RESEARCH ARTICLE

Phytochemical Investigation and Pharmacological Activity of *Solidago canadensis* L. against H1N1 Virus, involving the Separation and Identification of Three New Compounds

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ABSTRACT

Solidago canadensis L. (*S. canadensis*) is a member of the Asteraceae family, which comprises over a 100 species. The aerial portion of *S. canadensis* was defatted by maceration in hexane for 24 hours; the defatted plant components were extracted for 24 hours using a Soxhlet apparatus and aqueous ethanol 85%, and then fractionated by different solvents. The ethyl acetate, and chloroform fractions were examined using liquid chromatography-mass spectroscopy (LC-MS). The examination revealed the presence of several phenolics, coumarins, and flavonoid compounds. Preparative high-performance liquid chromatography (PHPLC) was utilized to isolate several compounds. Eugenol-o-glucoside, 2-hydroxy-4,5-dimethoxy-9,10-dihydrophenanthrene, and 2,5-dihydroxy-4-methoxy-9,10-dihydrophenanthrene (Hircinol) have been isolated and identified in the plant for the first time. High-throughput cytopathic effect (CPE) inhibitory tests for the H1N1 virus on vero cells were developed to investigate new potential antiviral agents. A crystal violet uptake assay was used to measure the cytotoxic and antiviral effects. The polyphenols in the ethyl acetate fraction showed high antiviral activities against H1N1 with a selective index (SI) = estimated CC_{50}/estimated IC_{50} = 23.6. Consequently, the tested samples are good candidates for further experiments as anti-influenza H1N1

Keywords: 2-hydroxy-4,5-dimethoxy-9,10-dihydrophenanthrene, Anti-influenza H1N1 virus, Eugenol-o-glucoside, Flavonoids, Hircinol, Phenolics, *S. canadensis.*

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INTRODUCTION

The Influenza A (IVA) virus is one of the most aggressive viruses ever discovered, having caused many worldwide outbreaks. Between 1918 and 1919, this virus claimed the lives of 50 million of the world's population. [1,2]

IVA is a member of the Orthomyxoviridae family, and its genome is made up of eight negative-stranded RNA segments. Spikes of major glycoproteins such as hemagglutinin (HA) and neuraminidase (NA), as well as a small population of matrix protein 2 (M2), are found on the viral envelope, which determines the serologic and antigenic characteristics of strains of the virus.[3]

The two proteins HA and NA, are used to differentiate IVA viruses into subtypes. There are 18 distinct subtypes of hemagglutinin and 11 different subtypes of neuraminidase (H1 through H18 and N1 through N11, respectively). While nature has identified over 130 IVA subtype combinations, the current subtypes of IVA viruses that frequently circulate in people are A (H1N1) and A (H3N2).[4]

Thousands of biologically active plants have been utilized in medicine throughout human history. According to a World Health Organization report, medicinal plants are used by nearly 80% of the world's population to take care of their health. [5]

Vaccination and medications such as neuraminidase inhibitors (*e.g.,* oseltamivir, zanamivir, as well as peramivir and laninamivir, and adamantanes) are among the available preventative and curative methods, due to viruses resistance, New techniques to prevent and treat influenza infections are required. Based on experimental and clinical research, polyphenolic compounds found in many plant components, have been identified as possible anti-influenza virus agents. [6]

The Asteraceae family contains over 25,000 species and 1,500 genres, and they are immensely diversified as subordinate plants, herbs, sub-shrubs, or trees, with 98% of the

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species being small. *Solidago virgaurea* L., *Solidago gigantea* L., *Solidago canadensis* L., and *Solidago chilensis* are the most researched species. [7]

S. canadensis L. is a member of the Asteraceae family, which comprises over 100 species. It is widespread in North American wildflowers and has more than a dozen species in South America, Europe, and Asia. [8]

S. canadensis has lately been cultivated in Iraq's Babylon and Diyala provinces.

S. canadensis (*S. Altissima*), is a 1–2.5 m tall upright plant with a range of around 1 m. The stems are pubescent throughout, with lanceolate, three-veined leaves that are roughish above and pubescent below. From August to November, broad plumose heads of yellow flowers make a large pyramidal panicle, making it an attractive fall border plant. [9]

Solidago has a variety of essential secondary metabolites, including saponins like *Canadensis saponin.*[10] Polysaccharides, flavonoids glucosides like quercetin, and rutin,[11–14] phenolic acids, such as caffeic acid and chlorogenic acid, and alkaloids, such as dictamnine-7-b-d-mannopyranoside and 8-methoxydictamnine-7-b-d-mannopyranoside.[15]

Flavonoids are polyphenolic compounds with 15 carbon skeletons (benzopyran) and a C6-C3-C6 backbone structure composed of two benzene rings, ring A and ring B, connected by a heterocyclic ring, ring C. More than 8000 polyphenolic compounds have been identified, and more than 4000 flavonoids have been recognized. Flavonoids are classified as flavones, flavonols, flavanones, flavanonols, isoflavones, and other classes based on the degree of oxidation, type, and mode of ring C substitution.[16]

Phenolic acids are compounds having C6-C1 skeleton-like gallic acid, protocatechuic acid, C6-C2, and C6-C3 skeleton-like caffeic acid, chlorogenic acid, 3,4-dicaffeoylquinic acid. [17]

Flavonoids like astragalin, quercetin, rutin, isorhamnetin, and isoquercetin, also phenolic acid-like chlorogenic acid and caffeic acid were shown to have a significant antiviral activity like the H1N1 virus, and measles virus.[18,19] antitumor, and other clinical effects. In this study, ultra-high performance liquid chromatography coupled with hybrid quadrupole-orbitrap mass spectrometry (UPLC-Q-Exactive/MS).

The CPE-inhibition test will be utilized to find antiviral candidates for the H1N1 virus. The dose-response test was created to assess the range of efficacy for the selected antiviral, as well as the range of cytotoxicity (CC_{50}) and the 50% inhibitory concentration. Antiviral activity in cell culture systems could be evaluated using this test.[20]

MATERIALS AND METHODS

Collection of Plant Materials

S. canadensis aerial parts were collected from a Babylon province farm in October 2020. The plant was identified and authenticated by Prof. Dr. Sukaena Abass, Department of Biology, College of Sciences, University of Baghdad. The aerial part was washed thoroughly, dried under shade, and ground in a mechanical grinder to a fine powder.

Equipment and Chemicals

The instruments used were rotary evaporator (BÙCHI Rotavapor R-205, Swiss), Sonicator (Branson Sonifier, USA), high-performance liquid chromatography (HPLC) (Waters 2690 Alliance HPLC system equipped with a Waters 996 photodiode array detector), All chemicals and solvents used were of analytical grade and obtained from Riedel-de Haen, Germany. Ethanol which is HPLC grade, was purchased from Sigma-Aldrich, Germany. The standard chlorogenic acid, caffeic acid, quercetin, astragalin, and rutin, were purchased from Chengdu Biopurify Phytochemicals, China (purity >97).

