incubated with the CDV (100 µl) of virus seed in each well. After adsorption for 60 min., the cells were overlaid with agarose containing the extract. The monolayer was observed for CPEs after 72 h. The viability of the infected and non-infected cells was evaluated using the MTT assay, as previously described [22]. The 50% inhibition concentration (IC_{50}) was also calculated.

2.6.7. Protocol C: Cell pretreatment (protective activity)

This protocol was used to test for virus entry into the host cells by blocking attachment to the cell surface [26,28]. A confluent monolayer of Vero cells in a 96-well flat-bottomed microtiter plate was inoculated with medium containing the extract for 48 h. Then, the cells were washed twice with phosphate-buffered saline and challenged with the CDV of each virus. After 60 min. of adsorption at 37°C, the monolayer was overlaid with agarose containing the culture medium and incubated. After 72 h, the virus-induced CPE was scored as above.

2.7. Statistical analysis

Data of the antiviral activity of L, F, S which stands for leaves, flowers, and stem, respectively, under three protocols (A, B and C) against HAV and HSV1 have been analyzed using Minitab 20 and SPSS 28. Data was cleansed before running any statistical analyses. Missing data and mistyping errors have been checked. Descriptive statistics including mean and standard error (SE) have been calculated from three replicates for each level. Inferential statistics have been used to compare results of different groups. All variables parametric assumptions have been tested and results showed significant violation of parametric assumptions. Box-Cox transformation for non-normal dependent variables was applied to the antiviral activity results using the optimal λ method. Different comparisons were done using One- and Two-way analysis of variance (ANOVA) under fit General linear model. Results showed a good fit for different models, while normal residual probability plots showed a linear attitude for all analyses after data transformation. P values were considered significant at $\alpha < 0.05$. Post hoc analyses of the interactions among all groups were done using Tukey test for pairwise comparisons. Results of the post hoc analyses are represented as letters where groups that share same letters are non-significantly different, while different letters express significant differences among different groups. For the HAV and HSV results, Two-way ANOVA was used to analyze the two categorical factors protocol (three levels), and extracts (four levels). For comparing different extracts to the doxorubicin against Vero, Panc1, Caco2, HepG2, and MCF-7, One-way ANOVA was used to analyze the one categorical factor extracts (four levels including the Doxorubicin as a positive control).

3. Results

3.1. UPLC/ESI-qTOF-HRMS/MS analysis

LC/qTOF-MS/MS analysis of the total methanolic leaves and flowers extracts of *A. latifolia* resulted in the tentative identification of 66 and 27 metabolites by the negative and positive mode of ionization, respectively. The BPC mass chromatograms for monitoring the separation processes of both extracts were illustrated (Figures 1a, b & 2a, b). Moreover, the output data i.e. R_t-values, molecular ion peaks, fragment ions and MFs for all metabolites and their identities are presented in tables 1 and 2. The peak numbers were assigned according to their elution order. The tentative identification of the constitutive metabolites was concluded by their R_t-values, monoisotopic masses of the molecular, adducts or fragments ions recorded in the corresponding MS^1 and MS^2 spectra (Figures S1–S31), based on the online comparison with the conventional library database and literature. The major identified metabolites were grouped into several types of secondary metabolites e.g. flavonoids, tannins, and phenolic acids.

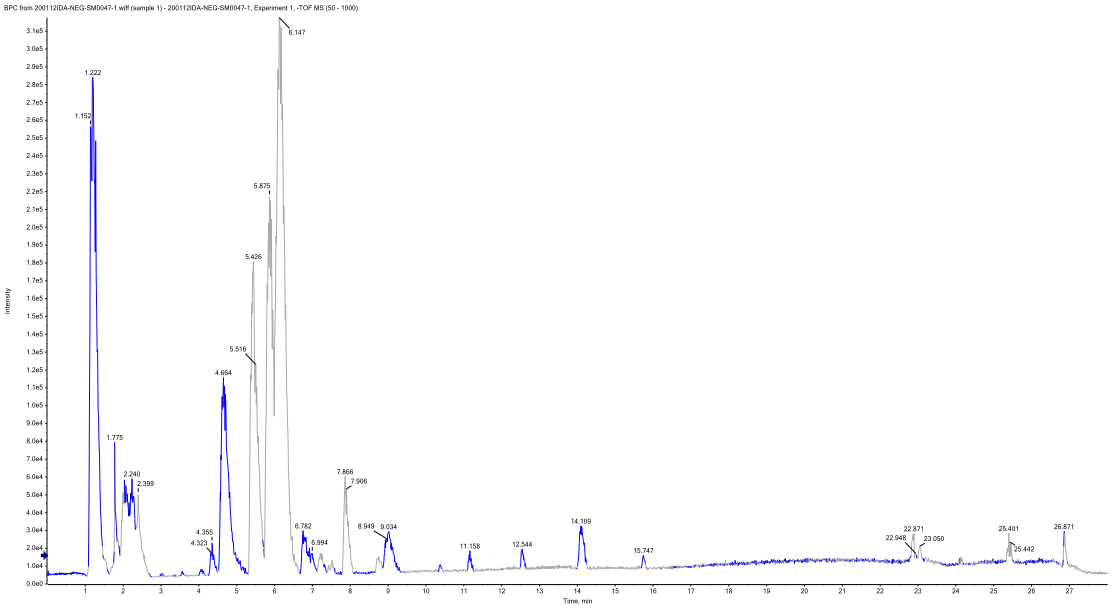

Fig. 1a. BPC mass chromatogram of the *A. latifolia* methanolic Leaves extract (negative ion mode)

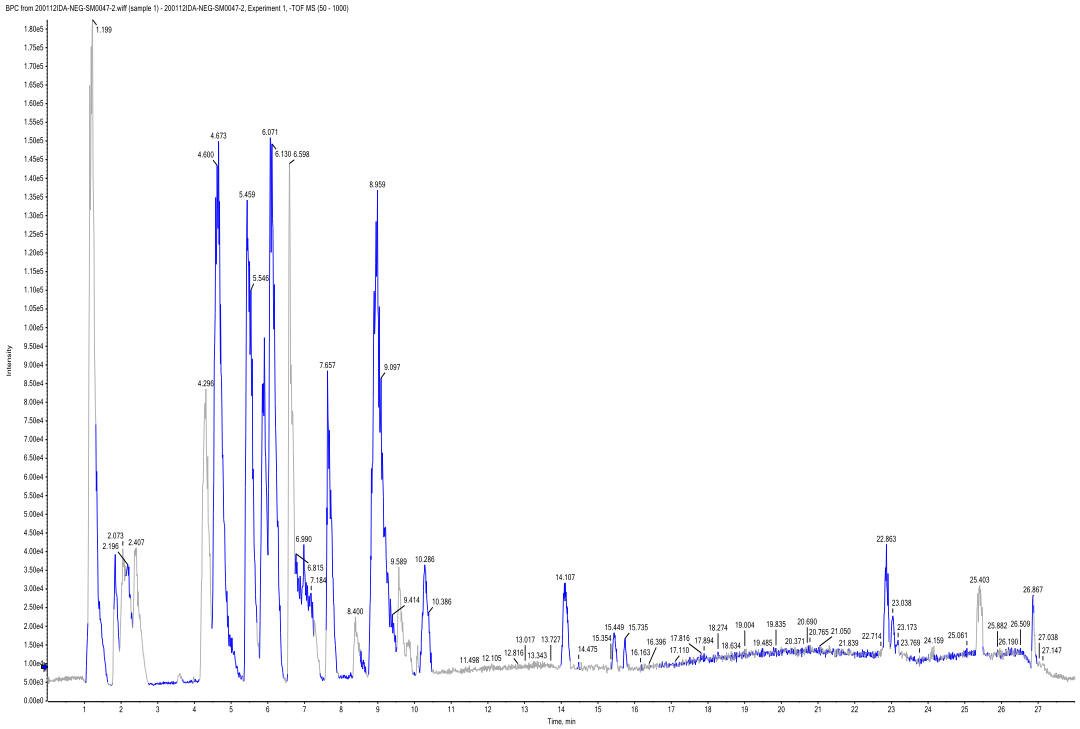

Fig. 1b. BPC mass chromatogram of the *A. latifolia* methanolic flowers extract (negative ion mode)

