

**Isolation of cytotoxic flavonoids from aerial parts of *Coronopus didymus* by  
bioassay-guided fractionation**

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**ABSTRACT**

*Ethnopharmacological relevance:* *Coronopus didymus* Linn. (Brassicaceae) is a medicinal plant used traditionally as antipyretic, expectorant, to purify blood and for alleviating symptoms of pain, inflammations, malaria, wounds and cancer.

*Aim of the study:* The present study was designed to isolate and identify the cytotoxic compounds responsible for anticancer activity from this traditionally useful medicinal plant.

*Materials and methods:* Bioassay-guided fractionation of the ethanolic extract of aerial parts of *C. didymus* allowed the isolation of compounds responsible for anticancer activity. Their structures were elucidated by UV Spectroscopy (with shift reagents), ESI-MS and NMR spectral data. Preliminary anticancer activity of ethanolic extract, different fractions and isolated compounds was assessed through MTT *in vitro* cytotoxicity assay in a concentration dependent-manner against human cancer cell lines (HeLa and LN18) and normal 293T cells.

24 *Results:* Three flavonoids namely 5,7,4'-trihydroxy-3'-methoxyflavone-4'-O- $\beta$ -D-  
25 glucoside (**1**), 5,7,4'-trihydroxy-3'-methoxyflavone-4'-O-(6"-acetyl)- $\beta$ -D-glucoside (**2**)  
26 and 5,7,4'-trihydroxy-3'-methoxy flavone (**3**), were isolated from aerial parts. Compound  
27 **1** was identified for the first time from the genus *Coronopus*. All the compounds **1-3**  
28 showed promising activity against HeLa cells with IC<sub>50</sub> values of 20.10, 0.32 and 1.10  
29  $\mu$ g/mL, respectively. Significant result was also obtained with compound **3** against LN18  
30 cells with IC<sub>50</sub> value of 13.99  $\mu$ g/mL.

31 *Conclusion:* The cytotoxic activity of the crude extract and fractions which may largely  
32 be due to its major isolated constituents, flavonoids **1-3**, against both HeLa and LN18  
33 cells provides a scientific basis for the ethnopharmacological use of *C. didymus* as  
34 anticancer agent.

35 *Keywords:*

36 *Coronopus didymus*

37 Cytotoxicity

38 HeLa and LN18 cell lines

39 Anticancer activity

40 Flavones

## 41 **1. Introduction**

42 Cancer is among the leading causes of death worldwide. According to National  
43 Cancer Institute, in 2016, about 16.8 million new cases are expected to be diagnosed and  
44 5.9 million cancer-related deaths just in the U.S. There are over 200 different known  
45 cancers that affect humans. So, tremendous resources are being invested in prevention,  
46 diagnosis, and treatment of cancer. The main focus of many pharmaceutical companies as

47 well as organizations, like the National Cancer Institute in the United States, the  
48 European Organization for Research and Treatment of Cancer, and the British Cancer  
49 Research Campaign is the discovery and development of anticancer agents by  
50 identification of cytotoxic compounds. For evaluating preliminary anticancer activity of  
51 natural products in terms of cell viability, the 3-(4,5-Dimethylthiazol-2-yl)-2,5-  
52 diphenyltetrazolium bromide (MTT) *in vitro* cytotoxicity assay is considered as a highly  
53 versatile, reliable, most economic, widely used and convenient method.

54 Over the last century, a number of top selling drugs have been developed from  
55 natural products *i.e.*, vincristine from *Vinca rosea*, silymarin from *Silybum merianum*,  
56 paclitaxel from *Taxus brevifolia*, resveratrol from *Vitis vinifera* *etc.*). 40% of the modern  
57 drugs in use have been developed from natural products (**Sarker et al., 2006**). According  
58 to the bulletin of the **World Health Organization (2002)** up to 80% of people in  
59 developing countries rely on plant-based traditional medicines for primary health care  
60 and its use is increasing. In some countries such as Singapore and the Republic of Korea  
61 where the conventional health-care system is quite well established, 76% and 86% of the  
62 respective populations still commonly use traditional medicines (**WHO, 2012**).  
63 According to **Newman and Cragg (2016)** in the area of cancer, over the time frame from  
64 around the 1940s to the end of 2014, of the 175 small molecules approved, 85, or 49%,  
65 are natural products or their derivatives. Hence, today major efforts are directed toward  
66 the identification of anticancer compounds from natural sources like plants.

67 *Coronopus didymus* Linn. (Swinecress) is a medicinal plant of the Brassicaceae  
68 family, found in wastelands, along roadsides, as well as a weed of cultivated areas.  
69 Previous studies of the secondary metabolites of *C. didymus* reported flavonoids,

70 glucosinolates, sterol glycosides, saponins and anthraquinones as the chemotaxonomic  
71 markers of the genus *Coronopus*. Perusal of literature reveals that *C. didymus* is used  
72 traditionally for the treatment of several diseases in which pain and inflammations are  
73 common (**Busnardo et al., 2010**) and for malaria, cancer, digestive, antipyretic and as  
74 expectorant (**De Ruiz et al., 1994**). The traditional use of this plant for anticancer activity  
75 in different parts of the world led us to explore the cytotoxicity activity of extract and  
76 fractions of this species, and to isolate and identify the active cytotoxic compounds.

77 In our previous work we reported the identification of 68 volatile compounds  
78 from the aerial parts and roots of this plant using Gas Chromatography-Mass  
79 Spectrometry (**Noreen and Farman, 2016**). In continuation of our exploration on *C.*  
80 *didymus*, we present here the activity-guided fractionation, structural elucidation and  
81 cytotoxic activity of isolated flavonoids (**1-3**), from aerial parts of this useful plant. These  
82 isolated compounds belong to class flavonoids particularly flavone. Their structures were  
83 elucidated by UV-Vis. Spectroscopy (with shift reagents), LC-ESI-MS analysis and  
84 NMR experiments. Detailed literature survey revealed that compounds **2** and **3** have  
85 previously been isolated from this plant (**Mishra et al., 2003; Prabhakar et al., 2002**),  
86 while compound **1** has never been isolated from *C. didymus* before, but it has been  
87 isolated from other Brassicaceae plants. The cytotoxic activity of the isolated flavonoids  
88 from *C. didymus* is carried out for the first time and is also compared with standard  
89 cytotoxic flavonoids and anticancer drug fluorouracil in this study.

## 90 **2. Experimental**

### 91 *2.1. Plant Material Collection and Identification*

The aerial parts of *C. didymus* were collected and dried at room temperature in spring 2015 from Sector I-8/1 Islamabad, Pakistan, from the plant identified by Dr. Mushtaq Ahmad, Department of Plant Sciences, Quaid-i-Azam University, Islamabad, Pakistan. A voucher specimen (No. 74) of this plant has been housed in the Herbarium of the same Department.

## 2.2. Extraction of the Plant Material

The dried aerial parts of *C. didymus* (1.0 kg) were chopped and extracted by maceration with ethanol ( $3 \times 10$  L) at ambient temperature for 7 days. The combined extracts were filtered and evaporated *in vacuo* using rotary evaporator (EYELA N-11 Rotavapor) at 45°C to afford a crude ethanolic extract (CDA\_11, 200g). Paper Chromatography (PC) of the ethanolic extract was done on Whatman® cellulose chromatography papers 1 Chr sheets (20 x 20 cm, Sigma-Aldrich) in 15% acetic acid and the spots after development were visualized under UV light at longer wavelength (365nm). The 200g of ethanolic extract was then dissolved in minimum amount of MeOH/ H<sub>2</sub>O (7:3, v/v) to get phenolic extract (CDA\_MW, 67 mL), for subsequent use in isolation of compounds by Size Exclusion Chromatography.