Extraction

Total of 750 g of shade-dried pulverized aerial part of *S. canadensis* were defatted by maceration with hexane for 24 hours and then allowed to dry at room temperature. The defatted plant materials were extracted by Soxhlet using aqueous ethanol 85% as a solvent for 24 hours. The extract was filtered, and the solvent was evaporated under reduced pressure using a rotary evaporator to get a dry extract. The residue of about 27 gm was suspended in 400 mL of water and partitioned successively with petroleum ether (B.P. 30–60), chloroform, ethyl acetate, and n-butanol (3×300 mL) for each fraction. The first three fractions were dried over anhydrous sodium sulfate, filtered, and evaporated to dryness.[21,22]

Preliminary Phytochemical Investigation

Phytochemical analysis for the screening and identification of bioactive chemical constituents in the medicinal plants under study was carried out on crude extracts, as well as powder specimens using the standard procedures as described by Harborne [23]

Test for Flavonoids

A few milligrams of aqueous ethanol plant extracts were suspended in ethanol, a few drops of 5% ethanolic KOH were added, and then a few drops of 5% HCl were added. The changes in colors were recorded.

Test for Phenols

A few milligrams of aqueous ethanol plant extracts were treated with a few drops of 1% $FeCl_3$. The formation of dark greenish-blue color indicates the presence of phenols.

Liquid Chromatography-Mass Spectroscopy LC-MS Analysis

LC-MS technique was applied for the detection of different constituents found in the ethyl acetate, and chloroform fractions of *S. canadensis* using a mobile phase composed of solvent A: Buffer (0.1% Formic acid in H_2O), solvent B: Acetonitrile, with the gradient program of elution shown in Table 1, Column used: GL-Science-C18-250 mm x 4.6 (5 µm particle size)-Japan, Column oven 35°C. Injection volume 10 µm, flow rate 1-mL/min, run time 25 minutes. LC-MS-Q-TOF model- X500 QTOF, software: AB-Siex-OS, Ionization mode: ESI Positive, Scan range 50–800 m/z, Ion source voltage

Table 1: The gradient flow program for HPLC analysis

No.	*Time*	*Flow*	*%A*	*%B*
1	0	1.00	90.0	10.0
2	5.00	1.00	90.0	10.0
3	10.00	1.00	10.0	90.0
4	15.00	1.00	10.0	90.0
5	20.00	1.00	90.0	10.0
6	25.00	1.00	90.0	10.0

5500V.

Qualitative Examining Bioactive Compounds in Ethyl Acetate, and Chloroform Fractions Using HPLC

Qualitative estimations were carried out for the identification of bioactive compounds present in all fractions were done by using Mass/Fragment Mass/RT/Isotope/Library/Formula/Ion Ratio) (NIST) Smart Confirmation. The identification of compounds was based on the comparisons of the obtained mass spectra with the NIST Library.

Isolation and Purification of Variant Compounds using Semi-preparative PHPLC

With acetonitrile: water mixes in varied ratios depending on the polarity of the fraction, HPLC (Pumps: -Shimadzu, LC-10-AD, Detector: SPD-20A-PDA, Column: RP-C18, 5 µm, 250 mmX 10 mm, Kromasil®, Sweden, Software: Shimadzu-lc-lab solutions) was used to accomplish the analysis.

Using gradient acetonitrile and 0.1% formic acid in water, 0–5 minutes, 30% acetonitrile, increasing the gradient to reach 100% acetonitrile in 35 minutes, and then isocratic detection for 10 minutes, the isolation of compounds S8 and S9 from the ethyl acetate fraction was achieved.

Using gradient acetonitrile and 0.1% formic acid in the water, 0–5 minutes, 70% acetonitrile, increasing the gradient to reach 100% acetonitrile in 25 minutes, and then isocratic detection for 10 minutes, the isolation of compounds S10 and S11 from the chloroform fraction was achieved.

Fraction photodiode array detectors were used for the detections. The ethyl acetate fraction was detected at wavelength 280 nm, while the chloroform fraction was detected at wavelength 235 nm. The flow rate was 5 mL/min, The temperature was kept at 30°C, and the volume of injection was 250 µL. the compounds isolated from ethyl acetate fraction symbolled S8, and S9, and the compounds isolated from chloroform fraction symbolled S10, and S11.

Identification and Structural Characterization of the Isolated Compounds

Liquid Chromatography-Electrospray Ionization Mass Spectrometry (LC/ESI/MS)

Due to its great selectivity and sensitivity, LC/ESI/MS has been widely utilized in a variety of studies. ESI/MS is particularly beneficial for the investigation of polar substances.[24] Negative ion mode was used for the identification of the isolated chemical compounds (Shimazu–LCESIMS-8045, Pumps: LC-2040, Detector: LC-2030/2040-PDA detector, Column oven: LC-2040, Column: Chimpack-RP-UPLC, C-18, 2.7 µM, Software: Lab solutions 3.2.).

Nuclear Magnetic Resonance (NMR)

Using Bruker®, AVANCE III HD, 400 MHz, Switzerland, 0.2 mL of a suitable deuterated solvent was used (Deuterated DMSO, Cambridge isotope®, U.S.A, Deuterated chloroform, $CDCl_3$, Cambridge isotope®, U.S.A) to dissolve the compounds, and then they were transported into a 3 mm NMR tube. The ^{1}H-NMR was measured at 400 MHz and the ^{13}C-NMR at 100 MHz. For proton experiments, the number of scans was set at 128; for the attached proton test (APT) experiments, it was 8000.

Determination of the Antiviral Activity of Phenolics and Flavonoids from the Areal Part of *S. canadensis* Obtained from the Ethyl Acetate Fraction by using the CPE

The antiviral activity of ethyl acetate fraction against the H1N1 virus was identified using the high-throughput cytopathic effect CPE-inhibition assay. The assay was developed to evaluate the range of efficacy, *i.e.,* the 50% inhibitory concentration (IC_{50}) and cytotoxicity for the chosen antiviral (CC_{50}), In cell culture systems, this assay is crucial for evaluating antiviral efficacy.[25]

Cell Lines and Culture

Nawah-Scientific, Egypt provided the H1N1 virus and Vero E6 cells, Vero E6 cells were grown in DMEM medium supplemented with 10% fetal bovine serum and 0.1% antibiotic/antimycotic solution. Gibco BRL provided the antibiotic and antimycotic solution, trypsin-EDTA, fetal bovine serum, and DMEM medium (Grand Island, NY, USA).[26]

Antiviral Activity Test

The crystal violet method was used to evaluate the antiviral activity and cytotoxicity assays according to Schmidtke *et al.,*.[25,26] One day before infection, Vero E6 cells were seeded at a density of 2x104 cells/well on a 96-well culture plate to achieve an MOI of 5 (MOI 5). The next day, the growth media was withdrawn and the cells were rinsed in phosphate-buffered saline. The crystal violet method was used to evaluate the infectivity of the H1N1 virus, which monitored CPE and allowed the percentage of cell viability to be measured. In mammalian cells, 0.1 mL of diluted H1N1 virus solution containing $CCID_{50}$ (50% cell culture infectious dose) of viral stock was added. This dosage was chosen to get the necessary CPEs. The cells were treated, with 0.01 mL of media bearing the required chemical concentration for compound treatments. The antiviral activity of each test sample was assessed using a ten-fold diluted concentration range of 0.1–100 g/mL. Cell controls and viral controls (virus-infected, non-drug-treated cells) (non-infected, non-drug-treated cells). Culture plates were incubated at 37°C with 5% CO_2 for 72 hours. The development of the cytopathic effect was monitored by light microscopy. Following a PBS wash, the cell monolayers were fixed and stained with a 0.03% crystal violet solution in 2% ethanol and 10% formalin.

