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Khaya senegalensis: phytochemical characterization using LC/HRESI-MS/MS and in vitro antiviral efficacy of extracts and two major metabolites

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Abstract

A growing number of individuals are being diagnosed with viral infections each year, and these diseases continue to be a major global source of morbidity and mortality. More potent antivirals are required, especially in light of the rise in antiviral medication resistance. Given that plants are a major source of structurally diverse bioactive chemicals for drug development, it is possible to generate natural products derived from plants with antiviral properties. The objective of this work was to examine the metabolic profile of leaf and stem extracts from *Khaya senegalensis* using UPLC-Q/TOF-MS. Moreover, the MTT assay was employed to assess the antiviral efficacy of *K. senegalensis* fruit (KF), stem (KS), and leaf (KL) extracts. As well, the antiviral effects were studied for the two major metabolites, *i.e.* quercitrin (1) and rutin (2), against Herpes simplex type I (HSV-1), hepatitis A (HAV), and coxsackie B4 (CoxB4). The antiviral activity of *K. senegalensis* is being examined for the first time in this study. Forty nine distinct metabolites were identified by UPLC-Q/TOF-MS. The phenolic kinds of these substances were divided into four groups: flavonoids (36), coumarins (4), phenolic acids (8), and organic acids (2). The main metabolites in the leaf and stem were rutin and quercitrin. Furthermore, metabolite 1 demonstrated greater antiviral activity with an IC_{50} Mean $\pm$ SD 33.38 ± 8.63 μ g/mL against HSV1. KS extract and metabolite 1 demonstrated the strongest antiviral activity against HAV (59.28 and 60.32 μ g/mL), which are almost equipotent to acyclovir (55.92 μ g/mL). Additionally, the greatest antiviral activity against CoxB4 was demonstrated by metabolites 1 and 2 (19.08, 17.52 μ g/mL). Following the computation of the Selectivity index (SI), it was discovered that metabolite 1 had a very high SI (14.34, 25.09) against HSV1 and CoxB4, but metabolite 2 had a high SI (23.97) against CoxB4.

Keywords *Khaya senegalensis*, Antiviral, CoxB4, HSV-1, HAV

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Introduction

Pathogenic microorganisms, including bacteria, viruses, fungi, and parasites, are the source of a wide range of illnesses and disorders known as infectious diseases that directly affect human health [1]. A major economic and health burden is caused by viral infections worldwide [2]. A virus's capacity for harm is indicated by its growing population, which shows its biological diversity and quick adaptation.

Viruses harm the host by replicating and destroying the structure and functionality of the host cell. They also change quickly in order to adjust to the internal environment of the host. Numerous virally induced illnesses are highly contagious, fatal, and challenging to treat [3].

Vaccinations are seen to be the most effective way to avoid viral illnesses. However, the creation of vaccines necessitates exacting, time-consuming procedures. Furthermore, antiviral medications must be used in addition to vaccines because immunization rates and outcome data are not outstanding in older populations [4]. There are very few antiviral medications that have been created, and they have serious drawbacks: Expensive, ineffective, and resistant to viral variations. Chemical compounds that originate naturally are known as natural metabolites. Their biological functions are diverse [5]. Numerous naturally occurring metabolites, including glycans, organic acids, flavonoids, quinones, alkaloids, and terpenoids, have generally a wide range of antiviral properties. According to Newman et al. [6], 63.1% of all small-molecule medicines created in the last 28 years depended on natural constituents [6].

The herpes simplex virus (HSV) is a serious human pathogen that replicates in cells at the site of infection [7]. The type 1 virus (HSV-1) is an enveloped virus that reproduces nuclearily and is usually acquired via direct contact with septic lesions or bodily fluids like saliva [8]. It can result in painful blisters or ulcers around the lips, in the eyes, on the oral cavity's mucous membrane, and in the genitalia in addition to asymptomatic infections [9]. So it is important to put a lot of effort into creating new anti-HSV drugs.

Major human pathogen Cocksackie B4 virus (CoxB4) typically produces mild, self-resolving symptoms such as fever, rash, and upper respiratory infection. In the pancreas, human enterovirus replicates quickly [10]. Numerous illnesses, including septic meningitis, myocarditis, hepatitis, gastroenteritis, pneumonia, and unexpected infant mortality, can be brought on by severe pancreatitis [11]. HSV-1 and CoxB4 infections are treated with medications called acyclovir and ganciclovir. Nevertheless, acyclovir resistance in viruses has become an issue

for treating viral illnesses, particularly in people with impaired immune systems [12].

Global public health continues to face challenges due to HAV-caused acute hepatitis [13]. HAV and enteric viruses can live for up to two months on environmental surfaces because they are extremely resistant to environmental factors such low pH and ambient temperature [14]. In food safety, the use of plant extracts as a substitute for chemical agents against enteric viruses has been proposed [15].

The mahogany family, *i.e.* Meliaceae, is a large family of flowering plants that is located throughout the pan-tropical region and is now spreading into the temperate zone. It comprises 49–50 genera and 620 species [16]. There are six recognized species in the genus *Khaya*, which is a member of the Meliaceae family. These species are highly significant both commercially and economically [17]. While the fruits and flowers of the *Khaya* species are identical, their leaflets differ most noticeably [18]. *Khaya senegalensis* (Desr.), popularly known as African mahogany, is one of these species. It is a sizable deciduous tree that is indigenous to Senegal and Uganda in the sub-Saharan savannah [19]. Its tree is easily identified by its 15–30 m height and its circular, dark-colored crown with glossy leaves all around. It produces fruit-like, woody capsules. It is frequently used to treat a variety of illnesses in Nigeria and other West African countries [19]. The bark stem of *K. senegalensis* is used to treat jaundice, hookworm, dermatitis and malaria [20]. Studies demonstrated that the stem bark from *K. senegalensis* has several properties, including antioxidant, anti-inflammatory, antimicrobial, hepatoprotective, anti-parasitic, anti-cancer, and hypoglycemic activity [20–25]. In addition to the anti-diarrheal qualities known for leaf aqueous extract, the leaves and seeds are used to treat fevers and headaches [26]. Moreover, *K. senegalensis* flowers showed antifungal activity [27]. Such plant potential for therapeutic use depends on its chemical profile, as tests on various plant sections have shown the presence of tannins, steroids, and other substances. Many studies, on various parts of the tested plant, revealed the presence of significant phytoconstituents from several classes, *e.g.* alkaloids, anthocyanins, anthraquinones, flavonoids, glycosides, hydroalcoholic, limonoids, oxalates, phlobatanins phytates, saponins, steroids, tannins and terpenoids [28–31].

In the current research, UPLC-Q/TOF-MS analysis was used to annotate the secondary metabolites of the leaf and stem plant extracts. Moreover, the antiviral activity was assessed by standard antiviral assays for three parts of *K. senegalensis* and two significant metabolites against CoxB4, HSV-1, and HAV viruses in comparison with the acyclovir, the standard antiviral medication.

Materials and methods

Plant material

Leaf, stem and fruit samples of *K. senegalensis* (KL, KS and KF, respectively) were collected in June 2019 at the Giza Zoo in Egypt, authenticated and dried as mentioned by Yassin et al. [32].

Extraction and isolation

Air-dried powder was extracted separately using 1.5 kg of leaves, 40 g of stem and 40 g of fruit using 70% aqueous methanol at 70 °C. This resulted in 400, 15, 12 g dry extracts of leaves, stem and fruits, respectively. A rotatory evaporator (Buchi Co., Switzerland) was used to dry each extract independently in vacuo at 60 °C. The extracts were defatted with hot petroleum ether under reflux at 40 °C after being individually dried in vacuo using a rotatory evaporator (Buchi Co., Switzerland) at 60 °C. Following that, sugar and inorganic salts were eliminated using hot pure methanol at 70 °C, leaving 300, 10, 9 g of dry, methanol-soluble leaves, stem and fruit parts, respectively. They are kept for biological research purposes. Separately, each of leaf and stem extracts were investigated on UPLC-MS/MS in negative ion acquisition modes, resulting in a tentative identification of several metabolites in each extract.

However, the leaf MeOH-soluble part (suspended in H₂O) was applied on a polyamide 6S (Reidel De Haen AG, Seeize, Hannover) column chromatography (CC). The elution was performed starting with H₂O, followed by a step-gradient of H₂O/MeOH up to 100% MeOH. Similar fractions are gathered into six groups (I–VI) using a Co-PC on Whatmann paper sheets (Whatmann Ltd., Maidstone, Kent, England), that are detected with UV light (254/365 nm, Desaga Heidelberg, Germany), and spray reagents like ammonia vapors and FeCl₃. Two pure metabolites, *i.e.* **1** (3 g) and **2** (2 g), were obtained by purifying the primary sub-fraction, which has two major spots, using a series of column chromatographic techniques with different solvent systems.

The two metabolites were analyzed with ¹H and ¹³C NMR spectroscopy at the NMR unit, Faculty of Science, King Abd El-Aziz University, Saudi Arabia, using a Bruker High-Performance Digital FT-NMR Spectrometer Advance III (850 and 213 MHz for ¹H and ¹³C, respectively).