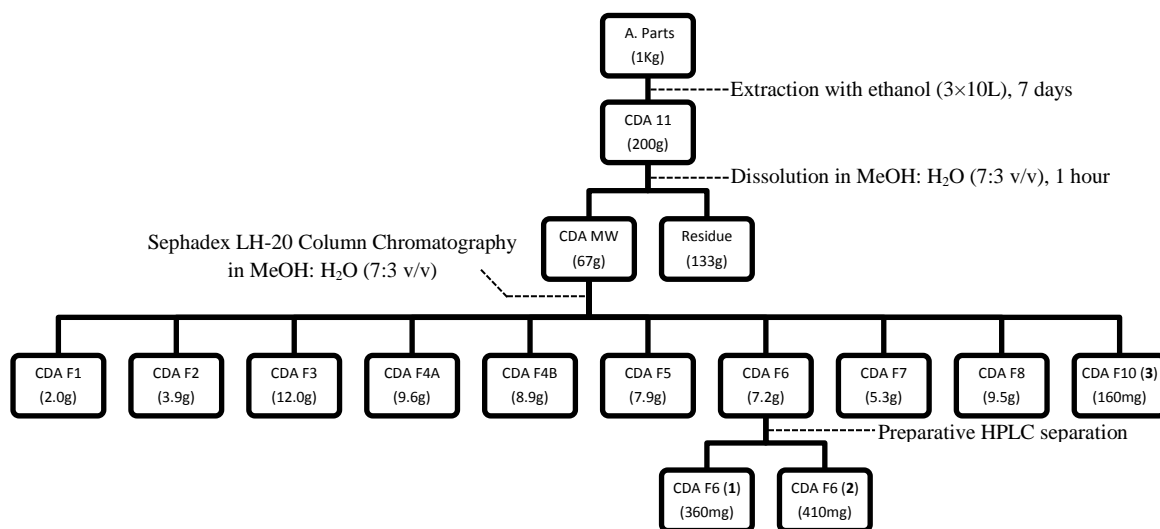
## 2.3. Purification by Size Exclusion Chromatography (SEC)

Purification of phenolic extract (CDA\_MW) was performed by Size Exclusion Chromatography on Sephadex LH-20 (Sigma Life Science, Sweden) column (90 cm × 2.2 cm) using Methanol/Water in a ratio of 7:3 (v/v) at room temperature as eluent, at a flow rate of 1 mL/min. to yield ten fractions (CDA\_F1-CDA\_F10) (**Fig. 1**) which were separated on the basis of the sizes of the eluting components. All the fractions were pooled together, concentrated *in vacuo* and submitted for LC-MS analysis and analyzed

115 by combined TLC analysis using pre-coated Silica gel 60 F<sub>254</sub> plates (0.25 mm, Merck).  
116 Pure fraction (CDA\_F10) containing one flavonoid aglycone (**3**) (160 mg) was obtained  
117 from SEC.

#### 118 *2.4. Preparative HPLC separation of phenolic constituents of fraction CDA\_F6*

119 Fraction CDA\_F6 was purified by Preparative HPLC on a Quattro HPLC system  
120 using reverse phase RP-18 endcapped Chromolith Performance column (100-4.6 mm), in  
121 negative-ion ESI mode using a capillary voltage of 3.0 kV, a cone voltage of 20 V,  
122 extractor voltage of 3 V, collision energy of 10 eV, desolvation temperature 200 °C and  
123 desolvation gas flow 250 (L/hr). The solvent system consisted of mobile phase A (milli-Q  
124 water) and mobile phase B (acetonitrile), at a flow rate of 2 mL/min. with gradient  
125 elution program: 0-1.0 min. isocratic 15% B, 1.0-8.0 min. linear gradient from 15% to  
126 30% B, 8.0-12.0 min. isocratic 30% B, 12.0-12.1 min. linear from 30% to 85% B, 12.1-  
127 13.0 min. isocratic 85% B, with 13.0-15.0 min. for initial conditions of 15% B for  
128 column equilibration. Total run time was 15min. using 15 µL sample injection volume to  
129 afford eight sub-fractions (CDA\_F6\_1N to CDA\_F6\_8N) which were separated on the  
130 basis of retention time. Chromatographic data was acquired using MassLynx V4.1  
131 software. Compound **1** (CDA F6 3N, 1.6 mg) and compound **2** (CDA F6 6N, 2.1 mg)  
132 were collected preparatively on this system from peaks eluting at 4.85 min. and at 7.28  
133 min. respectively, from 5 injections in yields of 31% and 45% from fraction CDA F6 and  
134 were pooled together and submitted for further analysis. The purity of the isolated  
135 compounds was checked by HPLC-ESI-MS. UV-Vis. spectra were recorded on UV-1700  
136 PHARMASPEC, Spectrophotometer (Shimadzu) and data was processed by UVProbe  
137 Version 2.00.



**Fig. 1.** Flow chart of bioactivity-guided fractionation of ethanolic extract of aerial parts of *C. didymus* showing separated fractions and isolated compounds.

## 2.5. UPLC-MS

LCMS analyses were performed on Waters ACQUITY Ultra-Performance Liquid Chromatography (UPLC) system using Purospher® STAR column packed with RP-18 endcapped (3 $\mu$ m), (Hibar® HR 5.0-2.1, UHPLC Column, Germany), directly coupled with Exactive Orbitrap Mass Spectrometer (MS). The solvent system consisted of mobile phase A (0.1% formic acid in milli-Q water) and mobile phase B (acetonitrile), at a flow rate of 0.2 mL/min. with gradient elution program: 0-1.0 min. isocratic 5% B, 1.0-8.0 min. linear gradient from 5% to 99% B, 8.0-10.0 min. isocratic 99% B, with 10.0-12.0 min. for initial conditions of 5% B for column equilibration. Total run time was 12 min. using 5  $\mu$ L sample injection volume. The Electro Spray Ionization (ESI) in negative mode with capillary voltage 2.5 kV, a cone voltage 15 V, collision energy of 5 eV, source temperature 350 °C, and desolvation gas temperature 190 °C was used. Samples were analyzed by LCMS using Single Reaction Monitoring mode (SRM) for identification of

152  $m/z$ , retention time and accurate masses of compounds **1-3**. Chromatographic data was  
153 collected and processed by Xcalibur software.

## 154 2.6. Nuclear Magnetic Resonance

155  $^1\text{H}$  NMR and One-Dimensional (1D) NOE spectra were acquired in  $\text{MeOH-d}_4$   
156 using a Bruker AVIII 700 spectrometer, 700-MHz NMR instrument equipped with a TCI  
157 cryoprobe at 298 K. Standard pulse sequences and parameters were used to obtain NMR  
158 spectra and the chemical shifts were expressed as  $\delta$  (delta) values.