After washing and drying, the optical density of individual wells was quantified spectrophotometrically at 540/630 nm. The percentage of antiviral activity of the tested compounds was calculated. using the following equation:

antiviral activity = [(mean optical density of cell controls−mean optical density of virus controls)/(optical density of the test−mean optical density of virus controls)] ×100%, using the Dynex Immuno Assay System DIAS reader.[27] Based on the results, the 50% CPE inhibitory dose (ID50) was calculated.

Cytotoxicity Test

Before this assay, we assessed the cytotoxicity; Vero cells were seeded at a density of $2x10^4$ cells/well in a 96-well culture plate. The next day, the culture medium containing serially diluted samples was added to the cells and incubated for 48 hours before being removed and the cells washed with PBS. The following steps were carried out in the same manner as described above for the antiviral activity assay.

The 50% cytotoxic concentrations (CC_{50}) and the 50% inhibitory concentration (IC_{50}) were determined using GraphPad PRISM Software (Graph-Pad Software, San Diego, USA).

RESULTS AND DISCUSSION

The Preliminary Phytochemical tests

The preliminary phytochemical tests on ethyl acetate and chloroform fractions were demonstrated in Table 2.

LC-MS Analysis

LC-MS analysis of ethyl acetate and chloroform fractions revealed the presence of variant bioactive compounds as demonstrated in Figures 1 and 2, Tables 3 and 4, respectively.

The Preparative HPLC Analysis (PHPLC)

High-quality liquid chromatography (HPLC) is a versatile and widely used technique for separating natural compounds. HPLC can be used to separate chemical substances by making use of the fact that different compounds move at different speeds in particular columns and mobile phases. The process of separation is called purification using HPLC.[28]

The PHPLC chromatograms of ethyl acetate and chloroform fractions are shown in Figures 3 and 4, respectively. The target peaks (S8, S9) from ethyl acetate fraction at a retention time of 18 and 28.11 minutes, and (S10, S11) from chloroform fraction at the retention time of 3.07 and 4.62 minutes were gathered by the fraction collector after they were monitored over time, *i.e.* time from the commencement of each peak appearance to peak disappearance.

Table 2: Phytochemical Screening of *S. canadensis* cultivated in Iraq

Phytochemicals	*Ethyl acetate fraction*	*Chloroform fraction*
alkaloids	_	_
terpenoids	-	+
flavonoids	+	+
phenols	+	+
steroids	-	-

(+), (-) represent the presence or absence of phytochemicals, respectively.

Identification of Isolated Compounds

LC/ESI/MS

The mass spectrum of the compounds S8, and S9 isolated from ethyl acetate fraction, and compounds S10 and S11 isolated from chloroform fraction are shown in Figures 5-8, respectively.

Nuclear Magnetic Resonance ^{1}HNMR and ^{13}CNMR

The ^{1}H-NMR and ^{13}C-NMR of the isolated compounds S8, S9, S10, and S11 are shown in Figures 9-16.

Interpretation and Structural Elucidation of the Isolated Compounds

- Compound S8 was isolated as an amorphous yellow powder having the chemical formula C21H20O11 as determined by the ESI-MS m/z value of 447 $[M-H]^-$ Figure 5, The molecular ion peak for compound S8 $[M-1]^-$ is 447 with major fragments (285, and 163) as shown in Figure 17. According to the ^{1}HNMR Figure 9 (3.15-3.73 (5H, m) 2″,3″,4″,5″,6″-H, 5.34 (1H, d) 1″-H, 5.72 and 5.89 (1H, S) 2″,4″,5″,6″- OH, 6.37 (1H, d) 6-H, 6.51 (1H, S) 3-H, 6.75 (1H, d) 8-H, 6.93, (1H, d) 3′-H, 7.41 (1H, dd) 6′-H, 7.45, (1H, d) 2′-H, 7.95, (1H, s) 3′-OH, 8.38, (1H, s) 4′-OH, 12.93 (1H, s) 5-OH) and APT ^{13}CNMR Figure 13 (60.07 (C-6″), 70.01 (C-4″), 77.06 (C-3″), 73.34 (C-2″), 76.40 (C-5″), 94.14 (C-8), 99.33 (C-6), 99.33 doublet (C-1″), 104.13 (C-10), 113.18 (C-2’), 116.27 (C-5’), 119.43 (C-6’), 121.72 (C-1’), 145.78 (C-3’), 149.04 (C-4’), 157.78 (C-9), 162.03 (C-5), 164.18

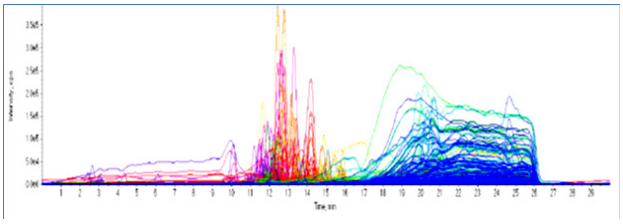


Figure 1: LC-MS spectrum of the Ethyl acetate fraction.

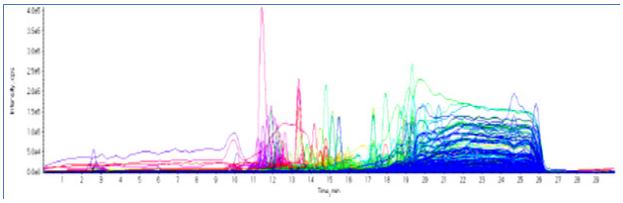


Figure 2: LC-MS spectrum of the Chloroform fraction.

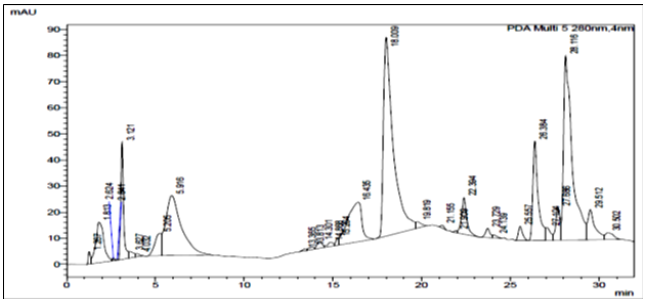


Figure 3: PHPLC chromatogram of ethyl acetate fraction

Table 3: The LC-MS analysis of detected bioactive compounds in the ethyl acetate fraction of *S. canadensis*.