UPLC/ESI-qTOF-HRMS/MS analysis

At the Children's Cancer Hospital (CCHE 57357), Cairo, Egypt, the proteomics and metabolomics team conducted the measurements. The extract was prepared, instrumented, and acquired for data processing on an HPLC standard interface (Exion LC, AB SCIEX, Concord, Canada) using a quadrupole time-of-flight (QTOF)

mass spectrometer (Triple TOF 5600 +, SCIEX) as described in Elkousy et al. [33]. Full details about the optimization conditions, *i.e.* preparation of the sample (final concentration), injected volume, type of pre- and main columns, flow rate, the eluents composition and their time gradient program and other details of LC/MS methodology were reported in the previous reference [33]. Using Respect negative (1573 records) reference databases in conjunction with the open-source software tools Analyst TF/1.7.1 and MS-DIAL/4.8, the determination and interpretation of MS data were completed.

Antiviral assay

Preparation of virus stock

HAV, HSV-1, and CoxB4 viral strains were graciously donated by the Laboratory of Virology, Microbiology Department, Faculty of Medicine (for Girls), Al-Azhar University, Cairo, Egypt. Each virus was propagated in Vero cells by infecting a confluent Vero cell monolayer as reported by El-Shiekh et al. [34].

Determination of MNTC

Maximum nontoxic concentration (MNTC) was ascertained for each extract by dilution of its sample ten times in MEM (minimal essential medium) containing FCS (Foetal Calf Serum), ranging from 100 to a 10⁻⁶ mg/mL dilution. Next, confluent Vero cells (ATCC CCL-81) were treated in four wells of a 96-well plate (Falcon; Corning, USA) with 0.2 mL of each dilution, with two rows left as the control. Distilled H₂O was used to dissolve acyclovir (Sigma-Aldrich, St. Louis, MO, USA) and MNTC was also assessed.

At 37 °C, the plates were incubated, and then an inverted microscope was used to observe the cells every day in order to ascertain the lowest concentration necessary to cause changes in the morphology of the cells. Even after seven days, there were no morphological changes between control Vero cells and the treated Vero cells at the MNTC. The MTT colorimetric assay (Bio Basic Inc., Canada) was carried out in accordance with the earlier instructions [35].

Determination of antiviral activity

Antiviral activity was assessed using MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) (Sigma Aldrich, USA). The experiment was performed according to Hansen et al. [36] with slight modification. Briefly 10,000 Vero cells (ATCC CCL-81) were plated in 96-well plate containing 200 µL supplemented DMEM media (Thermo Fisher Scientific, USA) with 3 wells left empty for blank controls. The cells were incubated overnight at 37 °C with 5% CO₂ to allow them to attach to the wells and develop a complete monolayer

sheet. An equal volume (1:1 v/v) of a non-toxic dilution of the tested sample and the virus suspension was incubated for 1 h. Then 100 μ L from the viral/sample suspension was added to each well, and the plate was placed on a shaking table at 150 rpm for 5 min. The plate was incubated at 37 °C with 5% CO₂ for 1–5 days to allow the virus effect. 2 ml or more of MTT solution per 96-well plate at 5 mg/ml in PBS was prepared. Then 20 μ L of MTT solution was added to each well, and the plate was placed on a shaking table at 150 rpm for 5 min to thoroughly mix the MTT into the media. The plate was then incubated at 37 °C with 5% CO₂ for 1–5 h to allow the MTT to be metabolized. The media was carefully dumped off, and the plate was dried on paper towels to remove any residue if necessary. Formazan, the MTT metabolic product, was resuspended in 200 μ L DMSO. The plate was placed on a shaking table at 150 rpm for 5 min to thoroughly mix the formazan into the solvent. The optical density was read at 560 nm using a multiwall microplate reader (Synergy HT, BioTek, France), and the background at 620 nm was subtracted. Optical density was directly correlated with the quantity of cells. The experiment was performed in triplicate, and the result was expressed as the mean $\pm$ SD.

Calculation of the selectivity index

Selectivity indices (SI) were computed by dividing the half maximum inhibitory concentration (CC₅₀) of normal Vero cells (ATCC CCL-81) by the specific IC₅₀ of virally infected cells [37].

Statistical analysis

Data of the cytotoxicity against Vero cells and antiviral activity of leaf, fruit, stem extracts, metabolite **1** and **2** against three viruses (HAV, CoxB4 and HSV1) have been analyzed using Minitab 19 and GraphPad prism software (GraphPad version 8.0.2. <http://www.graphpad.com/prism/prism.htm>). Data was cleansed before running any statistical analyses. Missing data and mistyping errors have been checked. Descriptive statistics including mean and standard deviation have been calculated from three replicates for each level. Inferential statistics have been used to compare the results of different groups. All variables' parametric assumptions have been tested. Different comparisons were done using one-way and analysis of variance (ANOVA) under fit General linear model. The *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.

Table 1 ¹H and ¹³C NMR spectral data (850 and 213 MHz, DMSO-*d*₆) of compounds **1** and **2**

NO	1		2	
	¹ H	¹³ C	¹ H	¹³ C
2	–	157.7	–	157.3
3	–	134.6	–	133.8
4	–	178.2	–	177.8
5	12.66 br s	161.7	12.59 br s	161.6
6	6.20 br s	99.2	6.20 d (1.8)	99.1
7	–	165.0	–	164.6
8	6.39 br s	94.1	6.39 d (1.8)	94.0
9	–	156.9	–	156.9
10	–	104.4	–	104.4
1'	–	121.2	–	121.6
2'	7.31 d (1.9)	116.1	7.54 br s	116.7
3'	–	145.7	–	145.1
4'	–	148.9	–	148.4
5'	6.87 d (8.3)	115.9	6.85 d (8.8)	115.6
6'	7.26 d (8.3)	121.6	7.55 dd, (hidden by H-2')	122.1
1"	5.26 br s	102.3	5.33 d (7.5)	102.1
2"	3.98 br d (1.2)	70.8	–	74.4
3"	3.52 (dd (9.0, 3.1)	71.0	–	76.9
4"	3.15 t-like (9.4)	71.6	3.08 t-like (9.2)	70.5
5"	3.22 m	70.5	–	76.2
6"	0.82 d (5.7)	17.9	3.70 br d (10.4) 6"a	68.5
1'''	–	–	4.39 br s	101.1
2'''	–	–	–	70.7
3'''	–	–	–	71.1
4'''	–	–	3.08 t-like (9.2)	72.4
5'''	–	–	–	70.2
6'''	–	–	0.99 d (6.1)	17.9

J-values are given in parentheses in Hz

Results

Identification of the isolated major compounds

The two major flavonoids, isolated from *K. senegalensis* leaf extract, were identified as two 3-*O*-glycosylquercetin depending on the chromatographic characteristics. Their structures were established as quercetin 3-*O*- α -L-¹C₄-rhamnopyranoside (quercitrin, **1**) and quercetin 3-*O*- β -D-rutinoside (rutin, **2**) by HRESI-MS, ¹H, and ¹³C NMR analyses alongside comparison with the corresponding published data of structurally related compounds (Table 1, Fig. 1).

UPLC/ESI-qTOF-HRMS/MS analysis

Base peak chromatograms (BPC) of *K. senegalensis* leaf and stem extracts revealed the presence of a large number of various polyphenolic-type metabolites (Figs. 2 and 3). Table 2 summarizes the retention times (*R*_t),

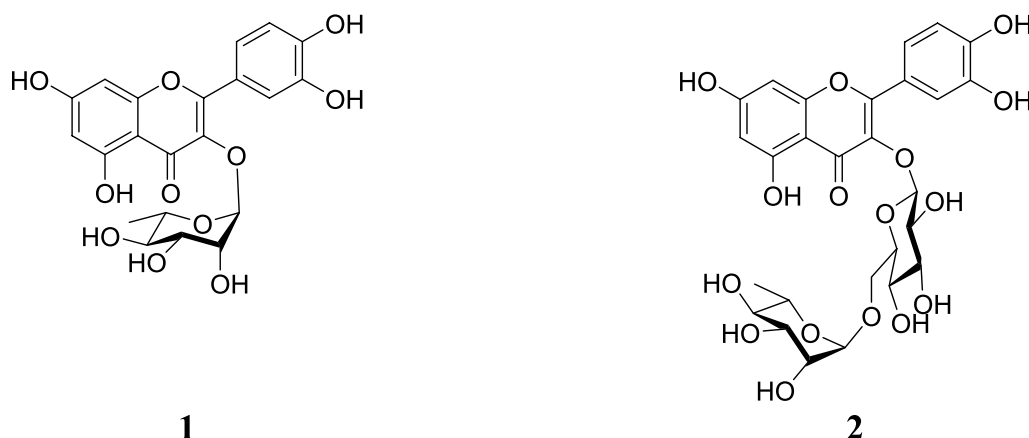


Fig. 1 Chemical structures of compounds 1 and 2

observed monoisotopic m/z masses of molecular, some selective fragments and/or adduct ions together with molecular formulas (MFs) and errors (ppm) values for the characterized metabolites. Overall, forty nine different metabolites were detected in both *K. senegalensis* extracts (Table 2). Their identification is reinforced by the verification of the corresponding extract ion chromatograms (XIC), molecular ions and tandem masses of selective fragments in MS^1 and MS^2 (two-stage fragmentation spectra). Further confirmation was achieved by a full matching with the corresponding convenient library database (MoNA-Mass Bank of North America) and reported data for each metabolite identified. These compounds were sorted according to their chemical structures into flavonoids (35), coumarins (4), phenolic acids (8), and organic acids (2),

Antiviral activity

Determination of MNTC

Vero cells were examined for any physical evidence of toxicity, such as partial or total monolayer loss, rounding, shrinkage, or cell granulation (cytopathic effect). The MNTC concentration of each sample was determined as the concentration of a sample that had no toxicity on viable cell number (100% viability). As indicated in Table 3, the least toxic tested samples were KL and metabolite 1, with MNTC values of 250 $\mu\text{g}/\text{mL}$, followed by KS, KF, and metabolite 2, which had MNTC values of 125 $\mu\text{g}/\text{mL}$. Acyclovir has MNTC of 125 $\mu\text{g}/\text{mL}$.