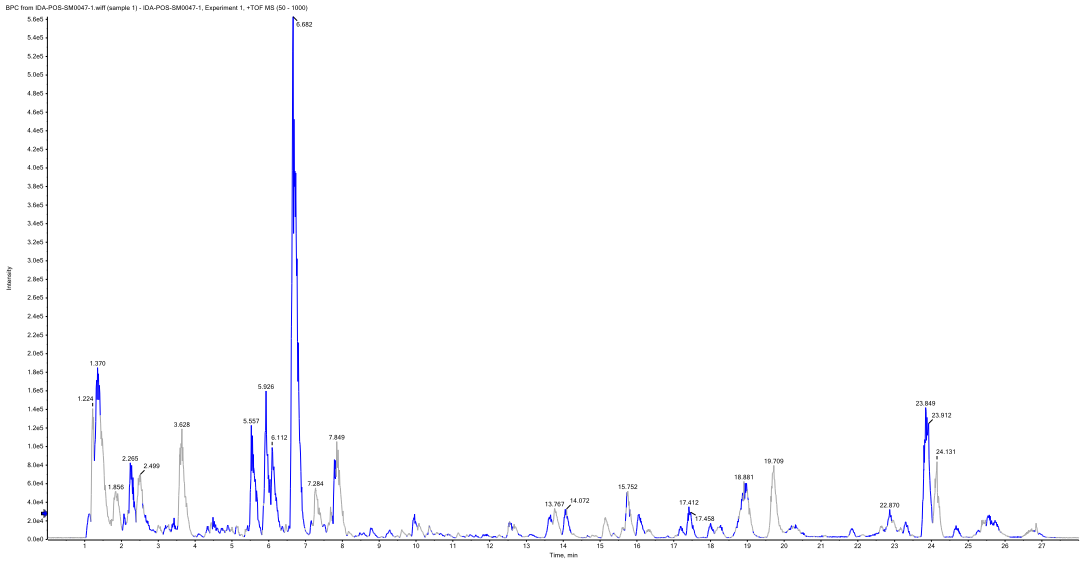

Fig. 2a. BPC mass chromatogram of the A. latifolia methanolic leaves extract (positive ion mode)

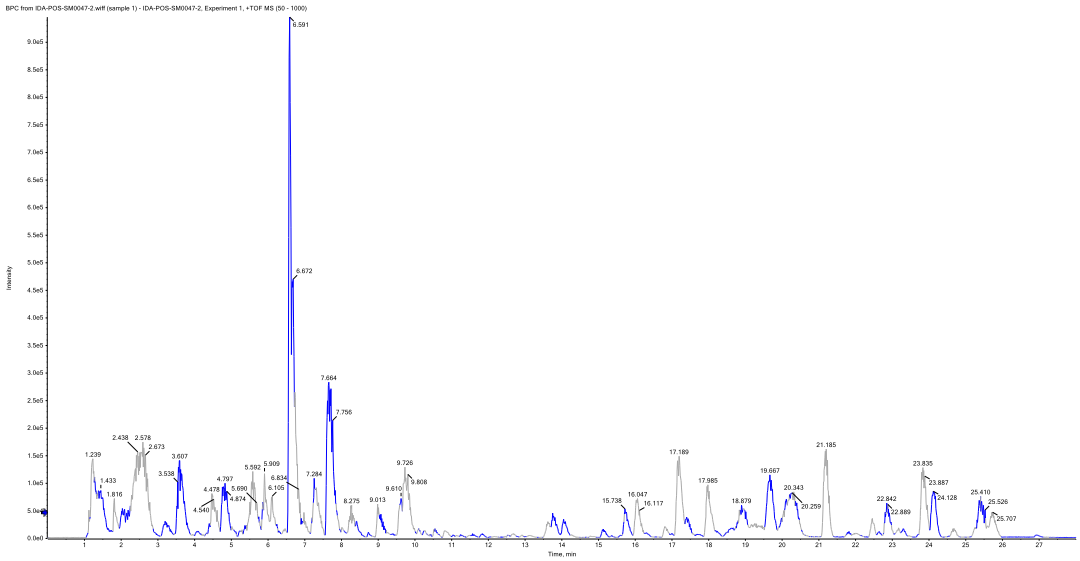

Fig. 2b. BPC mass chromatogram of the *A. latifolia* methanolic flowers extract (positive ion mode).

3.1.1. Characterization of metabolites detected in negative ion mode

Based on LC/MS output data, a total of 66 metabolites were detected by negative ion mode analysis of leaves and flowers extracts (Table 1). A total of 15 phenolic acids were identified for the first time in *A. latifolia* except for four, i.e. 3,4,3'-tri-*O*-methyl-flavellagic (**1**), ellagic (**3**), gallic (**10**) and 3,4,3'-tri-*O*-methylellagic acids (**29**), depending on their R_t values, XIC chromatograms, monoisotopic masses (*m/z*) of molecular ($[M–H]^-$) or some adduct ions and MS/MS specific fragments in MS^1 and MS^2 spectra (Figures S1–S31) for each peak in the BPC chromatograms (Figure 1). In addition, a total of 8 organic acids, 31 flavonoids, 8 tannins, 3 coumarins and a sugar were also tentatively identified (Table 1). The major flavonoids were identified as apigenin **65**, apigenin 8-*C*-glucoside **46**, apigenin 7-*O*-glucoside **57**, luteolin **26**, luteolin 6-*C*-glucoside **44**, and luteolin 7-*O*-glucoside **52**, that were tentatively identified for the first time in *A. latifolia*. Similarly, 3 major tannins, namely punicalagin **13**, digalloylglucose **21**, and monogalloylglucose **28,** were identified for the first time in this species. Ellagic (**3**) and quinic (**11**) were the major identified phenolic and organic acids, respectively.

Table 1. Metabolites identified from the methanolic leaf and flower *A. latifolia* extracts (negative ion mode)

Peak No.	R_t/min		$[M–H]^-$ (m/z)	MS/MS fragments/(m/z)	Area		MF	Metabolite
	Leaves	Flowers			Leaves	Flowers		
				Organic acids				
4	1.110	-	117.0118	73.0311, 117.0177, 117.0277	28811.03	-	$C_4H_6O_4$	Succinic acid
5	1.135	1.122	133.0112	71.01207, 72.99397, 89.03258, 115.00447, 133.00766,133.01418	741668.1	1141695	$C_4H_6O_5$	Malic acid
6	1.135	1.122	115.0076	71.01099, 71.01218, 115.0076	119114.6	102611	$C_4H_4O_4$	Maleic acid
9	1.160		173.0457	85.04115, 93.03416, 111.0429, 137.02295, 173.03842, 173.0439	516076.5	204594.2	$C_7H_{10}O_5$	Shikimic acid
11	1.221		191.0151	85.01099, 127.01218, 172.0076, 191.0151	1.58E+07	8562588	$C_7H_{12}O_6$	Quinic acid
12	1.224	-	105.0023	72.99042, 73.00248, 75.00077, 105.02399	30083.99	-	$C_3H_6O_4$	Glyceric acid
23	1.426	1.357	129.0374	55.01643, 58.99381, 68.99387, 85.0303, 129.03749,	121113.8	115826	$C_5H_6O_4$	Citraconic acid
24	1.737	-	130.99001	86.9915, 130.99001	209086.2	-	$C_5H_8O_4$	Methylsuccinic acid
				Phenolic acids				
1	1.068	1.375	359.0114	143.03133, 179.05981, 275.02942, 313.11874, 359.0114	10970.09	26698.45	$C_{17}H_{12}O_9$	3,4,3′-Tri-*O*-methylflavellagic acid
3	1.082	-	300.9911	255.23584, 300.9911	8657.007	-	$C_{14}H_6O_8$	Ellagic acid
10	-	1.197	169.0146	125.0235, 169.0146	-	13312.04	$C_7H_6O_5$	Gallic acid
15	1.249	1.879	353.1011	131.05583, 161.05629, 173.0457, 191.0652, 288.50473, 307.10123, 353.10443	103071.9	290610.9	$C_{16}H_{18}O_9$	Chlorogenic acid
19	1.270	-	153.01396	109.03123, 153.01396	16034.48	-	$C_7H_6O_4$	3,4-Dihydroxybenzoic acid
20	-	1.310	179.0556	58.00153, 75.0081, 85.39951, 135.06427, 159.03366, 161.0541, 164.0247, 179.05603	-	860464.9	$C_9H_8O_4$	Caffeic acid
25	1.800	-	163.0258	119.0258, 163.0258	46183.69	-	$C_9H_8O_3$	*E*-*o*-Coumaric acid
29	1.800	1.287	343.0117	169.0125, 181.0254, 343.0117	21041.79	37546.47	$C_{17}H_{12}O_8$	3,4,3′-Tri-*O*-methylellagic acid
30	-	1.854	163.03329	93.03122, 119.04946, 119.05254, 119.0540, 119.0587, 119.06024, 163.03329	-	31811.08	$C_9H_8O_3$	3-(4-Hydroxyphenyl) prop-2-enoic acid
35	-	2.267	167.0699	123.07515, 167.06992	-	52432.59	$C_8H_8O_4$	Homogenentisic acid
36	-	3.079	137.0259	93.03671, 120.03301, 137.02597	-	99615.09	$C_7H_6O_3$	*p*-Hydroxybenzoic acid
37	3.619	9.210	359.1421	359.14258	7991.25	27114.67	$C_{18}H_{16}O_8$	Rosmarinic acid
38	3.777	1.185	137.0218	93.0133,136.01503, 137.02318	66162.52	45474.34	$C_7H_6O_3$	Salicylic acid
39	3.830	-	151.0384	79.05724, 93.03708, 105.0313, 107.04847, 121.03809, 133.02759, 151.03804	16270.75	-	$C_8H_8O_3$	2-Hydroxyphenylacetic acid
51	6.619	6.608	167.0346	108.02675, 148.99373, 152.0307, 167.03406	140915.1	518611.3	$C_8H_8O_4$	5-Methoxysalicylic acid