No.	*Peak no.*	*Analyte Peak Name/ RT*	*Found At Mass*	*Fragment ions m/z*	*Library Hit (NIST) Smart Confirmation*
	515	595.2014/12.11	595.2015	-	Pelargonidin 3,5-diglucoside cation (NIST) Smart Confirmation
	533	271.0717/12.19 M+H+	271.0619	69.033, 91.054, 149.024, 192.98, 215.07, 243.065	Pelargonidin cation (NIST) Smart Confirmation
	549	581.1507/12.23	581.1506	287.06, 419.096	Naringin (NIST) Smart Confirmation
	608	449.1098/12.42	449.1089	127.039, 287.053	Astragalin (NIST) Smart Confirmation
	614	321.0958/12.46	321.0961	66.99, 153.01, 165.01, 229.04, 285.03, 303.04	Dihydromyricetin (NIST) Smart Confirmation
	619	611.1594/12.46	611.1593	85.028, 129.05, 303.047	Rutin (NIST) Smart Confirmation
	711	581.1500/12.76 M+H+	581.1500	133.049, 287.053	Cyanidin 3-O-sambubioside cation (NIST) Smart Confirmation
	719	465.1018/12.80 M+H+	465.1014	85.026, 127.039, 303.047	Isoquercetin (NIST) Smart Confirmation
	721	595.1661/12.80 M+H+	595.1659	65.087, 287	Datiscin (NIST) Smart Confirmation
	733	625.1775/12.83 M+H+	625.1775	317.064, 318.069	Narcissin (NIST) Smart Confirmation
	774	449.1802/12.99	449.1801	287.263	Luteolin-7-O-glucoside (NIST) Smart Confirmation
	821	317.0648/13.25	317.0646	153.01, 217.05, 285.04, 302.042	Quercetin 3'-methyl ether (NIST) Smart Confirmation
	824	479.1179/13.25	479.1177	145.028, 163.03, 317.063	Isorhamnetin 3-O-glucoside (NIST) Smart Confirmation
	854	951.2541/13.36 2M+Na+	951.2563	487.1206, 303.0504	Hyperoside (NIST) Smart Confirmation
	880	287.0564/13.51	287.0562	121.029, 153.019, 165.019, 213.055	Kaempferol (NIST) Smart Confirmation
	939	579.2067/13.86	579.2066	163.041, 325.092	Apigenin 7-O-neohesperidoside (NIST) Smart Confirmation
	941	291.0878/13.89	291.0879	119.047, 147.039	(+)-Catechin (NIST) Smart Confirmation
	400	355.1022/11.62 M+H+	355.1019	135.043, 145.027, 163.037	Chlorogenic acid (NIST) Smart Confirmation
	828	517.1347/13.25 M+H+	517.1344	163.075	3,5-Dicaffeoylquinic acid (NIST) Smart Confirmation
	1000	516.1491/14.12	516.1491	163.036, 333.095	3,4-dicaffeoylquinic acid
	129	277.0891/2.77	277.0906	-	6-Gingerol (NIST) Smart Confirmation
	466	197.1295/11.93	197.0823	70.065	4',6'-Dimethoxy-2'-hydroxy acetophenone (NIST) Smart Confirmation
	658	327.0986/12.61	327.0990	69.034, 97.028, 145.0282, 163.038	No Match
	791	489.2325/13.10	489.2324	-	Curculigoside (NIST) Smart Confirmation
	319	341.0878/11.24	341.0877	179.033	Esculin (NIST) Smart Confirmation
	469	423.1268/12.08 M+CH3OH+H+	423.1266	163.038	7-Hydroxycoumarinyl. gamma. -linolenate (NIST) Smart Confirmation
	568	339.1084/12.30 M+H+	339.1083	119.049, 147.043	Bergamotin (NIST) Smart Confirmation
	643	177.0550/12.53 M+CH3OH+H+	177.0552	78.04, 93.08, 121.02, 145.02, 153.01	8-Methoxycoumarin (NIST) Smart Confirmation
	833	147.0442/13.29	147.0444	89.043	trans-3-Coumaric acid (NIST) Smart Confirmation
	850	339.1068/13.36	339.1074	117.03, 145.02, 177.05	Demethoxycurcumin (NIST) Smart Confirmation

Table 4: The LC-MS analysis of detected bioactive compounds in the chloroform fraction of *S. canadensis*

No.	*Peak no.*	*Analyte peak name/ RT*	*Found at mass*	*Fragment ions m/z*	*Library hit (NIST) smart confirmation*
	23	240.8824/2.01	240.882	-	4,4'-Dimethoxystilbene (NIST) smart confirmation
	98	242.9265/2.39	242.926	-	No Match
	103	258.1107/2.54 M+H+	258.110	-	No Match

No.	*Peak no.*	*Analyte peak name/ RT*	*Found at mass*	*Fragment ions m/z*	*Library hit (NIST) smart confirmation*
	114	455.1167 / 2.62 M+K+	455.117	-	Anemosapogenin (NIST) Smart Confirmation
	197	289.0929 / 10.15 M+H+	289.093	127.039	2'-Hydroxy-.beta.-naphthoflavone (NIST) Smart Confirmation
	319	341.0878 / 11.24	341.088	89.05, 133.08, 179.03	Esculin (NIST) Smart Confirmation
	379	483.1484 / 11.55	483.148	-	Silybin (NIST) Smart Confirmation
	400	355.1022 / 11.62 M+H+	355.102	163.039, 193.049	Neochlorogenic acid (NIST) Smart Confirmation
	533	271.0717 / 12.19 M+H+	271.072	69.033, 151.089	Baicalein (NIST) Smart Confirmation
	649	369.1173 / 12.61 M+H+	369.117	145.028, 177.054	Curcumin (NIST) Smart Confirmation
	719	465.1018 / 12.80 M+H+	465.102	303.0513	Hyperoside (NIST) Smart Confirmation
	720	487.0846 / 12.76 M+Na+	487.085	135.045, 163.039, 303.050, 347.074	Isoquercitin (NIST) Smart Confirmation
	808	449.1076 / 13.17 M+H+	449.108	287.0548	Luteolin- 7-O-glucoside (NIST) Smart Confirmation
	809	471.0901 / 13.17 M+Na+	471.090	288.0597	Astragalin (NIST) Smart Confirmation
	824	479.1179 / 13.25	479.118	457.1099, 317.0661	Isorhamnetin 3-O-glucoside (NIST) Smart Confirmation
	828	517.1347 / 13.25 M+H+	517.135	-	3,5-Dicaffeoylquinic acid (NIST) Smart Confirmation
	922	193.0504 / 13.78	193.050	93.033, 122.03, 150.031, 178.026	Scopoletin (NIST) Smart Confirmation
	976	827.3477 / 14.04	827.348	263.0953	Stevioside (NIST) Smart Confirmation
	1205	403.1518 / 14.88	403.152	283.108, 343.128, 371.124	3',4',5',5,6,7-Hexamethoxyflavone (NIST) Smart Confirmation
	1241	303.0499 / 14.95	303.050	-	Delphinidin cation (NIST) Smart Confirmation
	1430	517.2405 / 15.79	517.241	163.04	3,4-Dicaffeoylquinic acid (NIST) Smart Confirmation
	1449	287.0564 / 15.94	287.056	-	Kaempferol (NIST) Smart Confirmation
	1462	131.0498 / 16.01	131.050	51.02, 103.05	Valeric acid ethyl ester (NIST) Smart Confirmation
	1489	317.0668 / 16.09	317.067	153.01, 302.04	3',4',5,7-Tetrahydroxy-3-methoxy flavone (NIST) Smart Confirmation
	1491	381.2265 / 16.09	381.227	-	Lactobionic acid (NIST) Smart Confirmation
	1527	459.2393 / 16.32	459.239	289.1029	Epigallocatechin gallate (NIST) Smart Confirmation
	1561	355.1182 / 16.62	355.118	163.0400	Chlorogenic acid (NIST) Smart Confirmation
	1703	209.1168 / 17.56 M+H+	209.117	79.05, 95.08, 135.11, 163.11	Ethyl trans-caffeate (NIST) Smart Confirmation
	1713	195.1388 / 17.60 M+H+	195.139	67.05, 79.05, 93.06, 117.07, 145.10	Caffeic acid methyl ester (NIST) Smart Confirmation
	1719	309.2421 / 17.60 M+CH3OH+H+	309.242	71.06, 99.08, 151.11, 249.22	6-Gingerol (NIST) Smart Confirmation
	1797	723.3354 / 17.94	723.335	61.06, 193.08	Prosapogenin A (NIST) Smart Confirmation
	1891	387.2536 / 18.47	387.254	299.20, 239.18	Harpagide (NIST) Smart Confirmation
	1950	435.2940 / 18.78	435.294	133.1, 147.1, 161.1, 253.1, 299.1	5,7-Dihydroxy 3,3',4',5',6,8-hexamethoxy flavone (NIST) Smart Confirmation
	1954	317.2121 / 18.81 M+H+	317.212	109.1, 119.1, 197.1, 299.1	Eicosapentaenoic acid methyl ester (NIST) Smart Confirmation
	1972	399.2520 / 18.89	399.252	83.04, 157.09,195.1, 299.1, 357.2	Loganic acid (NIST) Smart Confirmation
	1977	663.3162 / 18.93	663.316	-	Plantamajoside (NIST) Smart Confirmation
	1990	259.2070 / 19.04	259.207	70.06, 114, 170.1, 215.1	Isogentisin (NIST) Smart Confirmation
	2104	677.3725 / 19.46	677.372	331.2	Icariin (NIST) Smart Confirmation
	2151	433.2841 / 19.72	433.284	337.2	Isovitexin (NIST) Smart Confirmation