Cytotoxicity (CC_{50})

To ascertain cytotoxic concentration of each sample, the cytotoxicity assay was run. The tested sample concentration that will kill 50% of the cells is known as the CC_{50} . Descriptive statistics of the mean CC_{50} of the tested samples were performed by the one-way ANOVA followed

by Tukey's Post hoc test to determine the significant difference between each group. The mean of $CC_{50} \pm SD$ for each extract was calculated and presented in Table 3. KL, metabolite 1 and metabolite 2 showed the least cytotoxic effect with mean $CC_{50} \pm SD$ 606.81 $\pm$ 9.24 $\mu\text{g}/\text{mL}$, 478.81 $\pm$ 7.20 $\mu\text{g}/\text{mL}$ and 420.09 $\pm$ 4.95 $\mu\text{g}/\text{mL}$, respectively. They showed a higher safety pattern than the standard antiviral drug acyclovir that gave $CC_{50} \pm SD$ of 356.63 $\pm$ 4.61 $\mu\text{g}/\text{mL}$. All Groups showed highly significant differences between each other (p -value < 0.0001). Groups that share the same letters are non-significantly different, while different letters express significant differences among different groups Fig. 4.

Antiviral activity

Descriptive statistics of the mean IC_{50} of the tested samples against different viruses were performed by the one-way ANOVA followed by Tukey's Post hoc test to determine the significant difference between each group. The ideal antiviral drugs should have antiviral activity at very low concentrations i.e. low IC_{50} values [37].

Antiviral activity of tested samples against HSV-1 The IC_{50} value, as $M \pm SD$, for each sample against HSV1 was calculated and shown in Table 3. Metabolite 1 demonstrated the highest antiviral activity with an IC_{50} of 33.38 $\pm$ 8.63 $\mu\text{g}/\text{mL}$, followed by metabolite 2 and KS extract, which showed moderate activity against HSV1 with IC_{50} values of 56.34 $\pm$ 7.12 and 65.72 $\pm$ 4.9 $\mu\text{g}/\text{mL}$, respectively. KL and KF displayed the lowest activity when compared to acyclovir; their mean $IC_{50} \pm SD$ against HSV1 was 86.99 $\pm$ 7.18 and 80.97 $\pm$ 3.24 $\mu\text{g}/\text{mL}$, respectively.

All groups displayed extremely significant differences (p -values < 0.0001 , 0.001, and 0.01), with the exception of some groups that exhibited non-significant differences; 2 versus KS, KF versus KS, KF versus KL, KF versus

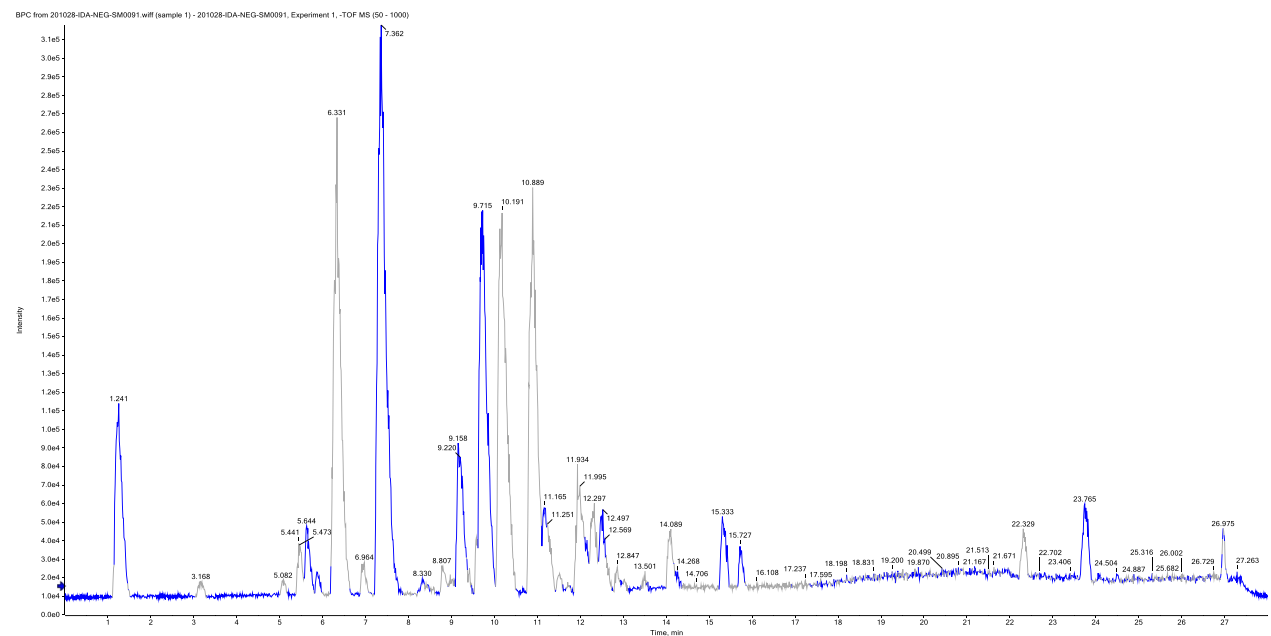


Fig. 2 BPC of *K. senegalensis* leaf extract (Negative ionization mode); Peak numbers agree with Table 2

acyclovir, and KL versus acyclovir). Groups that share the same letters are non-significantly different while different letters express significant differences among different groups Fig. 5.

Antiviral activity of tested samples against HAV Table 3 displayed the individual activity of every sample against HAV in the form Mean $\pm$ SD for IC₅₀. The antiviral potency of KS extract and metabolite 1 was found to be the high-

est one, with mean IC₅₀ $\pm$ SD values of 59.28 $\pm$ 2.95 and 60.32 $\pm$ 0.865 μ g/mL, respectively, that are almost equal to those of acyclovir (55.92 $\pm$ 14.63 μ g/mL). Except for some groups (metabolite 2 vs. acyclovir, KF vs. KS, KF vs. KL, and KF vs. acyclovir) the majority of groups displayed non-significant differences from one another. Groups that share the same letters are non-significantly different while different letters express significant differences among different groups Fig. 6.

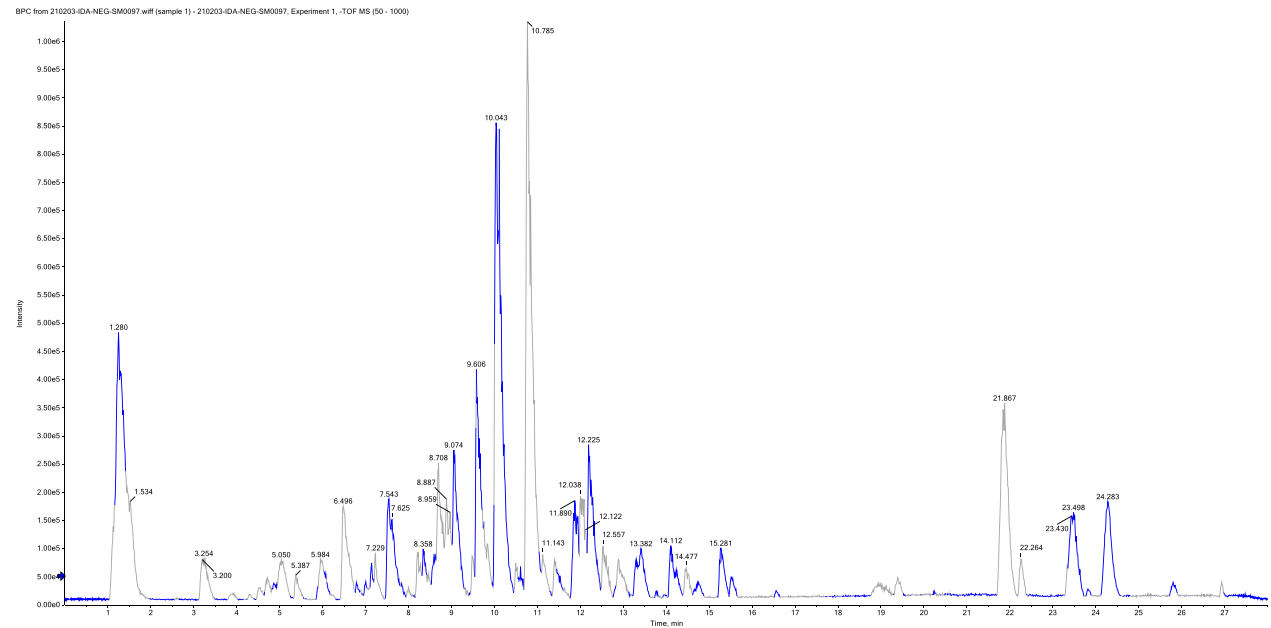


Fig. 3 BPC of *K. senegalensis* stem extract (Negative ionization mode); Peak numbers agree with Table 2