				Flavonoids				
16	1.249	-	591.1601	249.00897, 547.02393, 573.06213, 591.06738, 591.1601	16034.25	-	$C_{28}H_{32}O_{14}$	Acacetin 7-O-rutinoside
22	1.387	3.784	283.0808	130.05855, 283.08081	22410.64	39311.38	$C_{16}H_{12}O_5$	Acacetin
26	1.800	5.967	285.0614	152.07348, 285.06149	68272.23	36818.7	$C_{15}H_{10}O_6$	Luteolin
27	1.800	7.513	417.1237	286.90214, 307.0852, 390.90283, 417.10352, 417.12372	31583.26	36091.46	$C2_0H_{18}O_{10}$	Kaempferol 3-*O*-α-L-arabinoside
31	1.855		433.04608	297.35568, 299.98654, 321.0809, 389.03165, 391.04245, 433.04608	31700.7	10010.5	$C_{20}H_{18}O_{11}$	Quercetin 3-D-xyloside
32	1.931	1.961	447.05688	239.06255, 244.01151, 299.0232, 301.0042, 325.03751, 379.03564, 406.9359, 446.58228, 447.03897, 447.05688	231627.5	124.67.51	$C_{21}H_{20}O_{11}$	Quercitrin
33	2.020	9.230	300.9235	129.03758, 137.0291, 151.0026, 161.02888, 189.0477, 229.05092, 273.0443, 300.92357	1641337	920025	$C_{15}H_{10}O_7$	Quercetin
34	2.140	-	449.1132	169.0412, 271.04191, 449.07236, 449.11323	91809.25	-	$C_{21}H_{22}O_{11}$	Okanin 4'-*O*-glucoside
40	4.170	-	445.1236	161.02422, 443.68945, 445.12436	31987.16	-	$C_{21}H_{18}O_{11}$	Baicalein 7-*O*-glucuronide
41	4.250	7.045	415.12408	124.01266, 139.04224, 165.0124, 168.0081, 347.1251, 371.1401, 379.07703, 415.12408	151458.1	24217.82	$C_{21}H_{20}O_9$	Daidzein 8-*C*-glucoside
43	5.230		431.1917	179.0524, 287.9033, 431.1055, 431.14948, 431.19173	86109.97	113271.1	$C_{21}H_{20}O_{10}$	Kaempferol-3-*O*- α-L-rhamnoside
44	5.431		447.0899	133.02971, 198.0383, 255.0331, 285.03979, 299.05801, 310.5239, 325.0278, 327.39929, 357.01803, 363.8825, 371.0751, 387.07034, 397.41541, 411.09741, 425.07501,447.0899	1.05E+07	7487151	$C_{21}H_{20}O_{11}$	Luteolin 6-*C*-glucoside
45	5.666	8.319	609.1602	549.0744, 609.13239, 609.16028	29546.02	45807.12	$C_{27}H_{30}O_{16}$	Luteolin 7,3'-di-*O*-glucoside
46	5.866	5.893	431.0747	94.23771, 159.04955, 278.89978, 295.0577, 311.0528, 323.0563, 337.06854, 341.0754, 383.07855, 429.70956, 431.07477	1.06E+07	3929169	$C_{21}H_{20}O_{10}$	Apigenin 8-*C*-glucoside
47	5.962	6.261	609.1429	255.03503, 300.02313, 301.0293, 565.0255, 607.17188, 609.03143, 609.14294	118469.1	35662.13	$C_{28}H_{34}O_{15}$	Hesperetin 7-*O*-neohesperidoside
48	-	5.970	303.05823	125.02393, 175.03519, 189.0538, 193.0572, 201.01947, 217.04367,241.04228,258.9394, 259.0664, 275.0594, 285.0342, 303.05823	-	29345.1	$C_{15}H_{12}O_7$	(+-)-Taxifolin
49	6.062	-	463.0898	216.04095, 249.14554,260.75476, 286.9436, 300.03082, 301.0321, 326.9223, 349.0621, 394.91125, 430.09482, 463.08908	16784.5	-	$C_{21}H_{20}O_{12}$	Quercetin 4'-glucoside
52	6.754	6.782	447.0925	241.04922, 285.03732, 297.04126 324.6226, 357.05606, 429.08441, 445.6456, 447.09259	686589.5	1625637	$C_{21}H_{20}O_{11}$	Luteolin 7-*O*-glucoside