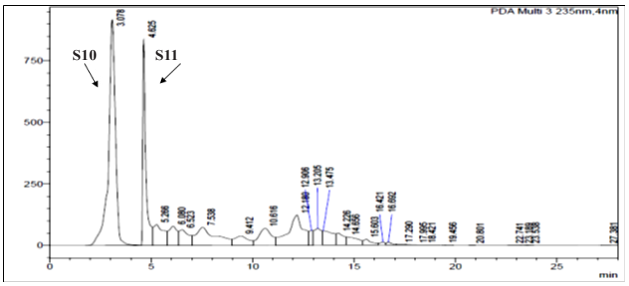


Figure 4: PHPLC chromatogram of chloroform fraction.

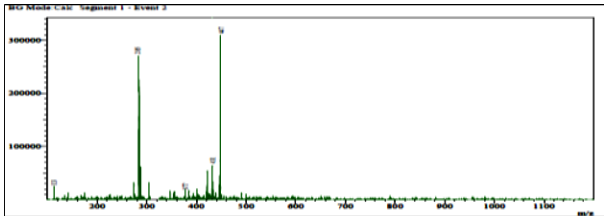


Figure 5: The LC-Mass spectrum of compound S8.

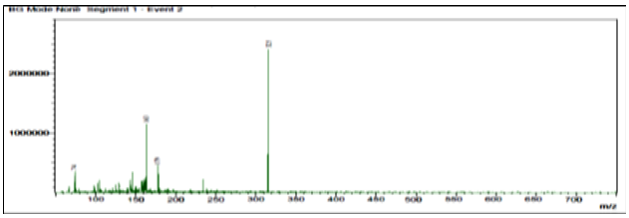


Figure 6: The LC-Mass spectrum of compound S9.

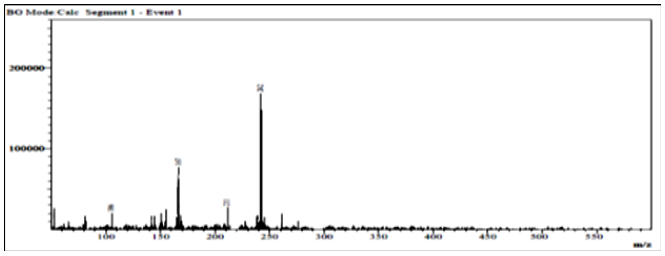


Figure 7: The LC-Mass spectrum of compound S10

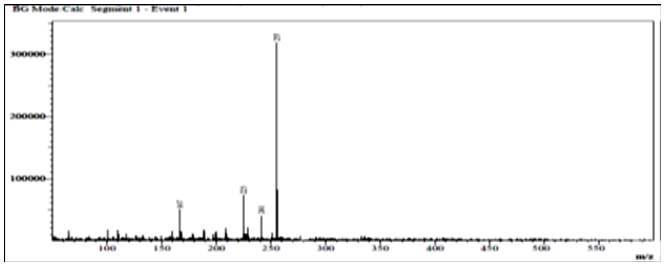


Figure 8: The LC-Mass spectrum of compound S11

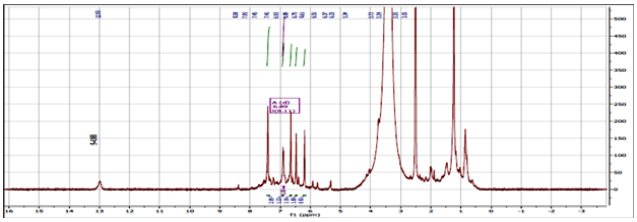


Figure 9: 1H-NMR spectrum of compound S8.

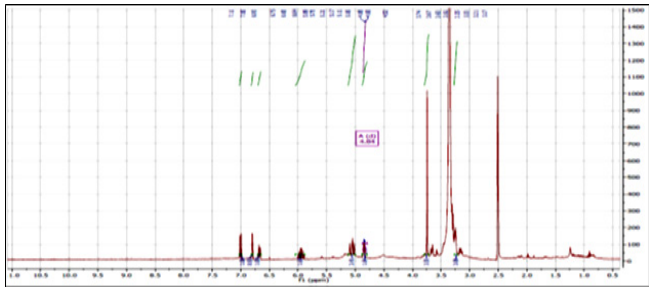


Figure 10: ^{1}H-NMR spectrum of compound S9.

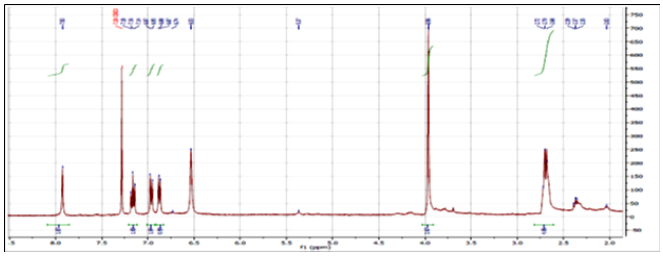


Figure 11: The ^{1}H-NMR spectrum of compound S10.