## 159 2.7. In vitro cytotoxic activity

### 160 2.7.1. Cell culture conditions

161 Human cervical cancer cells (HeLa), human glioblastoma cells (LN18) and  
162 normal human embryonic kidney cells (HEK-293T) were procured from internal cell  
163 bank, Chemistry Research Laboratory, University of Oxford, UK. Cells were grown in an  
164 incubator (BINDER CB150  $\text{CO}_2$  incubator, Akribis Scientific, UK) at  $37^\circ\text{C}$  and 5%  $\text{CO}_2$   
165 in Dulbecco's Modified Eagle's Medium (DMEM D6546, Fisher, UK) supplemented  
166 with 10% Fetal Bovine Serum (FBS) and 1% GlutaMAX<sup>TM</sup> (200 mM L-alanyl-L-  
167 glutamine dipeptide in 0.85% NaCl) (Gibco<sup>®</sup> by Life Technologies, Invitrogen,  
168 ThermoFisher Scientific, UK). At 85-90% confluence, HeLa cells and LN18 cells grown  
169 in T75 flasks were harvested using (1mL) 0.25% trypsin/ EDTA solution (Gibco<sup>®</sup> by Life  
170 Technologies, ThermoFisher Scientific, UK), while 293T cells were harvested with  
171 media but no trypsin (non-adherent). Cells were counted using Trypan Blue staining and  
172 an automated cell counter (Countess<sup>®</sup> II FL, ThermoFisher Scientific, UK).

### 173 2.7.2. Cytotoxicity assay



174           The cells were seeded onto 96-well tissue culture plates (Costar Flat Bottom  
175   Transparent Polystyrene), from 10-15 passages (P), with three plates of each cell line at  
176   plating density of  $1.17 \times 10^3$  cells/100  $\mu$ L/well for Hela cells (P-13),  $5.28 \times 10^3$  cells for  
177   293T (P-15) and  $3.52 \times 10^3$  for LN18 cells (P-14) in all wells except wells A1-H1  
178   (Blank, which do not contain any cells) and incubated for 24 hours at 37°C/5% CO<sub>2</sub> to  
179   allow cells to become adherent. Stock solutions of crude extracts, all the sephadex  
180   column chromatography fractions (see section 2.3), isolated compounds (see section 2.4)  
181   and standards were prepared in dimethyl sulfoxide (DMSO) at 10 mg/mL concentration  
182   and diluted in the respective medium (DMEM) containing 10% FBS and 1% GlutaMAX  
183   in four different concentrations; 50, 100, 200 and 400  $\mu$ g/mL. After 24 h of incubation at  
184   37°C/5% CO<sub>2</sub>, when cells reached more than 80% confluence, the old medium was  
185   removed by gently inverting and tapping the plates. After the plates were washed with  
186   100  $\mu$ L of Phosphate Buffer Saline (PBS), then three wells (A2-H10) from each cell line  
187   were treated with fresh medium (100  $\mu$ L) containing one of each of the various  
188   concentrations of plant extracts to be tested and incubated at 37°C/5% CO<sub>2</sub> for 24 h. In  
189   addition the same process was performed for the positive controls *i.e.*, fluorouracil, gallic  
190   acid, diosmetin, apigenin, luteolin and quercetin in the same concentration range in wells  
191   11A-11H. Control cells (12A-12H) were supplemented with 100  $\mu$ L of media while  
192   0.05% DMSO (v/v) was used as vehicle control. Blank wells (1A-1H) contained 100  $\mu$ L  
193   medium without cells.

#### 194   2.7.3. *MTT assay method*

195           After 24 h incubation, the supernatant was removed by gently inverting and  
196   tapping the plates. After the plates were washed with 100  $\mu$ L of PBS, 80  $\mu$ L of fresh

medium and 20  $\mu$ L of 5 mg/mL solution of 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) in PBS (buffer) was added to each well using a multichannel pipette and incubated for 3 hours at 37°C/5% CO<sub>2</sub>. After incubation, the supplement was removed by gently inverting and tapping the plates and wells were washed with PBS (100  $\mu$ L). The remaining dark blue formazan crystals under light protection were dissolved in 100  $\mu$ L of DMSO. The plates were shaken to enhance dissolution of MTT formazan and incubated at 37 °C for 30 min. The absorbance of each well was measured on a microplate reader (POLARstar OPTIMA, BMG LABTECH, Germany) at 544 nm wavelength. Cell viability at each plant extract concentration was calculated as a percentage of the control activity from the absorbance values using the following formula (1);

$$\text{Cell viability (\%)} = \frac{A_{\text{Sample}} - A_{\text{Blank}}}{A_{\text{Control}} - A_{\text{Blank}}} \times 100 \quad (1)$$

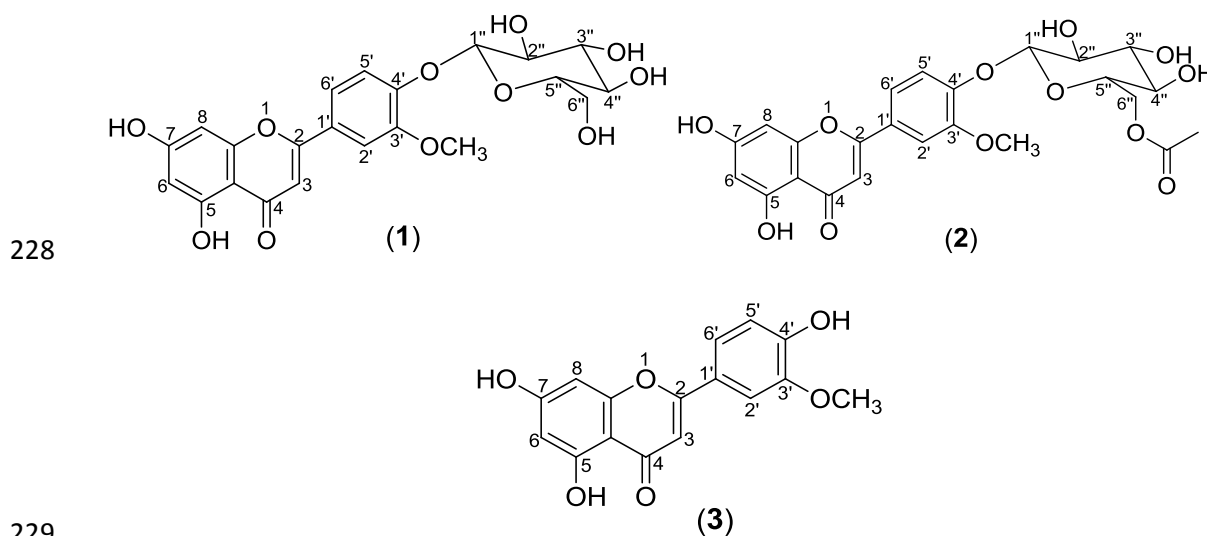
#### 2.7.4. Statistical analysis

All the data were presented as mean of three separate experiments, the standard error calculated from these measurements was provided as error bars. The dose-response data was best fitted to a straight line after logarithmic-transformation of the X-axis. The charts were plotted between percentage of cell viability and natural log (LN) of concentrations. The drug concentration that reduced the viability of cells by 50% (IC<sub>50</sub>) was determined from linear regression analysis using Microsoft excel with it's Data Analysis add-in. IC<sub>50</sub> values  $\leq$  300 were considered active.