53	6.762	7.070	507.1441	313.0458, 326.96968, 354.0148, 381.01258, 462.9871, 507.14414	65885.5	98746.6	$C_{23}H_{24}O_{13}$	Syringetin 3-*O*-galactoside
54	-	6.782	433.07358	177.02284, 258.9482, 271.06229, 329.0639, 370.88864, 387.21051, 433.07358	-	111574.2	$C_{21}H_{22}O_{11}$	Naringenin7-*O*-glucoside
55	6.820	6.790	477.0974	169.0514, 285.07639, 346.89249, 417.08151, 477.0974	805622.6	39469	$C_{22}H_{22}O_{12}$	Isorhamnetin3-*O*-glucoside
56	6.908		463.0855	286.9014, 301.03998, 326.01478, 354.2589, 463.08558	139190.4	73133.36	$C_{21}H_{20}O_{12}$	Myricitrin
57	-	7.120	431.09747	94.23771, 159.04955, 269.0432, 278.89978, 295.0577, 311.0528, 323.0563, 337.06854, 343.07855, 429.70956, 431.07477	-	921439.33	$C_{21}H_{20}O_{12}$	Apigenin 7-*O*-glucoside
58	-	7.221	449.0943	135.04457, 151.03925, 285.04614 286.03644, 287.04758, 299.03473, 430.72592, 447.67691, 449.0943	-	36692.3	$C_{21}H_{22}O_{11}$	Isookanin 7-glucoside
59	7.233	9.236	299.1052	183.04482, 267.19891, 285.0349, 299.05261	26379.3	63416.9	$C_{16}H_{12}O_6$	3,5,7-Trihydroxy-4'-methoxyflavone
60	7.284	7.221	577.0194	269.2012, 531.0258, 539.1259, 577.0194	27924.3	83.199.3	$C_{27}H_{30}O_{14}$	Rhoifolin
61	-	7.62	609.1745	183.04024, 242.05351, 286.038, 300.09268, 316.2092, 327.0506, 337.12265, 347.07895, 546.78082, 591.2506, 607.21387, 609.17456	-	14406.2	$C_{28}H_{34}O_{15}$	Hesperidin
62	7.632	7.829	461.1046	256.92499, 298.05109,324.9166, 392.89691, 392.90811, 415.22604, 461.10461	27375.4	79153.52	$C_{21}H_{18}O_{12}$	Kaempferol 3-Glucuronide
63	-	8.392	287.0567	107.02296, 112.9853, 134.03528, 150.9938, 190.9688, 218.94882, 286.92761, 287.05679	-	216497.7	$C_{15}H_{12}O_6$	3',4',5,7-Tetrahydroxyflavanone
64	9.290	6.474	593.1245	179.0258, 223.01548, 285.3694, 547.0258, 593.12458	148738.9	122650	$C_{27}H_{30}O_{15}$	Datiscin
65	10.275	10.280	269.0440	150.9958, 159.3698, 171.1258, 181.0256, 201.4589, 225.0147, 227.0158, 269.0440	437936	2346766	$C_{15}H_{10}O_5$	Apigenin
				Tannins				
2	1.068		633.0427	488.09848, 516.8988, 573.71405, 614.8319, 623.9838, 633.00427	17005.37	11011.63	$C_{27}H_{22}O_{18}$	Galloyl-hexahydroxydiphenoylglucose
7	1.090	1.185	781.0596	780.99408, 781.04932	184506.1	53649.31	$C_{34}H_{22}O_{22}$	Gallagalloyloyl-glucose (Punicalin)
8	1.160	1.324	481.0272	191.05739, 285.02536, 289.030, 481.0272	15679.95	2542667	$C_{20}H_{18}O_{14}$	Hexahydroxydiphenoyl glucose
13	1.236	1.160	541.0064 $[M–H]2^{-}$ =(M-2)/2	255.0010, 275.01352, 300.99472, 413.13248, 479.0379, 539.78528, 541.00647	37744.73	1213843	$C_{48}H_{28}O_{30}$	Punicalagin

17	1.261		933.0594	405.0947, 591.05341,600.99268, 721.0249, 781.0365, 927.85168, 930.19318, 933.05945	535392.4	411098.04	$C_{41}H_{26}O_{26}$	Hexahydroxydiphenoyl-valaneoylglucose
18	1.261		635.0519	131.04408, 169.00281, 203.0665, 243.0610, 300.99423, 611.89728, 635.05194	575041.3	75041.38	$C_{27}H_{24}O_{18}$	Ttigalloylglucose
21	1.362	1.317	483.0487	300.99472, 483.04874	41663.61	6321.243	$C_{20}H_{20}O_{14}$	Digalloylglucose
28	1.800	1.817	331.0425	168.0258, 193.0258, 241.0258, 271.3698, 313.01489, 331.0425	1090132	1048960	$C_{13}H_{16}O_{10}$	Monogalloylglucose
				Coumarins				
42	4.753	10.290	177.0182	93.03729, 133.02786, 149.0267, 177.01822	69255.8	60155.7	$C_9H_6O_4$	Daphnetin
50	6.582	-	339.1055	117.02938, 145.02492, 276.9234, 339.10553	66983.67	-	$C_{15}H_{16}O_9$	Esculin
66	10.29	-	177.023	117.0258, 135.4712, 145.2365, 162.2589, 177.0236	71890.95	-	$C_9H_6O_4$	6,7-Dihydroxycoumarin
				Sugars				
14	1.236	-	163.060	115.04859, 163.06613		-	$C_6H_{12}O_5$	L-(+)-Rhamnose

3.1.2. Characterization of metabolites detected in positive ion mode

Taking into account their R_t-values, XIC chromatograms, monoisotopic masses *m/z* of $[M+H]^+$ molecular or some adduct ions and MS/MS specific fragments of MS^1 and MS^2 spectra for each peak in the BPC (Figure 2), a total of major 27 flavonoids were tentatively identified (Table 2). Out of the 27 flavonoids,12 glycosides or aglycones are detected only in the positive ion mode analysis i.e. those of peak numbers **1**, **2**, **6**, **8**, **9**, **13**, **14**, **17**, **18**, **20**, **23** and **26,** see the two representative instances for apigenin 6-*C*-glucoside 7-*O*-glucoside (**13**, Figure S30) and vitexin 2"-*O*-rhamnoside (**20**, Figure S31). All identified metabolites were of flavone-, flavonol-, flavanone- or flavanonol-type or some of their *O*- and/or *C*-glycosides.

Table 2. Metabolites identified from the methanolic leaf and flower *A. latifolia* extracts (positive ion mode)

Peak No.	R_t(min)		$[M+H]^+$	MS/MS fragments (m/z)	Area		MF	Metabolite
	Leaf	Flower	(m/z)		Leaf	Flower		
1	2.373	7.554	319.0714	319.0714	17794.12	18295.98	$C_{15}H_{10}O_8$	Myricetin
2	3.120	-	449.0147	449.01472	13543.31	-	$C_{21}H_{20}O_{11}$	Luteolin 8-*C*-glucoside
3	3.888	7.123	417.1091	417.09147	24777.2	80639.8	$C_{21}H_{20}O_9$	Daidzein 8-*C*-glucosid
4	-	4.637	611.1168	207.03833, 355.0773, 377.13742, 565.00922, 611.11689	-	13271.98	$C_{27}H_{30}O_{16}$	Luteolin 7,3'-di-*O*-glucoside
5	5.100	6.323	433.1442	433.14423	35264.25	156855.1	$C_{21}H_{20}O_{10}$	Apigenin8-*C*-glucoside
6	5.891	6.042	579.1784	98.09348, 153.01781, 352.08667, 530.73151, 579.17865	374672.2	66725.22	$C_{30}H_{26}O_{12}$	Procyanidin B2
7	5.921		449.1058	229.25874, 339.02368, 353.0215, 377.0215, 383.01478, 413.0158, 431.0258, 449.1058	4485592	3101133	$C_{21}H_{20}O_{11}$	Luteolin 6-*C*-glucoside

8	6.174	-	741.1836	128.05722, 163.0636, 265.06839, 287.05637, 431.09525, 579.1588, 595.136117, 741.18365	27738.29	-	$C_{33}H_{40}O_{19}$	Kaempferol3-*O*-rutinoside 7-O-rhamnoside
9	6.850	6.770	465.1025	130.01258, 162.0258, 180.1258, 303.01258, 427.0125, 465.1025	105109.2	352514.8	$C_{21}H_{20}O_{12}$	Hyperoside
10	7.272	-	451.1536	289.10315, 451.15366	13325.76	-	$C_{21}H_{22}O_{11}$	Okanin4'-*O*-glucoside
11	7.275	-	465.1406	153.12617, 275.02252, 465.14069	72675.88	-	$C_{21}H_{20}O_{12}$	Quercetin 4'-glucoside
12	-	7.280	579.1759	114.89021, 173.13942, 263.15533, 271.06146, 379.04282, 417.15033, 433.11002, 502.13187, 505.18036, 579.15973	-	37519.11	$C_{27}H_{30}O_{14}$	Rhoifolin
13	7.391	8.731	595.1987	433.2475, 595.15057, 595.19879	85522.2	28122.17	$C_{27}H_{30}O_{15}$	Apigenin6-*C*-glucoside 7-*O*-glucoside
14	7.661	-	593.196	591.32513, 593.20142	374489.7	-	$C_{28}H_{32}O_{14}$	Acacetin7-*O*-neohesperidoside
15	-	7.667	611.191	303.0125, 449.0258, 465.0258, 611.1917	-	230505.9	$C_{28}H_{34}O_{15}$	Hesperidin
16	-	7.771	433.110	114.89656, 153.01505, 270.2164, 271.06195, 284.08456, 309.0722, 379.08191, 401.16843, 415.28427, 433.11655	-	473768.9	$C_{21}H_{20}O_{10}$	Apigenin7-*O*-glucoside
17	-	7.888	481.180	463.18317, 481.17484	-	16372.8	$C_{21}H_{20}O_{13}$	Gossypin
18	8.601	1.265	269.112	219.15686, 269.19962	13100.48	102290.9	$C_{16}H_{12}O_4$	Formononetin
19	9.262		287.055	153.0158, 287.0527	445180.6	3420926	$C_{15}H_{10}O_6$	Luteolin
20	9.652	9.633	579.146	147.09236, 313016943, 415.04508, 433.13324, 579.1461	13121.46	8711.215	$C_{27}H_{30}O_{14}$	Vitexin2"-O-rhamnoside
21	9.676		303.042	147.025, 153.0257, 229.0368, 233.0147, 287.01587, 303.0147	59032.5	173716.4	$C_{15}H_{10}O_7$	Quercetin
22	9.732	23.891	593.178	593.1816	16297.92	3056997	$C_{28}H_{32}O_{14}$	Acacetin7-*O*-rutinoside
23	-	10.786	273.073	68.99573, 107.0523, 145.10577, 153.01457, 167.06956, 174.03976, 258.10385, 273.07379	-	76488.2	$C_{15}H_{12}O_5$	Naringenin
24	-	10.836	271.059	153.0148, 271.0299	-	793716.1	$C_{15}H_{10}O_5$	Apigenin
25	-	11.130	301.071	121.02695, 153.01457, 168.009, 216.04961, 258.04492, 286.05338, 299.9306, 301.07132	-	14412.2	$C_{16}H_{12}O_6$	3,5,7-Trihydroxy-4'-methoxyflavone
26	12.265	21.265	317.041	317.04137	88210.84	24769.2	$C_{16}H_{12}O_7$	3,5,3',4' -Tetrahydroxy-7-methoxyflavone
27	13.739	-	289.110	83.05022, 167.04724, 289.11282	35200.15	-	$C_{15}H_{10}O_6$	5,7,3',4'-Tetrahydroxyflavanone