Table 2 Phenolic compounds identified in *K. senegalensis* leaf and stem using UPLC/ESI-qTOF-HRMS/MS analysis (Negative ionization mode)

No	RT	[M-H] ⁻ (m/z)		Formula	Area		Error		Fragmentation		Name
		Leaf	stem		Leaf	Stem	Leaf	Stem	Leaf	Stem	
1	4.685	4.301		417.1393	$C_{20}H_{18}O_{10}$	21730.4	24558.9	1.2	0.6	197.0535, 248.95038, 286.94247, 354.91254, 417.12863, 417.23535	Kaempferol 3-O- α -L-arabinoside
2	5.449	5.375		431.1907	$C_{21}H_{20}O_{10}$	20461.14	2090042.5	2.2	-0.7	227.03401, 255.0296, 257.06116, 284.03085, 285.03375, 294.9028, 347.06207, 362.8739, 364.26068, 430.86484, 431.09647	Kaempferol 3-O- α -L-rhamnoside
3	6.916	-		593.1535	$C_{30}H_{26}O_{13}$	148354.4	-	-2.3	-	256.02975, 267.04202, 284.03326, 284.08801, 285.04333, 529.18469, 533.19794, 547.18365, 591.2431, 593.14014	Kaempferol 3-O-(6- <i>p</i> -coumaroyl)-glucoside
4	-	15.88		461.3632	$C_{21}H_{18}O_{12}$	-	24772.8	-	0.4	461.15692, 461.16904, 461.23273	Kaempferol 3-O-glucuronide
5	2.008	2.868		593.1875	$C_{27}H_{30}O_{15}$	16918	144650.4	-3.5	-0.9	456.89294, 509.90958, 575.06415, 593.18079, 593.21863	Kaempferol 7-O-neo-hesperidoside
6	-	10.67		299.0554	$C_{18}H_{12}O_6$	-	28010.3	-	0.3	284.02579, 284.04483, 299.05121	3,5,7-Trihydroxy-4'-methoxy-flavone (Kaempferide)
7	8.231	-		447.2222	$C_{21}H_{20}O_{11}$	21018.9	-	0.3	-	300.01999, 301.0311, 334.90735, 343.07956, 438.13852, 447.08743, 447.21286	Quercetin 7-O-rhamnoside
8	6.658	6.825		463.091	$C_{21}H_{20}O_{12}$	513624.5	671350.8	-5.4	0.1	211.04919, 258.93927, 273.04315, 286.93328, 300.02005, 300.02493, 301.0434, 311.14117, 326.94092, 354.92252, 392.09848, 462.9234, 463.09052	Quercetin 4'-O-glucoside
9	8.57	8.595		433.1509	$C_{20}H_{18}O_{11}$	41491.9	101184.5	-0.3	-1.5	353.20016, 403.15375, 433.14795	Quercetin 3-O-D-xyloside
10	7.363	7.531		447.0947	$C_{21}H_{20}O_{11}$	23148990	4501151	-2.9	2.2	121.03425, 125.02856, 149.02052, 149.03604, 151.00621, 163.00879, 171.03885, 174.6071, 178.99545, 201.05066, 227.02625, 227.04326, 229.04367, 243.02573, 245.03329, 255.01228, 255.02356, 257.04376, 264.46191, 271.01987, 272.02518, 273.03232, 283.01855, 299.07495, 300.02576, 300.06488, 301.03442, 301.89508, 343.0412, 429.10831, 445.6084, 445.64417, 445.67697, 447.08829	Quercitrin
11	6.335	6.630		609.1446	$C_{27}H_{30}O_{16}$	13952193	268710.3	0.4	0.6	108.02499, 149.95956, 151.00247, 164.02161, 178.99704, 201.02029, 226.02264, 243.02975, 245.03291, 254.02957, 255.02768, 257.05014, 265.00607, 271.02179, 272.03174, 273.04126, 283.02051, 297.05569, 300.02774, 301.03397, 302.03207, 341.06613, 343.05118, 447.08173, 463.06927, 563.21216, 591.12616, 607.16882, 608.14008, 609.07031, 609.13654	Rutin
12	10.673	10.430		269.0440	$C_{13}H_{10}O_5$	16763.6	32138.2	2.8	-0.8	183.04961, 268.53735, 269.03979, 269.246	Apigenin
13	-	6.528		431.0956	$C_{21}H_{20}O_{10}$	-	145141	-	4.7	161.04713, 179.05186, 179.10478, 220.04214, 259.0405, 283.06448, 297.02689, 309.03195, 311.05136, 341.05865, 385.17746, 387.24533, 413.0972, 431.06064, 431.09875	Apigenin 8-C-glucoside (Vitexin)
14	4.242	4.263		577.1937	$C_{27}H_{30}O_{14}$	914552.7	164823.8	-1.5	-7.6	139.04366, 277.08368, 369.18011, 397.1597, 401.19736, 413.16873, 425.20544, 429.18393, 439.18896, 457.15631, 489.19254, 495.09186, 499.95865, 517.19434, 529.5163, 531.22382, 533.20795, 545.13763, 545.16071, 549.19427, 575.34613, 577.16321, 577.1971	Rhofolin (Apigenin 7-O-neo-hesperidoside)

Table 2 (continued)

No	RT	[M-H] ⁻ (m/z)		Formula	Area		Error		Fragmentation	Name
		Leaf	stem		Leaf	Stem	Leaf	Stem		
15	-	16.596	283.0995	C ₁₆ H ₁₂ O ₅	-	44768.5	-	3.7	111.04233, 129.05673, 150.02925, 164.0527, 179.03128, 183.08305, 192.04419, 241.08437, 255.10283, 283.0293, 283.09583, 283.22653, 283.26459	Acacetin
16	8.596	-	591.201	C ₂₈ H ₃₂ O ₁₄	30238.4	-	8.3	-	528.89191, 545.20288, 589.27319, 591.16022, 591.198, 591.30444	Acacetin 7-O-rutinoside
17	1.453	1.824	315.0476	C ₁₆ H ₁₂ O ₇	64920.9	78228.6	8.4	-2	300.02988, 315.0419	5,7,4'-Trihydroxy-3'-methoxyflavonol
18	7.095	-	623.1663	C ₂₈ H ₃₂ O ₁₆	42796.6	-	-2.2	-	180.97073, 579.24756, 591.18079, 623.151, 623.16864, 623.24268	Isorhamnetin 3-O-rutinoside
19	8.055	8.014	477.1773	C ₂₂ H ₂₂ O ₁₂	19553.9	41326.8	-1	-0.8	228.92807, 364.90839, 432.87866, 477.16714, 477.17947	Isorhamnetin 3-O-glucoside
20	8.179	8.057	415.1971	C ₂₁ H ₂₀ O ₉	135564	18045.8	-1	0.8	397.11993, 415.19748	Daidzein 8-C-glucoside
21	7.459	-	445.1343	C ₂₁ H ₁₈ O ₁₁	133725.3	-	-1.3	-	125.02068, 301.09082, 343.10376, 383.13699, 399.13351, 445.13483	Baicalin 7-O-glucuronide
22	-	6.516	301.2032	C ₁₆ H ₁₄ O ₆	20029.3	-	-	-0.7	186.93655, 255.24007, 301.19904	Hesperetin
23	1.568	1.122	289.1808	C ₁₅ H ₁₄ O ₆	44140.5	125492.3	-0.4	-3.6	84.99273, 112.98588, 180.97476, 217.01404, 245.10114, 289.1806	(+)-3,5,7,3',4'-Pentahydroxyflavan
24	-	7.016	303.0518	C ₁₅ H ₁₂ O ₇	-	98445.1	-	4.6	123.04258, 125.02138, 149.03276, 151.0014, 152.008, 171.05647, 175.03796, 177.02057, 178.99353, 179.00298, 185.12022, 189.06319, 190.94971, 199.04355, 217.0504, 218.94809, 225.11435, 257.14398, 258.94998, 259.05682, 275.04553, 275.07599, 285.04309, 303.05307, 303.13666, 303.21783	(+ -)-Taxifolin
25	-	5.062	507.1832	C ₂₁ H ₂₀ O ₉	-	112303.4	-	3.3	135.04027, 354.93021, 507.18622	Syringetin 3-O-galactoside
26	-	3.400	463.1458	C ₂₁ H ₂₀ O ₁₂	-	17260.6	-	-0.1	463.14276	Myricitrin
27	5.349	-	449.1115	C ₂₁ H ₂₂ O ₁₁	138405.9	-	2.1	-	135.04456, 151.00386, 241.14423, 255.15604, 287.05453, 324.94733, 381.13052, 403.20825, 449.1087, 449.19254	Isokanin 7-glucoside (Flavanomarein)
28	-	4.213	449.1061	C ₂₁ H ₂₂ O ₁₁	-	92500	-	-1.8	234.01761, 269.05997, 311.06839, 359.08475, 449.10968,	Okanin 4'-O-glucoside
29	1.241	1.205	611.1287	C ₂₈ H ₃₆ O ₁₅	50489.3	25063.3	-1	-5.9	257.03601, 265.09061, 287.07092, 323.05319, 333.07486, 341.09442, 481.01181, 548.99219, 553.17267, 565.15118, 575.13928, 611.05188, 611.12872, 611.19855, 186, 717.5	Neohesperidindihydrochalcone
30	-	6.503	609.1445	C ₂₇ H ₃₁ O ₁₆	-	9045257	-	0.5	151.00101, 163.00117, 178.995, 271.02386, 299.06772, 300.02841, 301.03476, 609.14398	Delphinidin 3-O-(6'-O-α-rhamnopyrano-syl)-β-glucopyrano-side)
31	10.285	10.161	271.0611	C ₁₅ H ₁₂ O ₅	110158.8	53212.1	0.9	3.2	83.01264, 151.00258, 177.02063, 177.0244, 271.04291, 271.05685	Naringenin
32	7.614	-	433.1139	C ₂₁ H ₂₂ O ₁₀	116698.6	-	0.9	-	217.95789, 253.06276, 270.03476, 270.05566, 315.06003, 387.20383, 433.07837, 433.10483, 433.19888	Naringenin 7-O-glucoside
33	4.893	-	435.1259	C ₂₁ H ₂₄ O ₁₀	31568.9	-	6.5	-	273.07373, 367.14508, 435.12595	Phlorizin
34	2.698	1.46	595.2053	C ₂₇ H ₃₂ O ₁₅	32207.9	54826.7	-1.1	0.9	549.17767, 595.19891	Eriodictyol 7-O-neo-hesperidoside