### 3. Results and discussion

#### 3.1. Structural determination

Size Exclusion Chromatography of the extract (CDA\_MW) followed by preparative HPLC of active fraction CDA\_F6 yielded compounds (**1-3**), as yellow amorphous solids. The identity of the isolated cytotoxic compounds **1-3** from *C. didymus* was confirmed by using UV Spectroscopy (with shift reagents), HPLC coupled with on-line Mass Spectrometry (MS) using an Electrospray Ionization source in negative ion mode and NMR spectral data, and were determined as 5,7,4'-trihydroxy-3'-methoxyflavone-4'-*O*- $\beta$ -*D*-glucoside (**1**), 5,7,4'-trihydroxy-3'-methoxyflavone-4'-*O*-(6''-acetyl)- $\beta$ -*D*-glucoside (**2**) and 5,7,4'-trihydroxy-3'-methoxy flavone (**3**) (**Fig. 2**). To the best of our knowledge this is the first report on the isolation of the compound **1** from this genus.

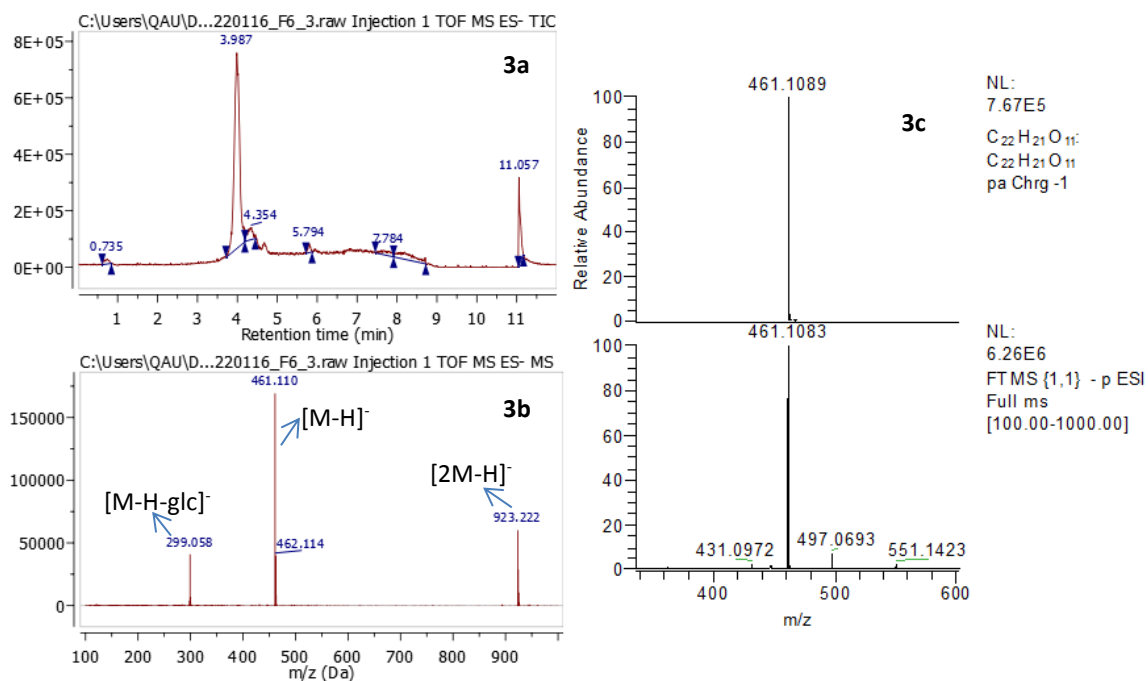


**Fig. 2.** Chemical structures of 5,7,4'-trihydroxy-3'-methoxyflavone-4'-*O*- $\beta$ -*D*-glucoside (**1**), 5,7,4'-trihydroxy-3'-methoxyflavone-4'-*O*-(6''-acetyl)- $\beta$ -*D*-glucoside (**2**) and 5,7,4'-trihydroxy-3'-methoxy flavone (**3**) isolated from *C. didymus* aerial parts.

Considering the possibility that compound **1** and **3** might have been formed through hydrolysis of compound **2** during separation procedures, but their presence on two Dimensional-Paper Chromatography of the crude ethanolic extract after development

236 with 15% acetic acid and the visualization of the spots under UV light at longer  
237 wavelength (365nm), indicates that this is not the case and all the three compounds  
238 appeared as deep purple color spots on PC. On spraying with ammonia these spots  
239 changed in color from deep purple to yellow-green, which is an indication of flavones  
240 with 5-OH group (Mabry et al., 1970).

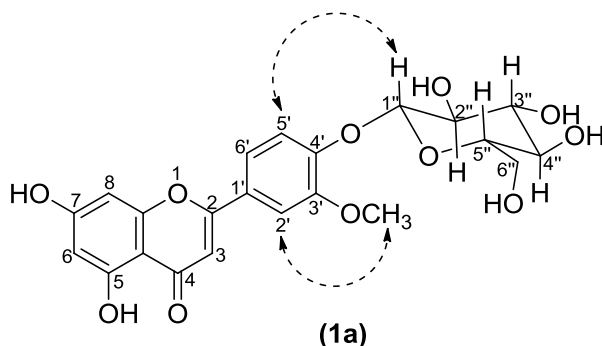
241 Compound **1** was isolated as yellow amorphous solid, appeared on Total Ion  
242 Current (TIC) chromatogram (**Fig. 3a**, tR: 3.99 min.) when the mobile phase composition  
243 was 40% acetonitrile (B) in water. This eluent composition indicated the presence of  
244 some hydrophilic moiety (sugar) in compound (**1**). The aglycone peak (**Fig. 3b**) at  $m/z$   
245 299  $[M-H-162]^-$ , appeared due to loss of 162 *a.m.u.* from molecular ion which indicated  
246 the presence of hexose as sugar moiety in the molecule. The High Resolution  
247 Electrospray Ionization Mass Spectrum (HR-ESI-MS) analysis (**Fig. 3b**) showed quasi-  
248 molecular ion peak at  $m/z$  461.1089  $[M-H]^-$ , consistent with the molecular weight 462,  
249 with Molecular formula  $C_{22}H_{22}O_{11}$ , within 5 ppm error of the theoretical  $m/z$  value  
250 ( $C_{22}H_{21}O_{11}$  calculated as  $m/z$  461.1083, lower spectrum **Fig. 3c**). Peak at  $m/z$  923  $[2M-H]^-$   
251 (**Huang et al. 1999**) attributed to the dimer of compound **1**.



**Fig. 3.** TIC (a), ESIMS (b) and HR-LC-ESI-MS (c) spectra of compound **1**.

Compound (**1**) was identified as a flavone, which is clear from the characteristic absorption pattern of Band II (269, 289sh) and Band I (334 nm) in MeOH and is also confirmed by the presence of free hydrogen (H3) at carbon number three (C3) by the presence of singlet due to 1H at  $\delta = 6.76$  ppm (aromatic proton) in  $^1\text{H}$ -NMR spectrum. The A-ring of the flavone **1** is represented by two meta-coupled resonances at  $\delta_{\text{H6}}$  6.236 (d,  $J = 2.1$  Hz) and  $\delta_{\text{H8}}$  6.499 (d,  $J = 2.1$  Hz), assigned to H-6 and H-8, respectively which confirmed the presence of substitution (hydroxylation) at 5 and 7 position. The assignments of all the other protons are listed in **Table 1**. 1D-NOESY experiment helps in determining the position of certain groups at particular position in the molecule. Irradiation of the three protons of methoxy group ( $\text{OCH}_3$ , at the C3' position) (**Fig. 4**) resulted in the increase in intensity of proton signal of C2' which showed connectivity of the methoxy group with the protons of the carbon C2' (dotted arrow) that was also present

on the B ring of flavone, which confirmed that OCH<sub>3</sub> and H2' are closer in space and that OCH<sub>3</sub> is also present on B ring at C3' position.