3.2. *In vitro studies*

3.2.1. Determination of cytotoxicity of A. latifolia extracts against cancer cell lines

The MTT assay was used to evaluate the cytotoxicity of *A. latifolia* three extracts (leaves (L), flowers (F), and stem (S)) against normal Vero (Table S1, Figure 3) and four human cancer cell lines of liver (HepG-2), colon (Caco-2), breast (MCF-7) and pancreas (Panc-1). The cytotoxicity experiment was performed 48 h after the extract samples were inoculated ranging from 31.25 to 1000 µ/ml. Doxorubicin was used as a positive control. The results showed that the tested samples; flower and stem extracts in particular exhibited non-cytotoxic activity to the normal cells [Nd] (CC_{50} value > 1000 µg/ml), which means that the samples are highly safe to normal Vero cells. Moreover, the tested samples inhibit the growth of the examined cancer cells in concentration dependent manner. As a result, the SI of the extracts to Vero and different cancer cells was more than that of doxorubicin >1, indicating that the tested samples are safer. The greater the SI value, the safer the drug. The sensitivity of the cell lines towards the samples was characterized by $IC_{50,}$ µg/mL (The concentration that causes 50% decrease in cell viability) and selectivity index (SI) values. In general, the drug potency is inversely proportional with the IC_{50} values.

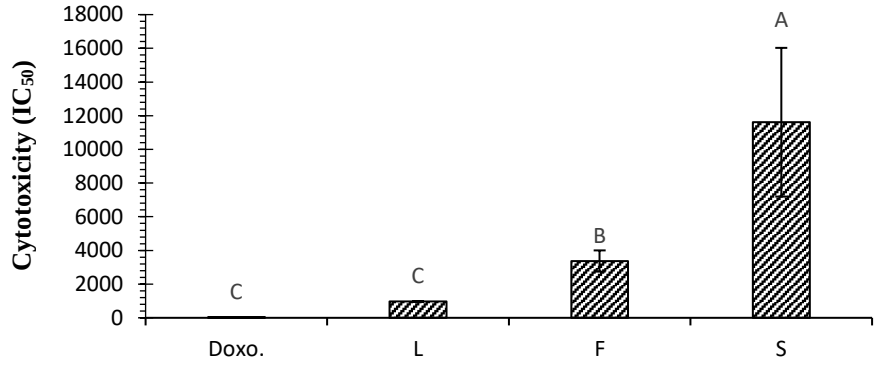

Fig. 3. Cytotoxicity of the different extracts against Vero cell line expressed as CC_{50} values (M±SE)

3.2.2. Cytotoxic effect of L, F and S extracts against different cancer cell lines

All extracts inhibited HepG-2, Caco-2/ATB-37, MCF-7/HTB-22 and Panc-1 growth in concentration dependent manner. Leaves extract showed the moderate activity against Caco-2 (IC_{50}=81.78±0.43 µg/ml & SI= 11.83) with significant difference vs. doxorubicin (IC_{50} = 31.91 ± 0.81& SI= 1) (Table S2, Figure 4a); it was noticed that stem, leaves and flowers extracts showed weak cytotoxic effect against HepG-2 cell line (IC_{50} > 100-1000 µg/ml) with no significant difference (IC_{50} = 203.3 ± 5.33, 221.71 ± 4.44, and IC_{50} = 329.35 ± 9.27 µg/ml, respectively) (Table S2, Figure 4b), indicating that the extracts have poor cytotoxic effect against liver cancer. However, doxorubicin demonstrated potent cytotoxic activity with significant difference (IC_{50}= 5.4± 0.22 µg/ml with high SI. However, almost no cytotoxic effects were recorded for any extract against Panc-1 cancer cell line (Table S2, Figure 4c). Their cytotoxic effects were recorded in the order of S (IC_{50} = 390.39 ± 5.64 > L (IC_{50} = 433.5 ± 11.1) > F (IC_{50} 568.11 ± 6.37) with significant difference vs. doxorubicin 19.07± 0.2. Commonly the high IC_{50} values described the high resistance of Panc-1 towards all examined extracts. Flower extract exhibited weak activity against MCF-7 (IC_{50}=288±20.1) with significant difference regarding the others and to doxorubicin 15.48±0.84 (Table S2, Figure 4d).

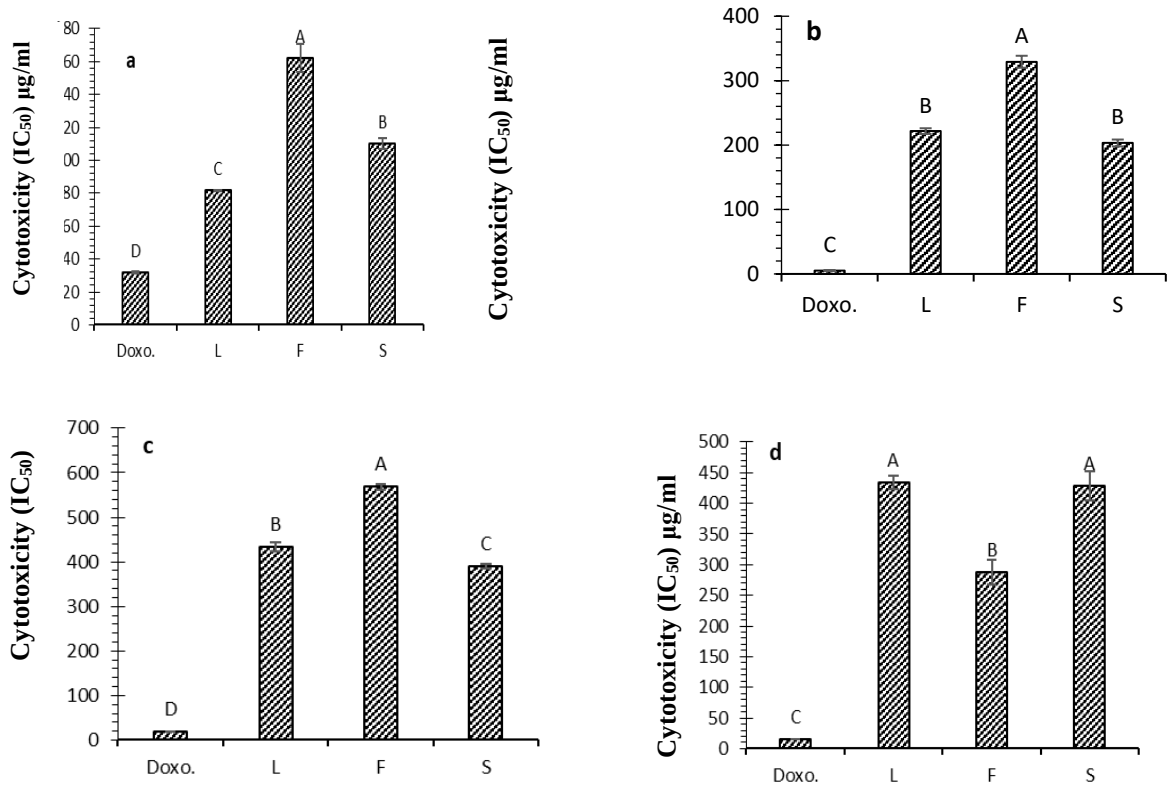

Fig. 4 Cytotoxicity of different extracts against Caco-2 (a), HepG-2 (b), Panc-1 (c), and MCF-7 (d) cell lines expressed as IC_{50} values (M±SE)