Table 2 (continued)

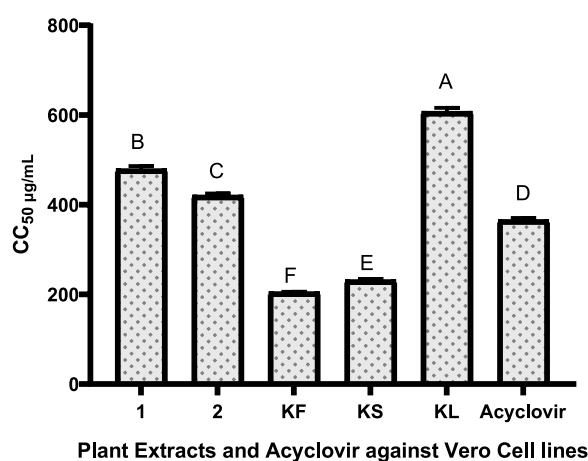
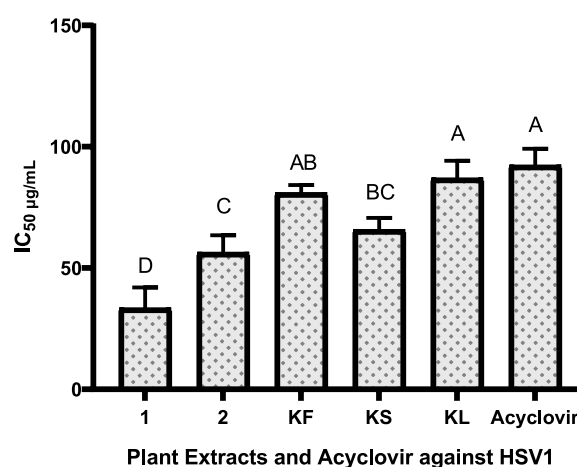
No	RT	[M-H] ⁻ (m/z)		Formula	Area		Error		Fragmentation		Name
		Leaf	stem		Leaf	Stem	Leaf	Stem	Leaf	Stem	
35	-	9.326	287.0557	C ₁₅ H ₁₂ O ₆	-	304383.9	-	0.9	125.02744, 135.04681, 150.52596, 150.99767, 151.00461, 218.94359, 286.12875, 287.0517		5,7,3,4'-Tetrahydroxy-flavanone, Eriodictyol
36	3.019	3.525	339.1675	C ₁₅ H ₁₆ O ₉	10079.4	115124.9	-1.6	0.2	173.07552, 176.02347, 294.91437, 339.0704, 339.09381		Esculetin
37	7.046	-	191.0376	C ₁₀ H ₈ O ₄	39560.9	-	-10.4	-	148.01656, 163.03705, 176.01453, 191.02974		Scopoletin
38	10.710	-	177.0558	C ₉ H ₆ O ₄	373248.8	-	0.7	-	117.02464, 117.03534, 118.03973, 121.03046, 133.02689, 134.04359, 145.02661, 162.03047, 177.02383, 177.05579		Daphnetin
39	-	10.633	177.0557	C ₉ H ₆ O ₄	-	313880.5	-	-0.5	89.0005, 133.0323, 149.0222, 177.02025		6,7-Dihydroxycouma-rin (Esculetin)
40	1.277	-	153.0161	C ₇ H ₆ O ₄	337403.7	-	15.5	-	91.01955, 108.02443, 109.02927, 153.01465		2,5-Dihydroxybenzoic acid
41	-	4.913	153.0202	C ₇ H ₆ O ₄	-	27714.5	-	-1	125.02119, 153.01945		3,4-Dihydroxybenzoic acid
42	1.374	3.375	137.022	C ₇ H ₆ O ₃	81737.9	184368.7	7.5	0	93.03008, 93.0328, 93.03825, 136.9846, 137.01932, 137.02428		P-Hydroxybenzoic acid
43	8.269	-	179.0325	C ₉ H ₈ O ₄	13467	-	10.6	-	179.03412		E-Caffeic acid
44	7.095	7.166	167.0332	C ₈ H ₈ O ₄	801599.2	2057572.9	-0.8	-7.6	152.00136, 167.02797		5-Methoxysalicylic acid
45	-	4.700	137.0239	C ₇ H ₆ O ₃	-	1481539.6	-	1	76.24986, 108.02089, 119.0181, 136.01372, 137.02534		Salicylic acid
46	3.896	3.934	359.1023	C ₁₈ H ₁₆ O ₈	39326.37	890984	-9.3	0.8	59.01382, 119.03741, 197.05084, 197.08455, 222.97284, 232.95122, 239.05836, 251.05302, 299.07388, 299.08609, 359.09821		Rosmarinic acid
47	1.411	1.561	353.0873	C ₁₈ H ₁₈ O ₉	80414.9	81565.2	0.2	5.3	135.03789, 163.00984, 36, 179.02679, 179.03624, 191.05687, 321.07379, 353.08362, 353.09689		Chlorogenic acid
48	1.193	1.222	191.0553	C ₇ H ₁₂ O ₆	2230388	14493942	2.2	0.7	72.99066, 85.02775, 87.00944, 93.03153, 127.04145, 171.03076, 171.03076, 173.0591, 190.44254, 191.06084		D-(-)-Quinic acid
49	-	1.198	173.0482	C ₇ H ₁₀ O ₅	-	290042.9	-	-10.3	73.02953, 85.02943, 93.03423, 137.01994, 155.03484, 173.04358		(-)-Shikimic acid

Table 3 Cytotoxic concentration 50% (CC_{50}), inhibitory concentration 50% (IC_{50}) and selectivity index SI ($SI = CC_{50}/IC_{50}$) of **1**, **2**, KF, KS and KL plant extracts

Extract	Vero cells (ATCC CCL-81) MNTC ($\mu\text{g/mL}$)	* CC_{50} ($\mu\text{g/mL}$)	HSV1 * IC_{50} ($\mu\text{g/mL}$)	SI	HAV * IC_{50} ($\mu\text{g/mL}$)	SI	CoxB4 * IC_{50} ($\mu\text{g/mL}$)	SI
1	250	478.81 $\pm$ 7.20	33.38 $\pm$ 8.63	14.34	60.32 $\pm$ 0.865	7.93	19.08 $\pm$ 2.39	25.09
2	125	420.09 $\pm$ 4.95	56.34 $\pm$ 7.12	7.45	77.03 $\pm$ 2.13	5.54	17.52 $\pm$ 5.48	23.97
KF	125	204.64 $\pm$ 1.33	80.97 $\pm$ 3.24	2.52	91.74 $\pm$ 4.56	2.23	65.6 $\pm$ 2.34	3.11
KS	125	231.84 $\pm$ 2.58	65.72 $\pm$ 4.9	3.52	59.28 $\pm$ 2.95	3.91	73.66 $\pm$ 2.73	3.14
KL	250	606.81 $\pm$ 9.24	86.99 $\pm$ 7.18	6.97	68.03 $\pm$ 8.76	8.91	61.11 $\pm$ 1.167	9.92
Acyclovir	62.5	365.63 $\pm$ 4.61	92.29 $\pm$ 6.85	3.96	55.92 $\pm$ 14.63	6.53	68.18 $\pm$ 2.24	5.36

* CC_{50} and IC_{50} are given as Mean $\pm$ SD

MNTC Maximum nontoxic concentration, HSV-1 Herpes simplex type I, HAV Hepatitis A, CoxB4 Coxsackie B4

**Fig. 4** Cytotoxicity (CC_{50}) assay of **1**, **2**, KF, KS, KL extracts vs acyclovir against Vero cells. Data were expressed as mean $\pm$ SD calculated from three replicates for each level using one way analysis of variance (ANOVA), Tukey post hoc test at $p < 0.05$. All groups showed significant differences to each other. Groups that share the same letters are non-significantly different, while different letters express significant differences among different groups**Fig. 5** Antiviral activity (IC_{50}) of **1**, **2**, KF, KS, and KL vs acyclovir against HSV1. Data were expressed as mean $\pm$ SD calculated from three replicates for each level using one way analysis of variance (ANOVA), Tukey post hoc test at $p < 0.05$. All groups showed significant differences to each other. Groups that share the same letters are non-significantly different while different letters express significant differences among different groups

Antiviral activity of tested samples against CoxB4 Table 3 records the mean of the $IC_{50} \pm SD$ values as a measurable parameter for every tested sample against CoxB4. It reveals that metabolites **1** and **2** show the highest activities, with mean $IC_{50} \pm SD$ values of 19.08 ± 2.39 and $17.52 \pm 5.48 \mu\text{g/mL}$, respectively. The mean $IC_{50} \pm SD$ extents of KF, KL, and KS were almost equal to the mean $IC_{50} \pm SD$ value of acyclovir.