**Fig. 4.** Key NOESY correlations of compound **(1)** from *C. didymus* aerial parts.

Anomeric proton (H1'') appeared as doublet at  $\delta_H$  5.073 with trans coupling  $J = 8.4$  Hz due to coupling with one proton on adjacent carbons of sugar at H2'', which confirmed the diaxial orientation (angle 180°) of proton at H1'' and H2'' and that the sugar has  $\beta$  configuration. Irradiation of the anomeric proton (H1'') of the *O*-glucoside group (**Fig. 4**) showed connectivity with the proton H5' (dotted arrow) that is present on the B ring of flavone, which showed that glucose moiety and H5' are closer in space, which confirmed that glucose is present on B ring at 4' position, this also indicates that anomeric proton is axial. All information from  $t_R$ , DAD, MS and NMR experiments indicated that the correct structure of compound is **1a** (5,7,4'-trihydroxy-3'-methoxyflavone-4'-*O*- $\beta$ -*D*-glucoside).

**Table 1**

<sup>1</sup>H NMR spectroscopic data of compounds **1-3**.

C/H	<b>1</b>		<b>2</b>		<b>3</b>	
	$\delta_H$ (ppm)	$J$ (Hz)	$\delta_H$ (ppm)	$J$ (Hz)	$\delta_H$ (ppm)	$J$ (Hz)
3	6.725, 1H, s	-	6.732, 1H, s	-	6.661, 1H, s	-

6	6.236, 1H, d	$^4J = 2.1$	6.236, 1H, d	$^4J = 2.1$	6.239, 1H, d	$^4J = 2.1$
8	6.499, 1H, d	$^4J = 2.1$	6.499, 1H, d	$^4J = 2.1$	6.498, 1H, d	$^4J = 2$
2'	7.582, 1H, d	$^4J = 2.1$	7.593, 1H, d	$^4J = 2.1$	7.516, 1H, s	$^4J = 2$
3'-OCH <sub>3</sub>	3.995, 3H, s	-	4.000, 3H, s	-	3.990, 3H, s	-
5'	7.331, 1H, d	$^3J = 8.4$	7.272, 1H, d	$^3J = 8.4$	6.965, 1H, d	$^3J = 8.5$
6'	7.630, 1H, dd	$^3J=8.4, ^4J=2.1$	7.622, 1H, dd	$^3J=7.0, ^4J=2.1$	7.544, 1H, dd	$^3J=8.5, ^4J=2$
1''	5.073, 1H, d	$^3J = 8.4$	5.074, 1H, d	$^3J = 7.7$	-	-
2''	3.512, 1H, t	-	3.567, 1H, m	-	-	-
3''	3.568, 1H, m	-	3.515, 1H, t	-	-	-
4''	3.51, 1H, m	-	3.427, 1H, m	-	-	-
5''	3.44, 1H, t	-	3.713, 1H, m	-	-	-
6''a	3.725, 1H, dd	$^2J=12.6, ^3J=5.6$	4.421, 1H, dd	$^2J=9.8, ^3J=2.1$	-	-
6''b	3.922, 1H, dd	$^2J=11.9, ^3J=2.1$	4.267, 1H, dd	$^2J=6.3, ^3J=5.6$	-	-
6''-OCOCH <sub>3</sub>	-	-	2.070, 3H, s	-	-	-

Spectra were obtained at 700MHz in MeOH-d<sub>4</sub>.

282

283 Compound **2** was isolated as yellow amorphous solid, appeared on HPLC  
284 chromatogram (tR = 4.46 min.) and showed quasi-molecular ion peak [M-H]<sup>-</sup> by high  
285 resolution ESI-MS at  $m/z$  503.1195 consistent with the molecular formula C<sub>24</sub>H<sub>24</sub>O<sub>12</sub>  
286 (MW = 504), within 5 ppm error of the theoretical  $m/z$  value (C<sub>24</sub>H<sub>23</sub>O<sub>12</sub> calculated as  $m/z$   
287 503.1187). The aglycone peak appeared at  $m/z$  299 [M-H-162-42]<sup>-</sup>, appeared due to loss  
288 of 204 *a.m.u.* from molecular ion which indicated the presence of acetyl hexose in the  
289 molecule.

290 The isolated compound **2** was identified as a flavonoid that was clear from its  
291 UV-Vis. absorption spectra in methanol with shift reagents. It was a flavone glycoside,  
292 which was clear from the characteristic absorption pattern of Band II (271, 290sh) and  
293 Band I (337 nm) in MeOH. A signal at  $\delta = 2.070$  appeared as a singlet due to three

294 protons in  $^1\text{H}$ -NMR confirmed the presence of acetyl group ( $6''\text{-OCOCH}_3$ ). The structure  
295 was confirmed based on NMR spectroscopic data (**Table 1**). All information from  $t_R$ ,  
296 DAD, MS and NMR spectroscopic data indicated that the correct structure of compound  
297 **2** is 5,7,4'-trihydroxy-3'-methoxyflavone-4'-*O*-(6''-acetyl)- $\beta$ -*D*-glucoside.

298 Compound **3** was isolated as a yellow powder, appeared on HPLC chromatogram  
299 ( $t_R$ : 5.02 min.) and showed quasi-molecular ion peak  $[\text{M-H}]^-$  by high resolution ESI-MS  
300 at  $m/z$  299.0561 consistent with the molecular formula  $\text{C}_{16}\text{H}_{12}\text{O}_6$  (MW = 300), within 5  
301 ppm error of the theoretical  $m/z$  value ( $\text{C}_{16}\text{H}_{11}\text{O}_6$  calculated as  $m/z$  299.0549).

302 Compound **3** was a flavonoid that was clear from its UV-Vis. absorption spectra  
303 in methanol with shift reagents. It was a flavone, which was clear from the characteristic  
304 absorption pattern of Band II (251, 269, 291sh) and Band I (347 nm) in MeOH. UV with  
305 shift reagent has been applied in studying various oxygenation patterns of this flavonoid.  
306 The presence of free 4'-OH was observed from bathochromic shift (+60 nm) as appeared  
307 in band I with the increase of intensity upon addition of sodium methoxide (NaOMe).  
308 The shift of 35 nm of band I with the decrease of intensity upon addition of aluminum  
309 chloride ( $\text{AlCl}_3$ ) was specific for a 5-hydroxyl substituent. Addition of aluminum chloride  
310 after neutralization with HCl gave the same 35 nm shift of band I with decrease in  
311 intensity (no change). This was due to complex formation with only the C-4 keto function  
312 and the 5-hydroxyl group which is very stable and do not break on addition of HCl and  
313 absence of catechol moiety in B ring. The shift of 23 nm of band II was observed with  
314 weak base NaOAc, which was characteristic for a free 7-hydroxyl group. On addition of  
315 boric acid reagent a very small shift of 2 nm in band I indicate absence of ortho-  
316 dihydroxyl groups on the B-ring which also confirmed the presence of some substituent