3.3. *Antiviral assay*

3.3.1. Determination of MNTC of A. latifolia different extracts

The MNTC is known as the maximum non-toxic concentration required to produce optimum biological activity and less cytotoxic effect. As shown in Table S3, it was determined for L, F, and S extracts to record 500, 500, and 1000 µg/ml, respectively vs. acyclovir (62.5 µg/ml) as a reference antiviral drug specific for HSV.

3.3.2. Antiviral activity of the different extracts against HAV and HSV1 using different protocols

The MNTC of each sample was demonstrated and used against each virus to evaluate the mechanism of its antiviral activity according to three protocols (Tables S4,S5 and Figures 5,6).

3.3.3. Comparison of the effect for the extracts against HSV1 vs. acyclovir with different protocols

The acyclovir antiviral activity was shown in the following order: protocol B (92.69 ± 1.32) > protocol A (83.76 ± 5.67) > protocol C (68.44 ± 7.62) as shown in Table S4 and Figure 5. On studying the mechanism of antiviral activities of *A. latifolia* different extracts against HSV-1, it was found that only protocol C (protective) showed moderate antiviral activity (65.53 ± 3.24) for flowers extract (F), with no significant difference with the leaves extract (L) (55.42 ± 4.86) and acyclovir (68.44 ± 7.62) , where both F and L extracts showed nearly the same weak activity using protocol A (anti-infectivity) and protocol B (anti-replication) (46.66 ± 3.85 and 40.94 ± 5.79) vs. (38.19 ± 5.38 and 36.46 ± 4.09), respectively, with no significant difference. However, acyclovir exhibited the maximum activity with protocol B and A (92.69 ± 1.32 and 83.76 ± 5.67, respectively). Thus, the flower extract showed moderate protective activity against HSV1 with no significant difference with acyclovir (65.53 ± 3.24 *vs* 68.44 ± 7.62). The least among the extracts was stem extract (S) showed no antiviral activity against HSV1.

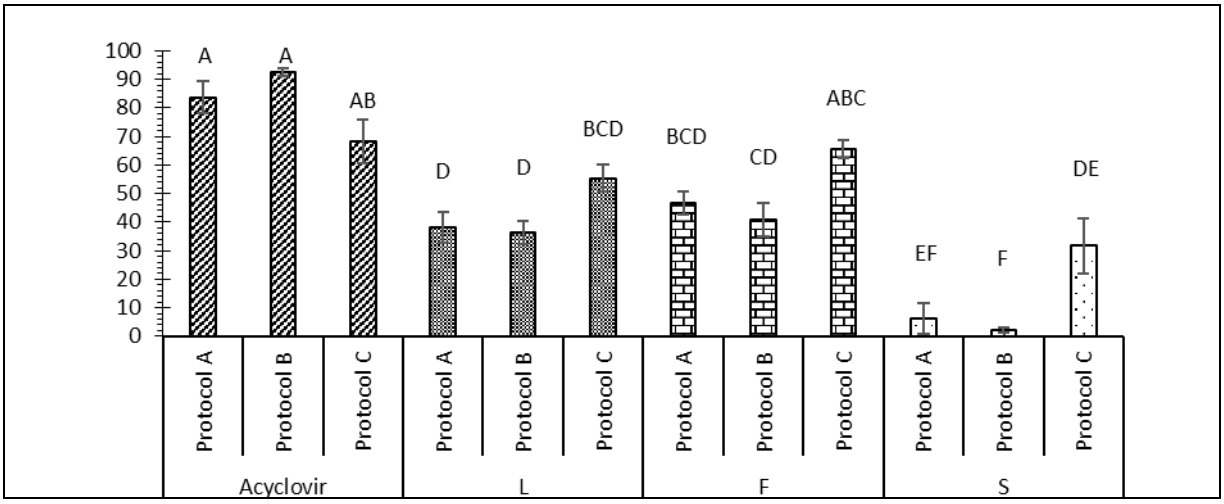

Fig. 5. Antiviral activity of the different extracts against HSV1 vs. acyclovir using different protocols expressed as antiviral activity % values (M±SE)

3.3.4. Comparison of the effect for the extracts against HAV vs. acyclovir with different protocols

On studying the mechanisms for the anti-viral activities of *A. latifolia* extracts against HAV (Table S5 & Figure 6), it was showed that the leaves extract (L) displayed a pronounced antiviral activity with HAV as follow: protocol C (82.99 ± 1.56) > protocol A (73.19 ± 3.1) > protocol B (63.63 ± 1.98), with no significant difference. For treatment with the flowers extract (F), the antiviral activity with HAV was for protocol C (54.16 ± 3.13) > protocol A (40.6 ± 2.61), with no significant difference, while it was the least for protocol B (22.84 ± 1.17), with significant difference. Thus, the leaves extract showed pronounced protective and anti-infectivity antiviral activity against HAV, and moderate anti-replicative activity with no significant difference. The least among the extracts was the stem extract had no antiviral activity against HAV

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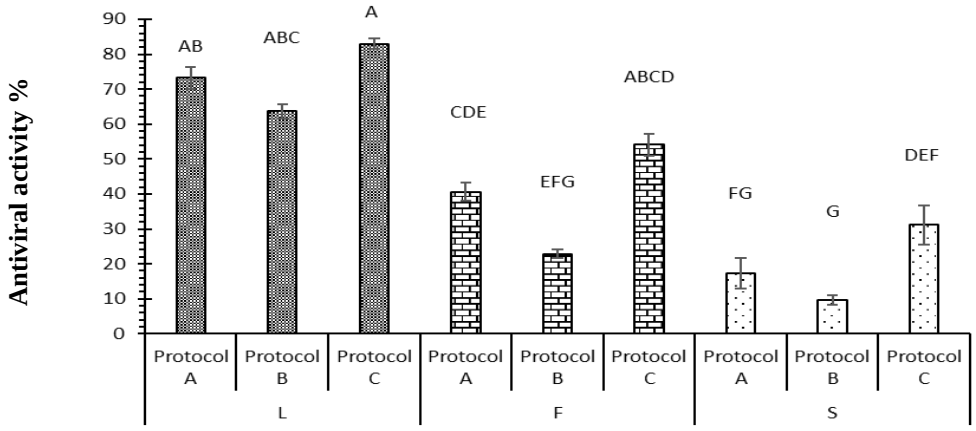

Fig. 6. Antiviral activity of the different extracts against HAV using different protocols expressed as antiviral activity % values (M±SE)