The majority of the groups differed significantly from one another (p -values were < 0.0001 , < 0.001 , and < 0.01), with the exception of some groups (KF vs. KS, KF vs. KL, KF vs. acyclovir, KS vs. acyclovir, KL vs. acyclovir). Groups that share the same letters are non-significantly different while different letters express significant differences among different groups as shown in Fig. 7

Determination of SI

The ratio of cytotoxic concentration for a sample to its effective antiviral concentration (CC_{50}/IC_{50}) is known as the selectivity index (SI). The higher the SI ratio, the more theoretically safe and effective a drug would be during in vivo treatment for a particular viral infection [37]. A selective bioactive sample must meet the acceptance threshold of SI, which is ≥ 10 . The SI values for **1**, **2**, KF, KS, and KL were displayed in Table 3. It was found that metabolite **1** had very high SI values against HSV-1 and CoxB4 ($SI = 14.34$ and 25.09), whereas metabolite **2** had high SI values against CoxB4 ($SI = 23.97$). KL had moderate SI values against CoxB4 and HAV ($SI = 9.92$ and 8.91).

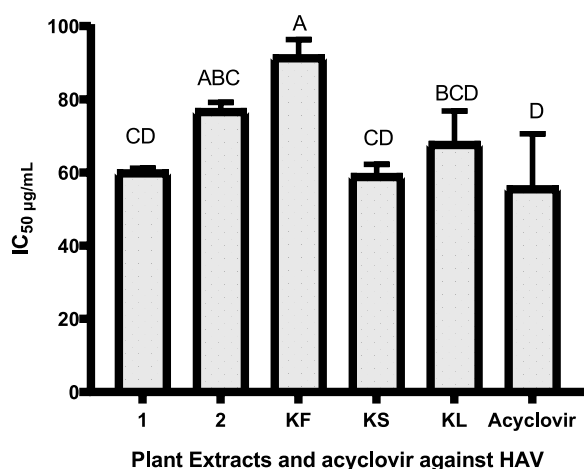


Fig. 6 Antiviral activity (IC₅₀) of 1, 2, KF, KS, and KL vs acyclovir against HAV. Data were expressed as mean $\pm$ SD calculated from three replicates for each level using one way analysis of variance (ANOVA), Tukey post hoc test at $p < 0.05$. All groups showed significant differences to each other. Groups that share the same letters are non-significantly different while different letters express significant differences among different groups

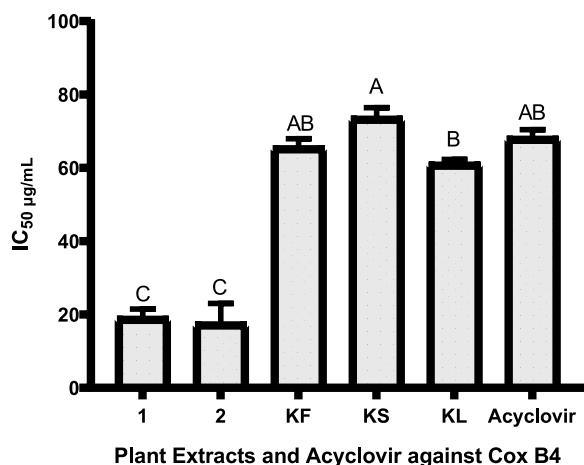


Fig. 7 Antiviral activity (IC₅₀) of 1, 2, KF, KS, and KL vs acyclovir against Cox B4. Data were expressed as mean $\pm$ SD calculated from three replicates for each level using one way analysis of variance (ANOVA), Tukey post hoc test at $p < 0.05$. All groups showed significant differences to each other. Groups that share the same letters are non-significantly different while different letters express significant differences among different groups

Discussion

Identification of the isolated major compounds

Compound **1** was predicted to be a 3-*O*-glycosylquercetin-like structure based on its chromatographic features (*R_f* value, fluorescence in UV light, and distinct reactivity towards different spray reagents) [38, 39]. Negative HRESI-MS² spectrum of **1** showed molecular ion peak

at m/z 447.0889 [M-H]⁻ *calcd.* for 447.0927 amu, MF of C₂₁H₂₀O₁₁-H and MW 448.1006 amu assignable for deoxyhexosylquercetin. It is afforded an intrinsic fragment ion at 301.0344 [M-H-146]⁻ together with its oxidized ion at 300.0280 [M-2H-146]⁻ that were characteristic for the loss of deoxyhexoside moiety. Thus, **1** was identified as quercetin 3-*O*-deoxyhexoside. NMR studies provided additional structural validation; the ¹H NMR spectral data (Table 1) revealed a distinctive ABM-spin coupling system for H-2', 6', and 5' at δ 7.31 (d, 1.9), 7.26 (dd, 8.3, 1.9) and 6.87 (d, 8.3) that was confirmative for 3',4'-dihydroxy ring B of quercetin. In addition, an AM-spin coupling system of two *meta*-coupled protons was assigned at 6.39 (br s) and 6.20 (br s) for H-8 and H-6 of a 5,7-dihydroxy ring A of the quercetin aglycone. The stereo structure of glycoside moiety was established as α -¹C₄-L-rhamnopyranoside because of the characteristic δ -values and splitting pattern of its proton signals at 5.26 (br s, H-1''), 3.98 (br d, $J = 1.2$, H-2''), 3.52 (1H, dd, $J = 9.0, 3.1$, H-3''), 3.22 (1H, m, H-5''), 3.15 (1H, t-like $J = 9.4$, H-4'') and 0.82 (3H, d, $J = 6$, CH₃-6''). ¹³C NMR spectral data (Table 1) showed typical 15 ¹³C-resonances of 3-*O*-substituted quercetin aglycone, particularly the key ones at δ 178.2, 148.9, 145.7, and 134.6, corresponding to C-4, C-4', C-3', and C-3, respectively [40]. Furthermore, an α -¹C₄-L-rhamnopyranosyl structure of the glycoside moiety was confirmed by six distinctive resonances, particularly those corresponding to the methyl and anomeric carbons, which were assigned at 17.9 and 102.3, respectively. All additional assigned resonances were consistent with the previously released information [40, 41]. Thus, **1** was identified as quercetin 3-*O*- α -L-¹C₄-rhamnopyranoside (quercitrin) (Fig. 1).

Meanwhile, **2** was predicted to be quercetin 3-*O*-glycoside based on the chromatographic behaviours [38, 39]. Negative HRESI-MS spectrum showed a molecular ion peak at m/z 609.1413 amu, corresponding to [M-H]⁻ ion of a quercetin deoxyhexosylhexoside structure. Furthermore, a fragment ion resulting from the loss of the glycoside moiety was detected at 301.0351 [M-H-308]. Further confirmation for the proposed structure was obtained from the ¹H NMR spectral data (Table 1), which showed an ABM-spin coupling system at ppm 7.55 (dd), 7.54 (br s), 6.85 (d, $J = 8.8$), interpretable for H-6', H-2' and H-5' of 3,4-dihydroxy B-ring. Another AM-spin coupling system was assigned for the two *meta*-coupled protons H-8 and H-6 at 6.39 and 6.20, respectively of 5,7-dihydroxy ring A. This evidence proved the aglycone to be quercetin. As well, the δ and J -values of the aliphatic ¹H-resonances, especially H-1'', H-1''' and CH₃-6''', were in complete accordance with a 3-*O*-rhamnopyranosyl-(1''' $\rightarrow$ 6'')- β -glucopyranoside [42]. Therefore, the structure

of **2** was established as quercetin 3-*O*- α -L-¹C₄-rhamnopyranosyl-(1'''' → 6''')-*O*- β -D-⁴C₁-glucopyranoside (rutin). In addition, ¹³C NMR spectral data (Table 1), demonstrated a set of 27 ¹³C-resonances characteristic for rutin, provided the final structural confirmation [40, 41]. As in the case of metabolite **1**, 15 ¹³C-resonances of quercetin moiety were interpreted including the key signals at ppm 177.8, 148.8, 145.1, and 133.8 of C-4, C-4', C-3', and C-3, respectively. Furthermore, the important ¹³C-resonances for the two anomeric carbons at δ 102.1 and 101.1 provided additional evidence for the rutinoside moiety. Confirmative evidence for the glycosidation at OH-3 and the interglycosidation at OH-6''' was concluded from the location of C-3 at 133.8 and C-6''' downfield shifted to 68.5 ($\Delta = + \sim 8$ ppm), respectively. Hence, metabolite **2** was finally confirmed as quercetin 3-*O*- α -L-¹C₄-rhamnopyranosyl-(1'''' → 6''')-*O*- β -D-⁴C₁-glucopyranoside or quercetin 3-*O*- β -D-rutinoside (rutin) (Fig. 1), which was in agreement with the data that had been previously released [43].