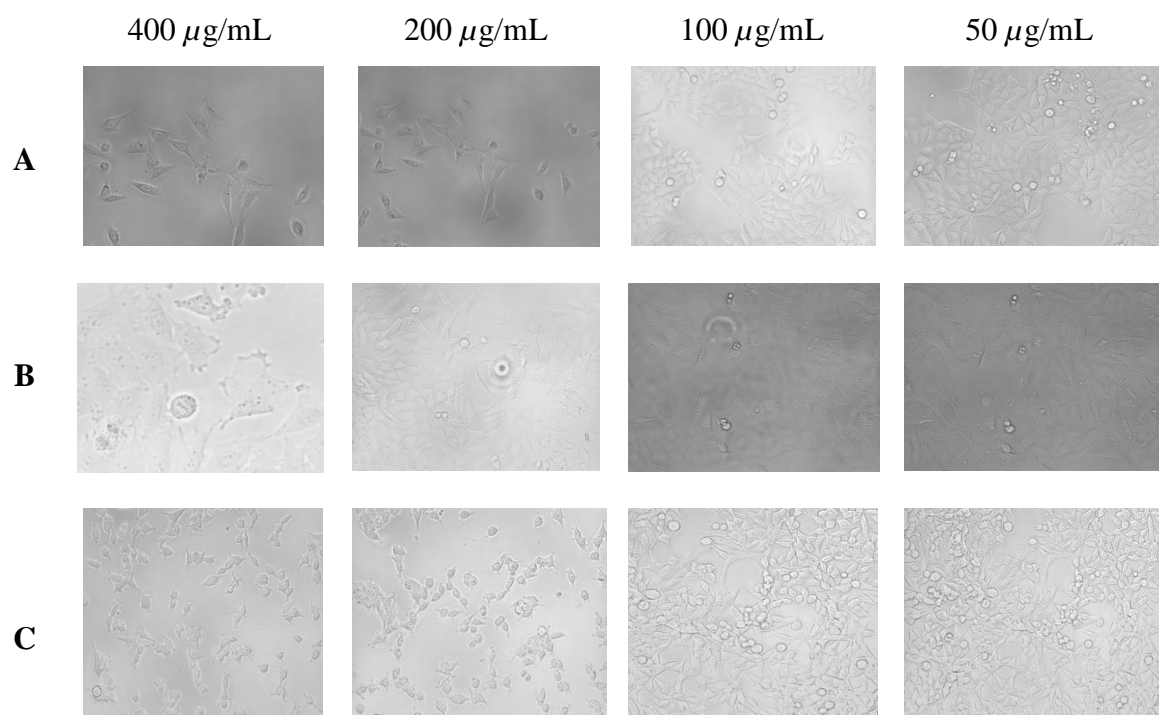
317 at 3'-OH. A signal at  $\delta = 3.99$  as a singlet in  $^1\text{H}$ -NMR also confirmed the presence of  
318 methoxy group (3'-OCH<sub>3</sub>). The structure was confirmed based on NMR spectroscopic  
319 data (**Table 1**). All information from  $t_R$ , DAD, MS and NMR spectroscopic data  
320 indicated that the correct structure of compound **3** is 5,7,4'-trihydroxy-3'-methoxyflavone.

### 321 3.2. Cytotoxic activity

322       Ethanollic extract of aerial parts (CDA 11) and phenolic extract (CDA MW) of *C.*  
323 *didymus* were found to possess good cytotoxic activity when screened against HeLa and  
324 LN18 cancer cells using MTT assay, while they did not show significant cytotoxic  
325 activity towards normal 293T cells. The reduction because of the treatment with the  
326 phenolic extract was more than the ethanollic extract. Therefore, the IC<sub>50</sub> value was  
327 102.91 and 111.72  $\mu\text{g/mL}$  for the phenolic and the ethanollic extract, respectively, against  
328 HeLa cells (**Table 2**). MTT assay was used to determine the drug response in cancer cells  
329 and overall cytotoxicity of compounds. MTT assays measured the mitochondrial  
330 enzymatic activity of cells which reduces a soluble tetrazolium containing yellow dye  
331 (MTT) into a purple colored insoluble formazan containing dye. Rapidly dividing cells,  
332 such as cancer tumor cells, show a high degree of metabolic activity and thus show a high  
333 degree of MTT reduction. The amount of formazan produced is directly proportional to  
334 the number of viable cells in the culture, indicating the degree of cytotoxicity of the  
335 compounds.

336       Cell morphology after treatment with phenolic extract against HeLa, LN18 and  
337 293T cells in a dose dependent manner (**Fig. 5**), showed clear evidence of decrease of  
338 cell viability against HeLa and LN18 cells while it was unable to decrease cell viability in  
339 293T cells. Detachments were visible in HeLa and LN18 cells at all the concentrations.

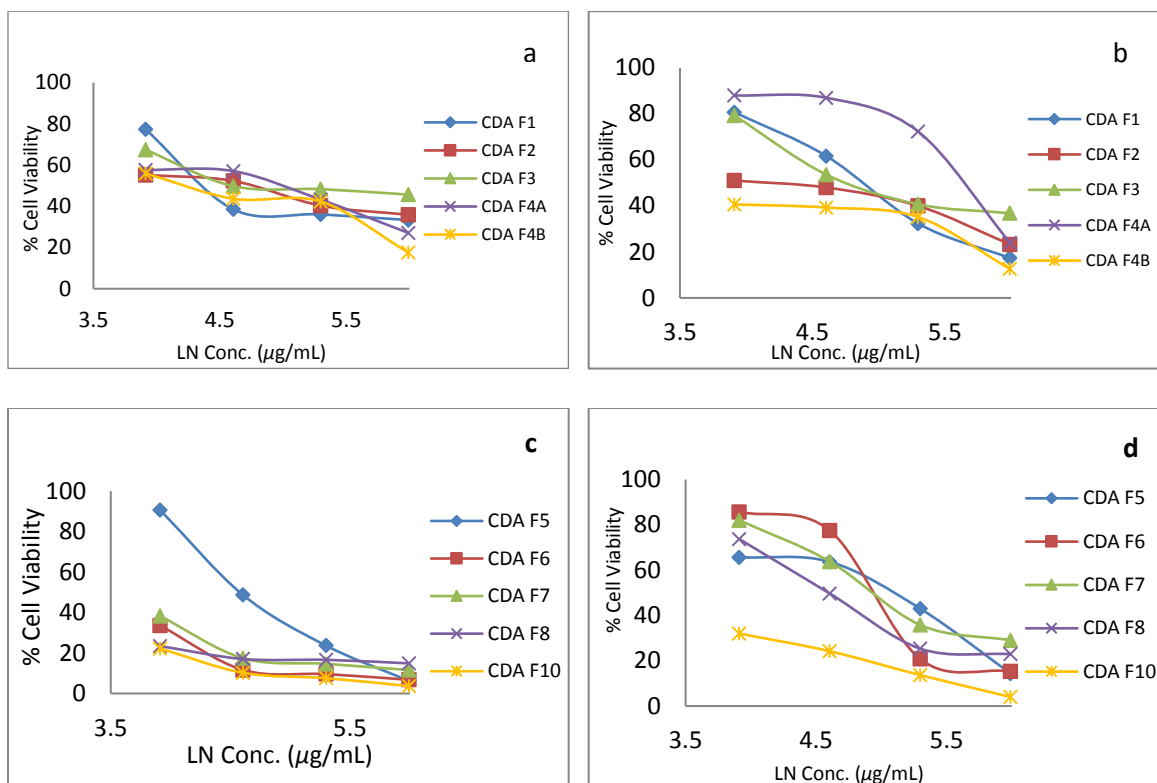
At the highest concentration of extract (400  $\mu\text{g/mL}$ ) (Natural log Conc. 5.991  $\mu\text{g/mL}$ ) there was the least number of viable cells while at the lowest concentration (50  $\mu\text{g/mL}$ ) (LN Conc. 3.912  $\mu\text{g/mL}$ ), the most viable cells were consistently reported across the two cancer cell lines (**Fig. 5**). The result confirmed that extract is selectively toxic to tumor cells.