4. Discussion

Figures 1 and 2 of BPC chromatograms revealed how much leaves and flowers *A. latifolia* extracts are crowded with different types of polyphenols. In addition, figures S1–S31 showed the high efficacy of the XIC chromatograms and corresponding monoisotopic masses in MS^1 and MS^2 spectra for the identification of different 31 metabolites examples. Metabolite **65** was detected in leaves and flower extracts (R_t≈10.28 min) with a molecular ion at *m/z* 269.0411 $[M–H]^-$and identified as apigenin by the matching with the conventional library database (Fig. S1). Metabolite **46** (R_t≈ 5.90) was recorded in both extracts and demonstrated a molecular ion peak at *m/z* 431.0963 $[M–H]^-$ with two characteristic fragment ions at *m/z* 341.0663 $[M–H–90]^-$and 311.0551 $[M–H–120]^-$for the loss of $C_3H_6O_3$ and $C_4H_8O_4$ moieties from the *C*-linked glucose. Thus, it was identified as apigenin 8-*C*-glucoside (Fig. S2). Metabolite **57** (R_t=7.12, flowers extract) revealed a molecular ion at *m/z* 431.0984 $[M–H]^-$ and its MS^2 spectrum showed the aglycone ion at *m/z* 269.0466 $[M–H–162]^-$ corresponding to the loss of hexoside group. Therefore, on matching with database it was identified as apigenin 7-*O*-glucoside (Fig. S3). Metabolite **26** exhibited its molecular ion peak at *m/z* 285.0404 $[M–H]^-$and a dimer adduct at 571.0887 $[2M–H]^-$ in its MS^1 spectrum that was in good agreement with the database of luteolin (Fig. S4). Metabolite **44** (R_t=5.431 in both extracts) gave a molecular ion at *m/z* 447.0930 $[M–H]^-$ in its MS^1 spectrum. At high CID, its MS^2 spectrum produced the fragment ions at *m/z* 357.0623 $[M–H–90]^-$, and 327.0501 $[M–H–120]^-$consisting with the loss of $C_3H_6O_3$, and $C_4H_8O_4$ moieties from the *C*-linked glucose. On matching with library database, it was identified as luteolin 6-*C*-glucoside (Fig. S5). Similarly, metabolite **52** (R_t≈ 6.8, leaves and flower extracts) displayed a molecular ion at *m/z* 447.0938 $[M–H]^-$ in its MS^1 spectrum, while it showed an aglycone ion at *m/z* 285.0404 $[M–H–162]^-$ corresponding to the loss of a hexoside group in the MS^2 spectrum. Finally, it was proved to be luteolin 7-*O*-glucoside on matching with the library database (Fig. S6). Among all MS ionization techniques, the multiple charging phenomenon was reported as a characteristic property for ESI-sources like other phenomena (e.g. cluster formation and consecutive oxidations) [29–32]. The real masses "m" of multiple charged ions carrying "n" charges can be calculated from m= (MW-n/n) or (MW+n/n) and the ions proper forms described as $[M–nH]^{n-}$ or $[M+nH]^{n+}$ in case of negative and positive ESI-MS, respectively. In this concern many polyphenols e.g. jacraninoside A [33] showed a double charged ion at *m/z* 623.19894 $[M–2H]^{2-}$, and a molecular ion at 1245.38880 $[M–H]^-$. In particular, also tannins [34,35] like grandinin [36] and punicalagin [37] gave their single and double charged ions at *m/z* 1065/ 532, for a MW of 1066 and 1083/541 for MW of 1066 Da. Accordingly, at high CID fragmentation potential, MS^2 spectrum of **13** (R_t≈1.2leaves and flower extracts) demonstrated a double charged ion at *m/z* 541.0240 $[M–2H]^{-2}$ together with two characteristic fragments at *m/z* 300.9987 [ellagic acid–H]$^-$and 781.0432 [M–H–ellagic acid]$^-$, that were matched with the output data of punicalagin (Fig. S17). Metabolite **21** (R_t≈1.3, leaves and flower extracts) showed a molecular ion at *m/z* 483.0205 $[M–H]^-$ and a characteristic fragment at *m/z* 300.9979 [ellagic acid–H]$^-$ that were in good consistent with a digalloylglucose data base structure (Fig. S18). Metabolite **28** (R_t≈1.8, leaves and flower extracts) revealed a molecular ion at *m/z* 331.0668 $[M–H]^-$, and its MS^2 spectrum showed characteristic fragment ion at *m/z* 169.0138 $[M–H–162]^-$= [gallate]$^-$ corresponding to the loss of deoxyglucose, hence it was identified as monogalloylglucose (Fig. S19). Metabolite **29** exhibited a molecular ion at *m/z* 343.1 $[M–H]^-$, along with a characteristic product ion at *m/z* 169.0135 $[M–H–174]^-$, which were in good agreement with 3,4,3'-tri-*O*-methylellagic acid (Fig. S20). Metabolite **3** (R_t = 1.082) afforded a molecular ion at *m/z* 300.9993 $[M–H]^-$ that was matched with the database of ellagic acid (Fig. S21). Metabolite **11** (R_t=1.22, leaves and flower extracts) showed typical molecular and dimeric adduct ions at *m/z* 191.055 $[M–H]^-$ and 383.1202 $[2M–H]^-$ that was in complete consistency with the database of quinic acid (Fig. S28). Other examples for the identification were given in the supplementary file based on the corresponding XIC and MS spectra.

Taken together, LC/MS analysis of the leaves and flowers *A. latifolia* extracts proved that they are rich in phenolic acids, flavonoids, and tannins, which may explain their significant antiviral activity. Seven types of flavonoids were identified in leaves and flowers extracts, namely, flavones, flavonols, flavanonols, isoflavones, flavanones, chalcone and flavan 3-ol including 16 aglycones and 36 of their corresponding *O*- and/or *C*-glycosides. This is the first time for the identification of 12 glycosylflavones from both leaves and flowers methanolic extracts of *A. latifolia*, except for acacetin 7-*O*-rutinosideand acacetin 7-*O*-neohesperidoside that were identified only from the methanolic leaves extract and apigenin 7-*O*-glucosideidentified from the methanolic flowers extract. Those glycosides were identified as acacetin 7-*O*-rutinoside, acacetin 7-*O*-neohesperidoside, luteolin 7,3'-di-*O*-glucoside, luteolin 7-*O*-glucoside,

luteolin 6-*C*-glucoside, luteolin 8-*C*-glucoside, apigenin 7-*O*-glucoside, apigenin 8-*C*-glucoside, rhoifolin, apigenin 6-*C*-glucoside 7-*O*-glucoside, vitexin 2"-O-rhamnoside and baicalein 7-*O*-glucuronide alongside their aglycones i.e. acacetin, apigenin and luteolin. Also 12 glycosylflavonols were currently reported for the first time in both investigated extracts except for quercetin 4'-*O*-glucoside and kaempferol 3-*O*-rutinoside 7-*O*-rhamnoside, which were identified from the leaves extract only and gossypin identified from the methanolic flowers extract. Their structures were identified as kaempferol 3-*O*-α-L-arabinoside, kaempferol 3-*O*-α-L-rhamnoside, kaempferol 3-*O*-glucoronoide, kaempferol 3-*O*-rutinoside 7-*O*-rhamnoside, quercetin 3-*O*-L-rhamnoside, quercetin 3-D-xyloside, quercetin 4'-*O*-glucoside, isorhamnetin 3-*O*-glucoside, myricetin 3-*O*-L-rhamnoside (myricitrin), syringetin 3-*O*-galactoside, hyperoside and gossypin together with their aglycones (kaempferol, quercetin, myrecetin), which have been identified before in *A. latifolia* and 3,5,7-trihydroxy-4'-methoxyflavone and 3,5,3',4'-tetrahydroxy-7-methoxyflavone. In addition to 4 glycosylflavanones were identified, i.e. naringenin 7-*O*-glucoside, isookanin 7-*O*-glucoside and hesperidin from flowers extract, and hesperetin 7-*O*-neohesperidoside together with 3',4',5,7-tetrahydroxyflavanone and naringenin aglycone from both extracts. Furthermore, the flavanonol taxifolin was isolated from the flowers extract, and the two glycosylisoflavones daidzein 8-*C*-glucoside and formononetin were isolated from both leaves and flowers extracts for the first time in this plant. Moreover, one chalcone i.e. okanin 4'-*O*-glucoside was identified from the leaves extract and one flavan 3-ol dimer i.e. procyanidin B2 identified from both extracts.