UPLC/ESI-qTOF-HRMS/MS analysis

In total, forty nine distinct metabolites were identified in the leaf and stem extracts of *K. senegalensis* (Table 2). Their identification is based on the verification of the retention times (*R_t*), observed monoisotopic *m/z* masses of molecular, some selective fragments and/or adduct ions in MS¹ and MS² two-stage MS/MS fragmentation spectra. In addition, their extract ion chromatograms (XIC), molecular formulas (MFs) and weights (MWs), together with the tandem mass spectral selective fragments and their full matching with the corresponding convenient library database (MoNA-Mass Bank of North America) reported for each metabolite identified. It is important to note and clarify that the blind matching of the output data from the LC/HR-MSⁿ hyphenation tools with the corresponding data from the relevant, powerful version of the ready, massively accumulated stored database is what determines the identity of the secondary metabolites. Therefore, in most circumstances, the identification, even if it is called tentative, is quite precise and sufficient to distinguish between various stereochemical properties if they were recorded and supported by already-identified data in such libraries. The identified metabolites were categorized based on their chemical structures into flavonoids (35), coumarins (4), phenolic acids (8), and organic acids (2).

The main components found in the leaf and stem extracts of *K. senegalensis* were flavonoids. The different types of the detected flavonoids were flavones, flavonols, isoflavones, flavanones, and some related glycosides. The presence of *O*- and *C*-glycosyl flavonoids in *K. senegalensis* was verified by observing their distinctive

fragmentation behavior during collision-induced dissociation. In their MS spectra, *O*-glycosyl flavonoids typically displayed ions at *m/z* [M-H]⁻, and fragment ions [M-H-162]⁻, [M-H-146]⁻ and [M-H-132]⁻, respectively, were found in *O*-hexosyl, *O*-deoxyhexosyl, and *O*-pentosyl derivatives [44]. However, in *C*-glycosyl flavonoids, the fragment ions were associated with crossing cleavage in the sugar units and had *m/z* values of [M-H-60]⁻, [M-H-90]⁻ and [M-H-120]⁻ [45].

Sixteen flavonoid glycosides were identified in the leaves, and stem extracts of *K. senegalensis* along with other eleven and eight were detected only in the stem, and leaf extracts, respectively. The major metabolite in the plant was quercetin 3-*O*-rhamnoside (quercitrin) as its peak areas were 23148990 and 4501151 in the leaf and stem extracts, respectively. However, its positional isomer (quercetin 7-*O*-rhamnoside) was also identified (*R_t* = 8.231) with peak areas of 21018.9. The first displayed a molecular ion at *m/z* 447.0947 [M-H]⁻ and its MS² spectrum showed the aglycone ion at 301.0344 [M-H-146]⁻ (with its oxidative form at 300.0280 [M-2H-146]⁻), *calcd.* for 447.2222, 301.0311, and 300.0258, corresponding to the loss of rhamnosyl moiety. Another characteristic oxidative fragment from the aglycone after the loss of CO molecule from ring C was observed at 271.0231 [M-rhamnosyl-CO-3H]⁻ in MS²-spectrum, *calcd.* for 271.01987 (Fig. 8a). Its 7-positional isomer showed similar peaks at *m/z* 447.0787 [M-H]⁻, 301.0459, and 300.0216 amu (Fig. 8b), *calcd.* for 447.2222, 301.0311, and 300.01999. Thus, both compounds were tentatively identified as quercetin 3-*O*- α -L-rhamnoside (quercitrin) and its 7-rhamnoside analogue according to their reported data [46] and matching with the library database. The second major metabolite in the plant was also a quercetin glycoside, i.e., rutin (peak area 13952193, 268710.3) that is identified from the leaf and stem. It revealed a molecular ion at *m/z* 609.1455 [M-H]⁻ and characteristic aglycone fragments at 301.0355 [M-H-rutinoside]⁻ and its oxidative one, i.e. 300.0276 [M-2H-rutinoside]⁻ that were matched with the calculated and database values (Table 2, Fig. 8c). Hence, it was identified as quercetin 3-*O*-rutinoside (rutin) [47].

Based on the peak area, the third flavonoid revealed a molecular ion peak at *m/z* 431.1915 [M-H]⁻ *calcd.* for 431.1907 amu, with the aglycone fragment ion at *m/z* 285.03375 [M-H-146]⁻ due to the loss of rhamnosyl moiety. In addition, two characteristic aglycone fragments were recorded at 179.0571 and 161.0490 amu according to the RDA-fragmentation pattern. Hence, this component was tentatively identified as kaempferol 3-*O*- α -L-rhamnoside (Fig. 8d) [48].

One of the *C*-glycosyl flavonoids that were identified, had a molecular ion at *m/z* 431.0986 [M-H]⁻ *calcd.*

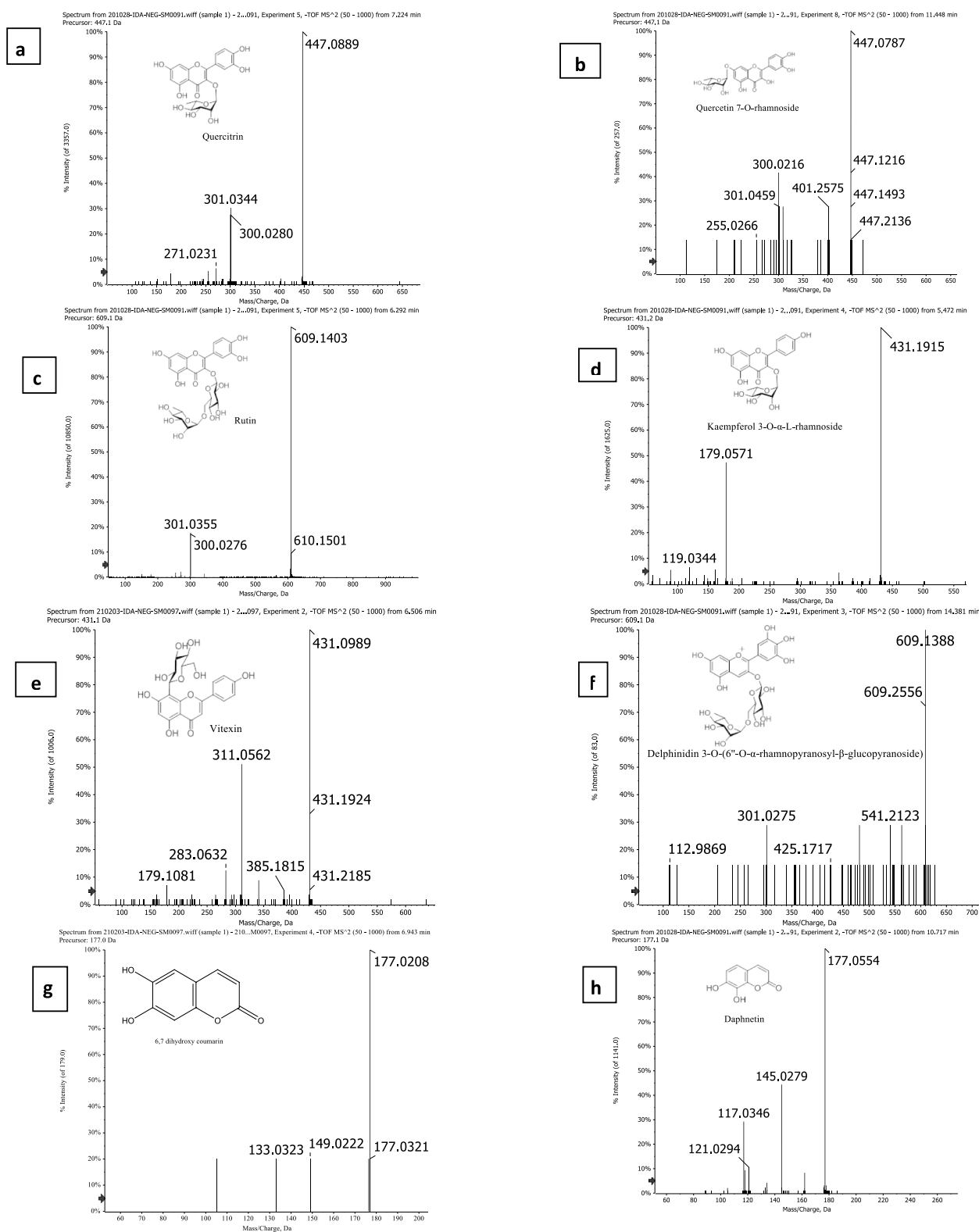


Fig. 8 MS² spectra of some major identified metabolites in *K. sengelansis* leaves and stem **a.** Quercitrin, **b.** Quercetin 7-O-rhamnoside, **c.** Rutin, **d.** Kaempferol 3-O- α -L-rhamnoside (Vitexin), **e.** Delphinidin 3-O-(6''-O- α -rhamnopyranosyl- β -glucopyranoside), **f.** 6,7 dihydroxy coumarin (Esculetin), **g.** Daphnetin

431.0978, for a MF $C_{21}H_{20}O_{10}$ and MW of 432.1056 amu. At high CID potential (MS^2 spectrum, Fig. 8), two fragments were recorded at 341.0598 $[M-H-90]^-$ (lower intensity) and 311.0562 $[M-H-120]^-$ (higher intensity), due to the losses of $C_3H_6O_3$ and $C_4H_8O_4$ from the $[M-H]^-$ ion, by the cross-ring cleavages of hexose unit. This property discriminated the 8-C-isomer from 6-C-type according to previously reported data [49]. As well, another distinct fragment for C-glycosides was observed at 283.0632 $[M-H-glucoside + CH_2]^-$. All above-discussed data were matched with the library database of apigenin 8-C-glucoside (vitexin), (Fig. 8e).