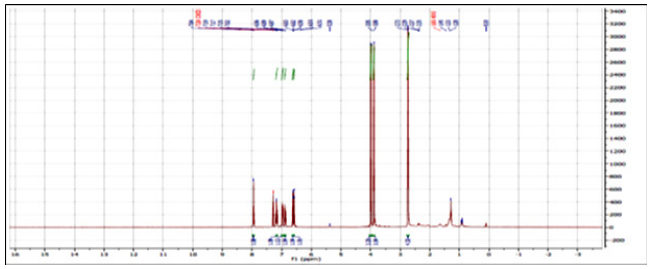


Figure 12: The ^{1}H-NMR spectrum of compound S11.

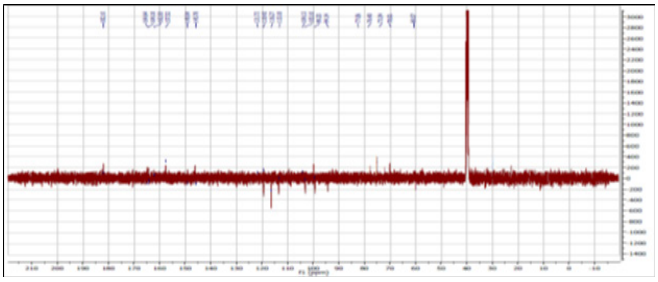


Figure 13: The APT ^{13}C-NMR spectrum of compound S8.

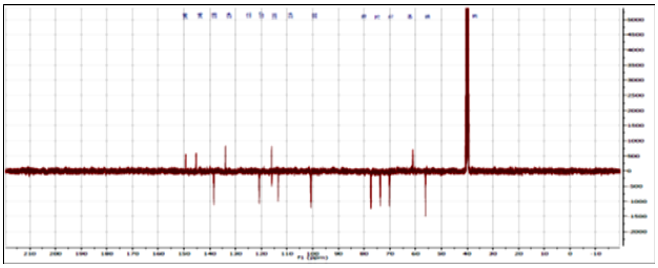


Figure 14: The APT ^{13}C-NMR spectrum of compound S9.

(C-7), 164.64 (C-2), 182.10 (C-4)), solvent used DMSO, S8 contains a flavone skeleton with 15 carbon atoms, and the compound was identified as luteolin-7-O-glucoside. The result was compatible with other published data.[29,30]

- Compound S9 was isolated as an amorphous powder

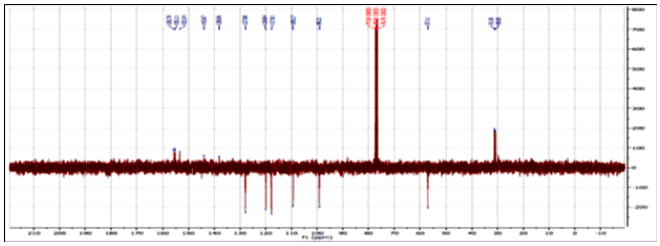


Figure 15: The APT ^{13}C-NMR spectrum of compound S10.

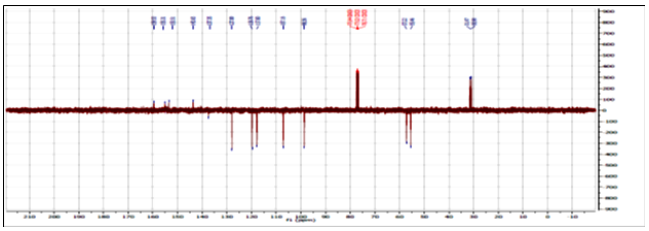
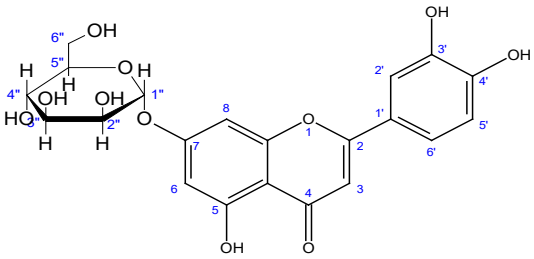


Figure 16: The APT ^{13}C-NMR spectrum of compound S11.



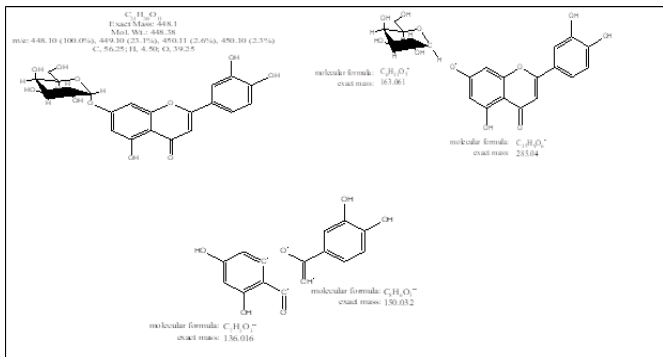
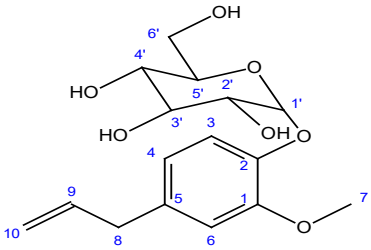


Figure 17: The Mass fragments of compound S8.

having the chemical formula C6H22O7 as determined by the ESI-MS m/z value of 325 [M-H]$^-$ Figure 6, The molecular ion peak for compound S9 [M-1]$^-$ is 325 with major fragments (179, 163, and 147) as shown in Figure 18. According to the ^{1}HNMR Figure 10 (3.17 (1H, m) H-4'and 5', 3.21 (1H, dd) H-2'and 3', 3.35 (2H, d) H-8, 3.67 (1H, d) H-6', 3.74 (3H, s) H-7, 4.52 (1H, s) 4' OH and 6' OH, 4.85 (2H, dd) H-10, 5.05 (1H, d) H-1' α, 5.11 (1H, s) 3' OH, 5.57 (1H, s) 2' OH, 5.89 (1H, m) H-9, 6.65 (1H, d) H-4, 7.05 (1H, d) H-3), and APT ^{13}CNMR Figure 14 (39.1 (C-8), 56.5 (C-7), 64.7 (C-6'), 71.3 (C-4'), 75.9 (C-3', C-5'), 78.2 (C-2'), 101.0 (C-1'), 114.1 (C-10), 116.7 (C-6), 117.2 (C-3), 121.1 (C-4), 134.6 (C-5), 138.3 (C-9), 146.2 (C-2), 149 (C-1), solvent used DMSO, S9 contains a skeleton with 15 carbon atoms, and the compound was identified as eugenyl-O-glucoside. The result was compatible with other published data.[31]