For a small fraction of today's known viral diseases, there are vaccines that can be successfully applied. Beside the limited number of medicaments used, in most cases, their use is accompanied by the appearance of side effects or the formation of resistant viral mutants, making therapy ineffective. Therefore, turning to nature to find effective therapies is a good solution to this problem. Thus, the need to discover novel antiviral herbal remedies has been mandated [1]. There have been numerous studies on the cytotoxic and antiviral activities of natural products, including flavonoids, tannins, and other phenolic compounds. This study displayed the optimization of UPLC-MS/MS tool for rapid identification of the phytoconstituents in *A. latifolia* leaves and flowers extracts. In addition, it described the evaluation of the cytotoxic activity for the leaves, flowers, and stem methanol extracts against four cancer cell lines (HepG-2, MCF-7, Caco-2 and Panc-1) and their antiviral mechanisms against HAV and HSV-1 viruses. We evaluated the antiviral activity and the mode of action of the plant extracts at different viral replication stages by adding the extract to an infected cell culture, incubating the plant extract with the cell culture before inoculation with the virus suspension or incubating the plant extract with the virus before infection. This was designed for each extract to measure the anti-replicative, protective, and anti-infective activity, respectively, where each experiment was repeated three times for consistency of the results. All the antiviral tests were performed using the MNTCs of each extract vs. acyclovir as a reference drug that was used as a specific positive control for HSV-1. It was worth mentioning that the investigated extracts exhibited a broad cytotoxic activity range against the examined cancer cell lines. Such variation in cytotoxicity from one cell to another can be explained due to the differences in their origin, morphology, and genomes, resulting in susceptibility difference to chemotherapeutic agents. Currently, LC/MS analysis has tentatively identified variety of bioactive compounds, including flavonoids, phenolic acids, and tannins that were reported to possess strong anticancer activity by different mechanisms including free radical sequestration, electron donation, metal ion chelating, and gene expression regulation [38]. Flavonoids are reported to inhibit many different protein kinases leading to inhibition of signal transduction for cell proliferation [39]. The antitumor mechanism of luteolin was associated with the protection against carcinogenic stimuli, inhibition of tumor cell proliferation and induction of apoptosis [40]. Thus, the pronounced cytotoxicity of the examined extracts can be directly correlated to their high content of different polyphenolic types. It is displayed that the leaves extract showed moderate activity and high SI against Caco-2 cancer cell line with significant difference vs. doxorubicin. Simultaneously, all extracts showed nearly weak to poor cytotoxic activity against HepG-2 with no significant differences. Furthermore, the flowers extract demonstrated the weak activity against MCF-7 cancer cell line, while the high IC_{50} values described the high resistance of Panc-1 towards all examined.

The antiviral activity of pure flavonoids or flavonoid-containing plant extracts has been extensively studied [41,42]. A large body of evidence suggests the antiviral activity of flavonoids against many viruses e.g., human cytomegalovirus [43]; HSV-1 and HSV-2 [41]; influenza virus, respiratory syncytial virus, and adenovirus [44]; coronavirus, parainfluenza virus, Coxsackie virus [45], and rotavirus [46]. The major flavonoids (quercetin, hesperidin and luteolin) were tested for their anti-HSV effect and found to directly inactivate HSV [47]. This study identified several tannins for the first time in *A. latifolia,* namely galloylhexahydroxy-

diphenoylglucose, gallagalloylglucose (punicalin), hexahydroxydiphenoylglucose, punicalagin, hexahydroxydiphenoylvalanoyl-glucose, trigalloyl-glucose, di-galloylglucose and mono-galloylglucose. Tannins exhibited antiviral activity in different stages of viral replication, including the extracellular virions themselves, their attachment to the cell, their penetration into the cell and the replication process in the host cell; additionally, the assembling of new viral particles, transport proteins, polysaccharides, and viral enzymes. In almost all of the above-mentioned stages, the tannin activity is due to their ability to bind permanently to the proteins of the capsid, either to specific viral enzymes required for viral replication or to newly synthesize viral proteins involved in the composition of the new viral particles [45,48]. Early research showed that the high molecular weight tannins inhibited both influenza A virus (IAV) receptor binding and neuraminidase activity. However, those tannins with low molecular weight inhibited neuraminidase but not hem- agglutination [49]. Ellagitannins isolated from *Tuberaria lignosa* inhibited HIV virus entry into MT-2 cells [50]. There is evidence that ellagitannins suppressed HIV replication by inhibiting reverse transcriptase [51]. Also, it was found that the hydrolysable tannins showed a great inhibitory action against HSV-1 by targeting the virus glycoproteins and finally reducing cellular attachment and host cell entrance [52]. Putranjivain A isolated from *Euphorbia jolkini* inhibits the entry of the virus and the late stages of HSV-2 replication, *in vitro* [53]. Accordingly, it can be concluded that the high content of ellagitannins that were identified in the leaves and flowers extracts, with other constitutive phenolic types, played an essential role in their strong antiviral effects against tested HAV and HSV-1 viruses. The current phytochemical analysis of the *A. latifolia* leaves and flowers extracts showed that they were rich also in phenolic acids such as gallic, chlorogenic, 3,4-dihydroxybenzoic (protocatechuic), caffeic and salicylic acids. As consistence with our findings [54], reported that the aqueous extract of *Plantago major*, which is used for treating viral hepatitis in Chinese traditional medicine, showed strong antiviral activity that was mainly attributed to its rich phenolic content and caffeic acid. Additionally, gallic, chlorogenic, quinic, and caffeic acids have been evaluated for their antiviral activity and showed high activity against HSV1, HSV2, and influenza virus [47]. This study proved that the flowers extract showed nearly promising protective antiviral activity against HSV-1 with no significant difference with acyclovir (IC_{50}= 65.53 ± 3.24 *vs* 68.44 ± 7.62 μg/ml, respectively), while the leaves extract showed moderate protective activity (55.42 ± 4.86 μg/ml) with no significant difference, but the stem extract showed no activity. Moreover, the leaves extract showed pronounced protective and anti-infectivity antiviral activity against HAV with (IC_{50} 82.99 ± 1.56 and 73.19 ± 3.1 μg/ml, respectively). However, the flowers extract showed moderate protective activity (IC_{50}= 54.16 ± 3.13 μg/ml) against HAV, while the stem extract did not show any activity.

The findings provide experimental evidence that the leaves extract inhibited the two viruses in the order of HAV >HSV-1 for protocol A (anti-infectivity) and C (protective), furthermore, HSV-1>HAV for protocol B (anti-replicative) at maximum concentration of 500μg/ml. Moreover, it showed higher antiviral activity than acyclovir against HAV through protocol A and C. Acyclovir, as a positive control, is one of the approved drugs for first line in treatment of HSV infections acts by inhibition of viral DNA polymerase and DNA synthesis through targeting of viral thymidine kinase and thus inhibiting viral replication [55]. The flowers extract, at maximum concentration of 500 μg/ml, inhibited the two viruses in the order of HSV-1 >HAV for protocol A and C. This activity was related to the presence of flavonoids as the predominant constituents and the richness of the plant in other polyphenolic types e.g., phenolic acids and tannins.

5. Conclusions

LC/MS tool showed, in high efficacy, that both leaves and flower *A. latifolia* extracts are rich in phenolic compounds, particularly flavonoids, phenolic acids and tannins. This would be logic explanation for their significant cytotoxic and antiviral effects measured. This study showed that the plant extracts exhibited high variation of the cytotoxic effect against tested cancer cell lines. The moderate activity was only detected for the leaf extract against Caco-2 cancer cell line. However, the high IC_{50} values described the high resistance of different cell lines towards all examined extracts. Furthermore, this study is considered to be the first report for the evaluation of the antiviral activity for *A. latifolia* species. It was proved that the leaves extract showed the highest protective antiviral activity against HAV, while the highest protective antiviral activity against HSV-1 was recorded by the flowers extract. In addition, anti-infectivity and anti-replicative activity were exerted by the leaves extract against HAV virus. Further biological research is recommended for the interpretation of the antiviral mechanisms and quantitative structural activity relationship (QSAR) to the constitutive phenolics content and types of *A. latifolia* extracts to clarify if a pre-clinical study is needed.

Conflicts of interest

There are no conflicts to declare.

Supporting Information

Additional Supporting Information may be found in the online version of this article: Supplementary Material. Output data of LC/MS analysis of the plant extracts, i.e. XIC and MS spectra are presented in the supporting information as 31 figures (S1-S31) and five tables for biological activities results (Tables S1-S5).

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