**Fig. 5.** Cell morphology of the HeLa (a), LN18 (b) and 293T (c) at four different drug concentrations for phenolic extract.

The phenolic extract was subjected to sephadex column chromatography to afford ten fractions. The percentage cell viability of all the ten sephadex fractions differed between the two cancer cell lines *i.e.*, HeLa and LN18 (**Fig. 6**). All fractions showed a significant reduction in cell viability in a concentration dependent-manner following 24 hours of exposure. There was inverse relationship between increasing concentration of different fractions and the percentage cell viability (**Fig. 6**). Among all fractions, fraction

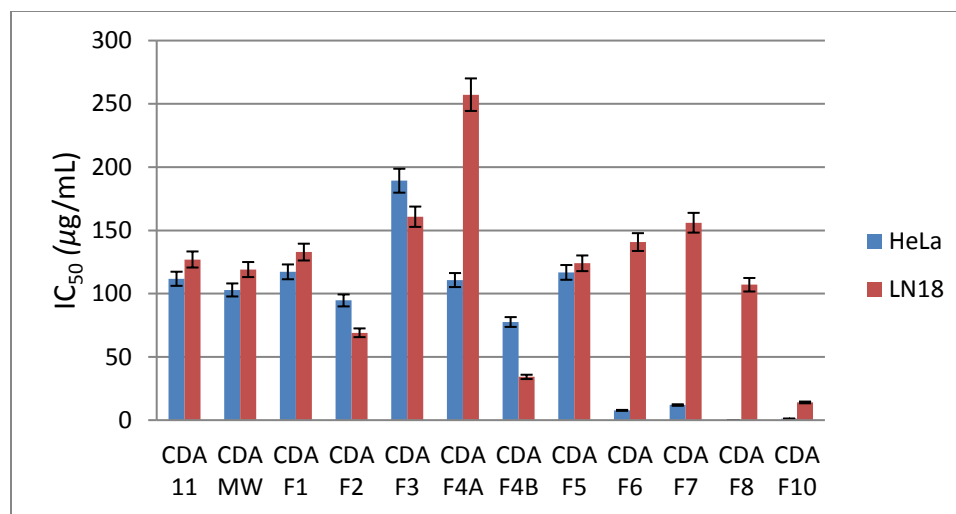
CDA F6 and F10 showed significant anti-cancer effects on HeLa cells even when the concentration was 50  $\mu\text{g/mL}$  (**Fig. 6**).



**Fig. 6.** Plot of percentage cell viability vs natural log of concentrations ( $\mu\text{g/mL}$ ) for Sephadex fractions CDA F1-F4B against **a)** HeLa and **b)** LN18 cells and for fractions CDA F5-F10 against **c)** HeLa and **d)** LN18 cells.

The calculated  $\text{IC}_{50}/24 \text{ h}$  values for all the fractions are given in **Table 2**. As a general rule lower  $\text{IC}_{50}$  values indicate higher cytotoxic effects while higher  $\text{IC}_{50}$  values indicated lower cytotoxic activity. All the fractions were found to be cytotoxic to HeLa and LN18 beyond negative controls. In particular, four out of ten sephadex fractions namely CDA F6, F7, F8 and F10, possessed a promising activity against HeLa cell with  $\text{IC}_{50}$  values of 7.71, 11.9, 0.02 and 1.10  $\mu\text{g/mL}$ , respectively (**Fig. 7**), while fractions CDA F2, F4B and F10 showed the highest activity against the LN18 cancer cells with

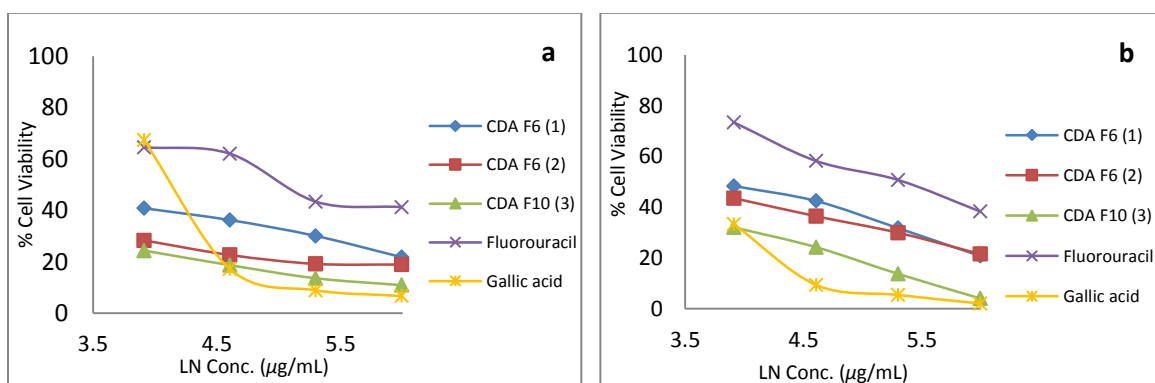
IC<sub>50</sub> values of 69.0, 34.19 and 13.99  $\mu\text{g/mL}$ , respectively. These results demonstrated good cytotoxic activity of these fractions which may be due to the presence of phenolic compounds particularly flavonoids which is confirmed by LCMS analyses of these fractions. There have been reports where flavonoids are found to inhibit human breast cancer cell proliferation and delay of mammary tumorigenesis (So et al., 1996).

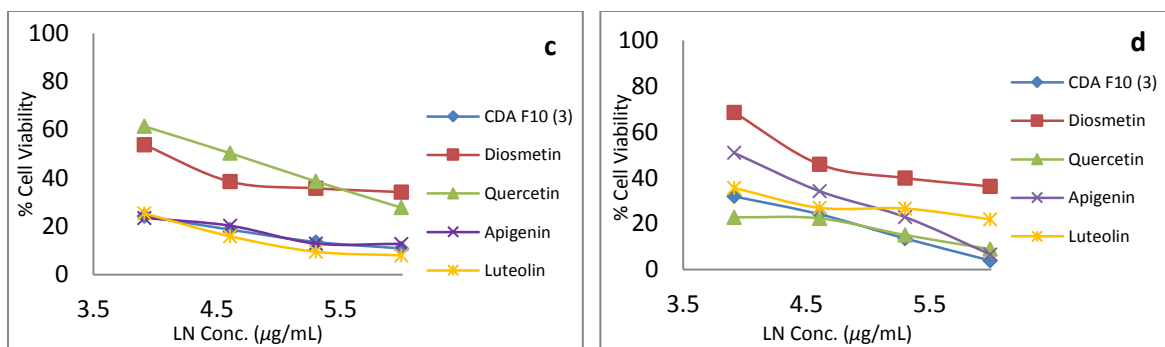


**Fig. 7.** Plot of IC<sub>50</sub> values of different extracts and sephadex fractions against HeLa and LN18 cells.

The active fraction CDA F6 was further subjected to Preparative HPLC to afford two cytotoxic compounds (**1** and **2**). The isolated compound **1** showed a promising activity against HeLa and LN18 cells with lower IC<sub>50</sub> values of 20.1 and 49.2  $\mu\text{g/mL}$  respectively (**Table 2**). However, the compound **2** showed selective high activity against HeLa cells with an IC<sub>50</sub> value of 0.32  $\mu\text{g/mL}$  which is even more than compound **1** (IC<sub>50</sub> 20.1  $\mu\text{g/mL}$ ). It can also be hypothesized that the cytotoxic activity of compound **2** is enhanced by the presence of acetyl group on the C-6" position. It is also noteworthy that cytotoxic activity did not occur in all cell line for the same flavonoid.