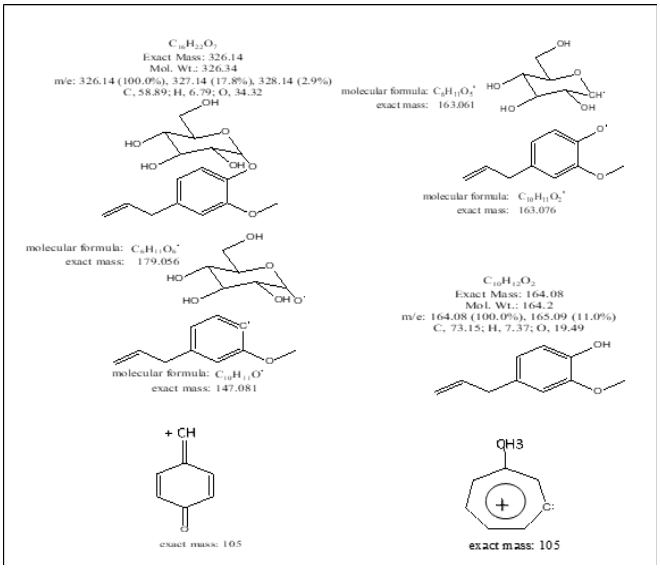
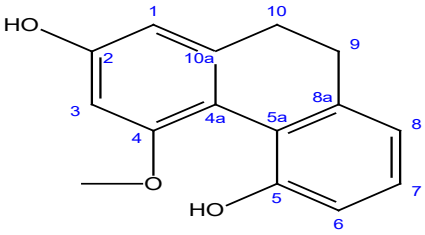


Figure 18: Mass fragments of compound S9.



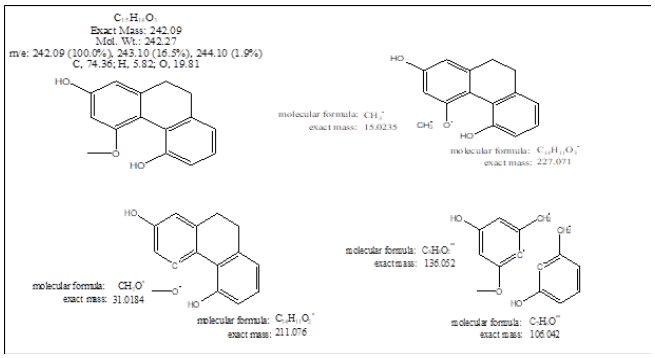


Figure 19: Mass fragments of compound S10.

- Compound S10 was isolated as an amorphous powder having the chemical formula C15H16O3 as determined by the ESI-MS m/z value of 242 [M-H]$^-$ Figure 7, The molecular ion peak for compound S10 [M-1]$^-$ is 242 with major fragments (211, 167, and 106) as shown in Figure 19. According to the ^{1}HNMR Figure 11 (2.35-2.39 (1H, m) H9, 2.68-2.72 (1H, m) H10, 3.96 (3H, s) O-CH3 on C4,

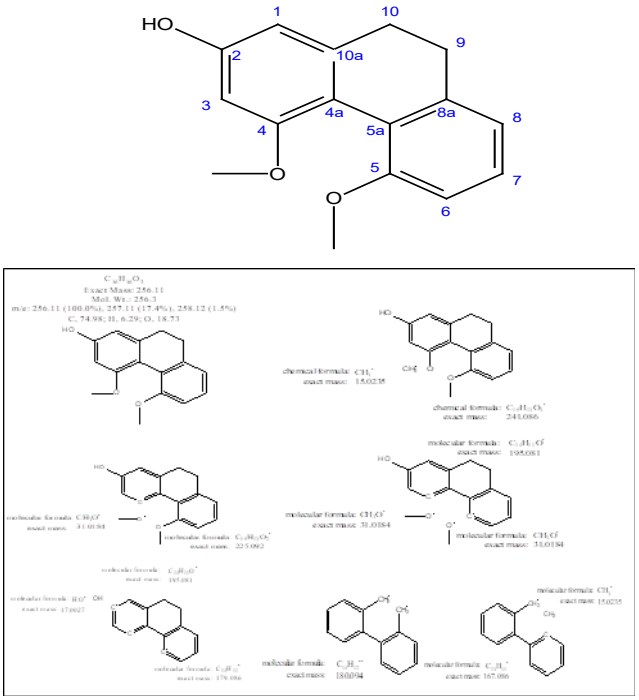


Figure 20: Mass fragments of compound S11.

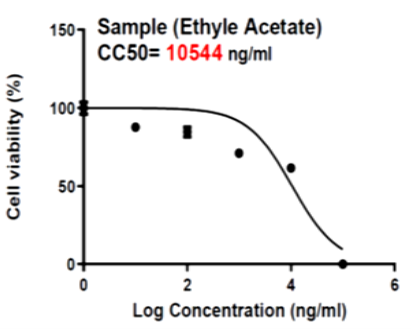


Figure 21: Cytotoxic concentration 50% CC_{50}.

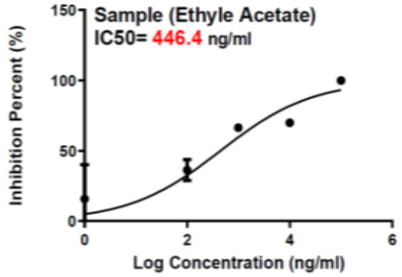


Figure 22: Inhibitory concentration 50% IC_{50}.

5.37 (1H, s, broad) C2-OH, 6.53 (1H, s) H-1, 6.74 (1H, s, broad) C5-OH, 6.87-6.88 (1H, dd) H-8, 6.95-6.97 (1H, dd) H6, 7.14-7.18 (1H, dd) H-7, 7.93 (1H, s) H-3) and APT ^{13}CNMR Figure 15 (30.83 (C9), 31.16 (C10), 57.11 (O-CH3 on C4), 99.21 (C3), 109.27 (C1), Small peak at 113 (C5a), 117.81 (C6), 119.84 doublets (C8), 119.84 (C4a), 127.88 (C7), 138.06 (C8a), 143.87 (C10a), 153.34 (C2), 155.11 (C5), 155.70 (C4)), solvent used HDO, CDCL3, S10 contains a skeleton with 15 carbon atoms, and the compound was identified as 2,5-dihydroxy-4-methoxy- 9,10-dihydrophenanthrene (hircinol), The result was compatible with other published data.[32]

- Compound S11 was isolated as an amorphous powder having the chemical formula C16H16O3 as determined by the ESI-MS m/z value of 255 $[M-H]^-$ Figure 8, The molecular ion peak for compound S11 $[M-1]^-$ is 255 with major fragments (167, 241, and 255) as shown in Figure 20. According to the ^{1}HNMR Figure 12 (2.35-2.37 (1H, m) H9, 2.39-2.73 (1H, m) H10, 3.88 (3H, s) OCH3 on C5, 3.95 (3H, s) OCH3 on C4, 5.38 (1H, br. s) OH, 6.51 (1H, S) merged with proton 8 peaks H-1, 6.55-6.63 (1H, dd) H-8, 6.87-6.96 (1H, dd) H-6, 7.02-7.19 (1H, dd) H-7, 7.94 (1H, s) H-3) and APT ^{13}CNMR Figure 16 (30.90 (C9), 31.47 (C10), 55.46 (O-CH3 on C5), 57.22 (O-CH3 on C4), 98.56 (C3), 107.16 (C1), Small peak at 112 (C5a), 117 (C4a), 117 doublets (C6), 119.76 (C8), 127.89 (C7), 137.35 (C8a), 143.65 (C10a), 155.51 (C2), 153.31 (C5), 159.53 (C4)), solvent used HDO and CDCL3, S11 contains a skeleton with 16 carbon atoms, and the compound was identified as 2-hydroxy-4,5-dimethoxy- 9,10-dihydrophenanthrene. The result was in agreement with some other published convergent data.[32]

Cytotoxic Effect

The cytotoxic effects of the polyphenolic compounds in the ethyl acetate fraction of *S. canadensis* had to be tested on Vero cells to rule out non-specific activities, and the maximal non-toxic concentration for the Vero cells was also calculated.