It is noteworthy to notice that just one anthocyanin-type flavonoid was identified in the stem ($R_t = 6.503$), as it represents the most major metabolite with a peak area of 9045275. It showed an oxidative molecular ion at m/z 609.1454 $[M-2H]^-$, because of its ready positively charged form in nature. It is calculated as 609.1456 for MF $C_{27}H_{31}O_{16}$ and MW 611.1612. At high CID fragmentation energy, it produced a characteristic aglycone fragment at m/z 301.03476 $[M-H-308]^-$ corresponding to the loss of rutinoside moiety together with other two oxidative fragments at 300.02841 $[M-2H-308]^-$ and 299.06772 $[M-3H-308]^-$. In addition, a C-ring cleavage resulted in three known fragments at 151.00101, 163.00117, 178.995, and 271.02386 amu. The identification was further reinforced by matching with the library database and previously reported data [50] for delphinidin 3-O-(6"-O- α -rhamnopyranosyl)- β -glucopyranoside (Fig. 8f).

Four coumarins were found in the extracts of *K. sengelansis* leaves and stems; two were detected in the leaf alone, and one was detected in both extracts. The fourth, was detected only in the stem. The major identified coumarin in the stem was 6,7-dihydroxycoumarin (esculetin) that displayed a deprotonated ion at m/z 177.0192, *calcd.* 177.0188 for MF $C_9H_6O_4$ and MW 178.0266. In its MS^2 spectrum, two fragment ions were assigned at m/z 149.0222 $[M-H-28]^-$ and 133.0323 $[M-H-44]^-$ corresponding to the loss of CO and CO_2 neutral molecules, (Table 2, Fig. 8g), that were in complete agreement with the library database and reported data of Esculetin [51]. However, the major identified one in leaf extract was assigned to daphnetin that showed a molecular ion peak at m/z 177.0558 $[M-H]^-$ (Table 2), *calcd.* 177.0188. Its MS^2 spectrum showed a characteristic fragment ion at m/z 133.02689 $[M-H-44]^-$ corresponding to the loss of CO_2 (Table 2, Fig. 8h). The compound was tentatively identified as daphnetin according to reported data [52] and the library database.

All identified phenolic acids showed the deprotonated molecular ion $[M-H]^-$ peak as the energetic precursor ion for some of the characteristic fragment ions in MS^2

spectra such as decarboxylation ($-CO_2$, 44 mu) and dehydration ($-H_2O$, 18 mu) fragment ions. Eight phenolic acids were detected in the leaf and stem extracts of *K. sengelansis*. 5-Methoxysalicylic and salicylic acids are the two major identified ones. The organic acids, normally, give more or less the same behavior as phenolic acids under ESI-MS/MS fragmentation potential (collision-induced dissociation potential, CID). As well, the most common fragment ions released due to the loss of 18 and/or 44 amu corresponding to the loss of neutral H_2O or CO_2 molecules from the molecular ion/s produced. Two organic acids were identified in *K. sengelansis* leaf and stem, *i.e.* quinic and shikimic acids.

Antiviral activity

Numerous studies have been conducted on the findings of novel antiviral drugs since viral resistance is becoming a more significant concern for people's health. Studies on the antiviral properties of natural products, such as flavonoids, phenolic acids, and other phenolic compounds, have also been conducted extensively for the same reason [53]. Moreover, natural antivirals fight against the common viruses including the influenza virus, respiratory viruses, hepatitis, and herpes virus in humans. Because of their mode of action, they have no negative side effects and are economical [54]. The natural bioactive compounds of plants act by disrupting the invasion, replication, and host contact of viruses at different phases of their life cycle [55]. The typical method for finding naturally occurring biologically active plant-derived compounds is bioactivity-guided fractionation. Prior to isolating and identifying the bioactive components, crude extracts are initially evaluated for antimicrobial activity. Then fractionation procedures are carried out repeatedly until the bioactive components are isolated and recognized [56]. The antiviral properties of *K. sengelansis* crude extracts and their constituents have been the subject of numerous published research, many of which are based on the uses of plants in traditional medicine [33].

A range of bioactive metabolites, including flavonoids, coumarins, phenolic acids, and organic acids that have been shown to have potent antiviral activity, have been tentatively identified by LC/MS analysis of *K. sengelansis* leaves and stem extracts.

Antiviral activity of flavonoid derivatives has been documented against a variety of viruses, including poliomyelitis virus, rotavirus, rhinovirus, HIV, Coxsackie B virus, coronavirus, cytomegalovirus, poliovirus, sindbis virus, and rabies virus [53]. From the most prevalent flavonol molecule with significant antiviral properties in nature is the quercetin [57]. According to Guo. et al. [58], rutin has been demonstrated to have antiviral action against influenza viruses, human noroviruses and HBV.

Likewise, anthocyanins have been reported to possess antiviral therapeutic qualities that can help prevent and treat viral infections; they have also been proven to exhibit anti-HSV activity [59]. Delphinidin has been shown to be one of the most promising anthocyanin antiviral agents. It obstructs HCV adsorption and attachment during the inoculation phase [60]. Additionally, coumarins are employed as therapeutic agents. They are stable, soluble, low molecular weight compounds without any hazardous or negative side effects. They therefore appear to be promising candidates for use as medications to treat a variety of bacterial and viral diseases. A variety of pathways and enzymes required for the entrance, survival, and infection of viruses can be targeted by coumarin and its derivatives. Numerous investigations have exhibited its suppressive impact on the replication of various viruses, such as coxsackie virus A16 (CVA16), influenza, HCV, and HIV [61]. Furthermore, scopoletin demonstrates strong virucidal and antiviral action against SARS-CoV-2 infection, even at low concentrations, according to Baggieri et al. [62].

In the current study, the antiviral activity of the total methanol extract of *K. senegalensis* leaves (KL), stem (KS) and fruit (KF) had been tested against three viruses, *i.e.* HSV-1, CoxB4, and HAV, at different concentrations. Also, the two major isolates that were isolated from leaves extract (quercitrin, **1** and rutin **2**), have been tested against the same viruses. The MNTC and the IC_{50} of all tested samples were determined on Vero cells prior to determining antiviral activities. The findings revealed that **1** showed higher antiviral activity against HSV1 with IC_{50} value $33.38 \pm 8.63 \mu\text{g/mL}$, followed by KS extract and then **2**, which showed moderate activity with IC_{50} values of 65.72 ± 4.9 and $56.34 \pm 7.12 \mu\text{g/mL}$, respectively. However, KL and KF extracts showed the lowest antiviral activity in comparison to acyclovir, with IC_{50} values 86.99 ± 7.18 and $80.97 \pm 3.24 \mu\text{g/mL}$, respectively. Moreover, KS extract and **1** showed the highest antiviral potency against HAV based on their own IC_{50} values of 59.28 ± 2.95 and $60.32 \pm 0.865 \mu\text{g/mL}$, which are nearly equipotent to acyclovir ($55.92 \pm 14.63 \mu\text{g/mL}$). Furthermore, **1** and **2** showed the highest activity against CoxB4 with IC_{50} values 19.08 ± 2.39 and $17.52 \pm 5.48 \mu\text{g/mL}$ respectively; meanwhile KF, KL and KS gave raise nearly equipotent effects relative to acyclovir, in parameters of IC_{50} .

The ideal drug should have a relatively high cytotoxic concentration but with a very low bioactive concentration. Evaluation of SI value (for any research on herbal drugs and/or isolated compounds) is very crucial for determining whether further research studies can be continued or not. The higher the SI ratio, the theoretically more effective and safe a drug would be during in vivo treatment for a given viral infection [37]. The

results of the computation of SI showed that quercitrin had a very high SI (25.09) followed by rutin (23.97) against CoxB4, also quercitrin had a good SI (14.34) against HSV1.

Concerning the novelty in the current study, it can be said that with respect to the phytochemistry, LC/MS–MS tool was used, with high accuracy, for the identification of large number of various structural phenolics including rutin and quercitrin as the most major ones from *K. senegalensis*. Simultaneously, they are few identified previously from different parts of *K. senegalensis* and *grandifoliola* [27, 63–66], and isolated in this study from the *K. senegalensis* leaf. However, from the biological point of view antiviral activity has been concerned in few reports on other types of viruses differ from HSV-1, HAV and Cox-B4 [67–70]. Accordingly current promising new findings for significant antiviral activity against HSV-1, HAV and Cox-B4 can be explored new future insights about the probability for pharmaceutical preparation/s and/or drug/s after further extensive in vivo and clinical trials on quercitrin, rutin and examined total extracts.

Supplementary Information

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Additional file 1.

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Author contributions

All authors contributed to the study's conception and design. Data collection and analysis were performed by all authors. All authors participated in the writing of the manuscript. All authors read and approved the manuscript.

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Declarations

Competing interests

The authors certify that this publication does not include any conflicts of interest. The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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