Graphs of cytotoxicity concentration 50% (CC_{50}) and the 50% inhibitory concentration (IC_{50}) on Vero E6 cells and H1N1 virus, Figure 21 and 22, respectively.

DISCUSSION

The phytochemical method used revealed the separation of four biologically active compounds; compound (S8) luteolin-O-glucoside, (S9) eugenyl-O-glucoside from ethyl acetate fraction, (S10) 2,4-dihydroxy-5-methoxy-9,10-dihydrophenanthrene (hircinol), and (S11) 2-hydroxy-4,5-dimethoxy-9,10-dihydrophenanthrene. Compounds S9, S10, and S11 are isolated and identified from *S. canadensis* for the first time.

The tested samples (ethyl acetate) showed moderate to high antiviral activities against H1N1 with selective index = estimated CC_{50}/estimated IC_{50} = 23.6.

The selectivity index (SI) is a ratio that divides the antiviral activity (AVA) value by the cytotoxicity (TOX) value (AVA/TOX) to determine the window between cytotoxicity and antiviral activity. The greater the SI ratio, the more successful and safer a medicine should be for treating a viral infection *in-vivo*. The ideal medicine would be cytotoxic at extremely high doses yet antiviral at very low concentrations, resulting in a high SI value (high AVA/low TOX) and the ability to destroy the target virus at concentrations much below its cytotoxic concentration. A compound's selectivity index is a commonly used metric to represent a compound's in vitro efficacy in inhibiting viral reproduction.[33]

One of the most commonly used ways of detecting cell viability is crystal violet (CV). CV is a triarylmethane dye that binds to ribose-type compounds in nuclei, such as DNA. Dead adherent cells will normally detach from cell culture plates and be washed away from the live cell population. CV staining may be used to measure cell viability following treatment with a virus and test substance by quantifying the total DNA of the residual population. At 570 nm, the CV staining is proportional to the cell biomass and may be quantified. For comparison, the cytotoxicity of the test substances is also measured in uninfected cells.[34]

The tested sample (ethyl acetate fraction) showed moderate to high antiviral activity against H1N1 with a selective index = estimated CC_{50}/IC_{50} = 23.6.

The obtained result reveals a linear connection between the concentration of the tested material and the degree of cytoprotecting (antiviral effect) against H1N1 virus cytotoxicity. The CC_{50}% was 10544 ng/mL, but the IC_{50}% was only 446.4 ng/mL, indicating that the tested material had no cytotoxic impact against vero cells in the control test.

The result revealed that polyphenols of *S. canadensis* at a concentration of 100 µg/mL have antiviral activity against the H1N1 virus with a viability% of reaching 99%.

Polyphenols show high effectiveness against the influenza virus due to their ability to reduce the level of reactive oxygen species (ROS) and increase the level of glutathione (GSH) in infected cells.[35]

Flavonoids, isoquercetin, and rutin interact primarily through the forming of multiple hydrogen bonds between hydroxyl groups with the key amino acids in the catalytic core of the neuraminidases enzyme (which is crucial for the release of progeny virions from the host cell), with the sugar portion bound at the center of the enzyme's active site, while flavonoid scaffolds help maintain this bonding preference.[36,37]

Rutin is a potent inhibitor for the SARS-CoV-2 main protease (Mpro) enzyme.[38]

Rutin displays antiviral effectiveness against murine norovirus MNV-1,[39] enhancing antibacterial activity,[40] antioxidant activity,[41] anticancer activity,[42] and anti-inflammatory.[43]

Isoquercetin (quercetin-3-b-D-glucoside) has antiviral properties against the H1N1 virus *in-vitro* and *in-vivo* by inhibiting multiplication with an effective dose ED_{50} of 1.2 lM and a high therapeutic index.[44]

Astragalin has various pharmacological activities like antioxidant,[45] antitumor,[46] and antiinflammatory.[47] Astragalin shows high binding energy in interaction with SARS-CoV-2 (main protease and spike protein).[48]

Luteolin-O-glucoside is a flavonoid that exhibits cancer chemo-preventive and cancer-fighting properties and anti-inflammatory and antioxidant activities.[49,50]

Quercetin shows a diversity of pharmacological activities like antioxidant, anticancer, anti-inflammatory, cardioprotective, antibacterial, wound healing, and antiallergic.[51-53]

In IVA Virus-infected cells, quercetin suppressed the production of HSPs such as HSP70 and HSPA8.[54] Another study demonstrated the ability of quercetin to inhibit IAV replication by regulating the expression of heat shock proteins, fibronectin 1, and prohibitin.[55]

Quercetin has the potential to interact with a surface protein (envelope protein hemagglutinin (HA)) that plays a key role in the viral entrance, blocking virus entry and suppressing viral infection.[56]

Chlorogenic acid (CHA) demonstrates anti-inflammatory, antipyretic, analgesic, antibacterial, antiulcerogenic, antioxidant, and antiviral activities.[57-62]

CHA may be useful in the treatment of influenza virus infection, It suppresses the expression of the H1N1 NP protein, inhibits the release of freshly generated virions from infected cells by the suppression of neuraminidase activity, and suppresses influenza virus at the late stages of the infectious cycle.[63]

Caffeic acid possesses several bioactivities like anticancer,[64] antibacterial,[65] neuroprotective,[66] antimalarial,[67] and anti-inflammatory.[68]

The methyl ester of caffeic acid showed antiviral properties against respiratory infections.[69]

Caffeic acid suppresses influenza virus multiplication by direct binding and interacting with key enzymes required for viral RNA replication in infected cells.[70]

3,4-DCQA increased the expression of tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) mRNA, whereas H1N1 hemagglutinin (HA) mRNA was somewhat decreased, resulting in improved viral clearance.[71]

3,5-DCQA possesses anti-human respiratory syncytial virus (RSV) activities by inhibiting viral cell fusion at the early stage of the RSV replication cycle and inhibiting cell-cell fusion at the end of the viral replication cycle.[72]

Eugenol and other eugenol derivatives have a wide range of biological activities in the treatment of bacterial and fungal infections, as well as in the treatment of cancer.[73]

CONCLUSION

Plant extracts are complex combinations of a variety of natural chemicals, synergistic strategies have been proposed to explain why plant complexes are frequently more effective than their most common individual components.

The activity of polyphenolic compounds detected in the ethyl acetate fraction of the *S. canadensis* L. plant demonstrates different mechanisms of action and properties that appear to have synergistic or additive effects against the H1N1 virus.
The tested samples are good candidates for further experiments as anti-influenza H1N1.

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