381 The active fraction CDA F10 containing the highest amount of compound **3**  
 382 exhibited the high cytotoxic activity with IC<sub>50</sub> values of 1.10 and 13.9  $\mu\text{g/mL}$  for HeLa  
 383 and LN18 cells, respectively, while 400  $\mu\text{g/mL}$  for normal 293T cells (**Table 2**).  
 384 Compound **3** showed the most significant cytotoxic impact (least viable cell number)  
 385 even at lowest concentration with HeLa and LN18 cells (**Figure 8**). Specific structural  
 386 features are involved for biological properties of this compound. Flavonoids with 2,3-  
 387 unsaturation in the C-ring of flavonoids significantly increase the anti-proliferative effect  
 388 of these compounds (**Williams et al., 2004; Benavente-García et al., 2007; Benavente-**  
 389 **García and Castillo, 2008**). Flavonoids with 5- and 7-OH groups in the A ring  
 390 significantly inhibit lipogenesis (**Brusselmans et al., 2005**). **Cardenas et al., 2006**  
 391 reported that the hydroxyl group at 4'-OH in B-ring improves the anti-proliferative effect  
 392 of the flavonoids when apigenin (with 4'-OH) and chrysin (without 4'-OH) were  
 393 compared. All the above structural features *i.e.*, 2,3-unsaturation, 5, 7 and 4'-OH are  
 394 present in this compound **3**, which are together responsible for the cytotoxic activity of  
 395 this compound against HeLa and LN18 cells.





**Fig. 8.** Plot of percentage cell viability vs natural log of concentrations ( $\mu\text{g/mL}$ ) for isolated compounds (**1-3**) and standards against **a**) HeLa and **b**) LN18 cells and for isolated compound (**3**) and cytotoxic flavonoid standards against **a**) HeLa and **b**) LN18 cells.

It was observed that the isolated compounds **1-3** exhibit a stronger cytotoxic activity than the sephadex fractions which have more activity than ethanolic extract. Suggesting the potential of flavonoids as anticancer agents. The compounds **1-3** exhibited about 160 times more potent cytotoxic activity in comparison to the positive control drug fluorouracil against HeLa cells in MTT assay. These results suggest the importance of flavonoid glycosides in anticancer activity.

**Table 2**

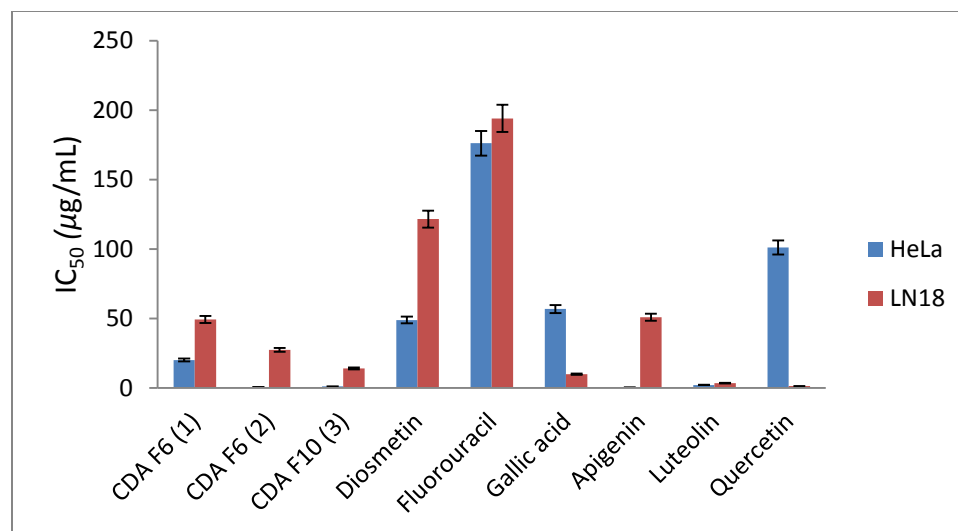
$\text{IC}_{50}$  values for extracts, fractions, isolated compounds and standards against HeLa, LN18 and 293T cells.

Crude extract	Fractions	Isolated compounds	Standards	$\text{IC}_{50}$ ( $\mu\text{g/mL}$ )		
				HeLa	LN18	293T
CDA 11				111.72	126.97	> 400
CDA MW				102.91	119.02	> 400
	CDA F1			117.21	132.82	> 400
	CDA F2			94.63	69.06	> 400
	CDA F3			189.23	160.77	> 400
	CDA F4A			110.71	257.23	> 400

CDA F4B	77.55	34.19	> 400
CDA F5	116.76	123.96	> 400
CDA F6	7.71	140.75	> 400
CDA F6 (1)	20.10	49.25	> 400
CDA F6 (2)	0.32	27.35	> 400
CDA F7	11.93	156.02	> 400
CDA F8	0.02	107.01	> 400
CDA F10 (3)	1.10	13.99	> 400
Diosmetin	48.90	121.51	> 400
Fluorouracil	176.09	194.02	> 400
Gallic acid	56.82	9.93	> 400
Apigenin	0.46	50.95	> 400
Luteolin	2.17	3.46	> 400
Quercetin	101.19	1.38	> 400

409

410 HPLC–MS analysis showed that these flavonoids (**1-3**) were major compounds in  
411 the crude aerial parts extract. Bioactivity guided fractionation led to the isolation of  
412 flavonoids (**1-3**) as major components of active fractions CDA F6 and F10. Nevertheless,  
413 other fractions obtained at lower quantities during the bio-guided fractionation also  
414 displayed interesting activities and the compounds they contain may also account for the  
415 overall activity of the crude extracts and would be worth being further isolated and  
416 identified.



417

418 **Fig. 9.** Plot of IC<sub>50</sub> values of isolated compounds from *C. didymus* and standard  
419 polyphenols against HeLa and LN18 cells.

420 All the isolated compounds **1-3** were methylated flavonoids so it was desirable to  
421 check their cytotoxic activity in comparison to unmethylated flavonoids and methylated  
422 flavonoid standards. From the comparison of isolated methylated flavonoids (**1-3**), one  
423 standard methylated flavonoid (diosmetin) and unmethylated flavonoids standards  
424 (apigenin, luteolin, quercetin) it has been shown that methylated flavonoid compounds  
425 showed high cytotoxicity towards HeLa and LN18 cells with lower IC<sub>50</sub> values (**Fig. 9**)  
426 than unmethylated flavonoids. Previous studies by **Walle et al. (2007)** also showed that  
427 methylated flavonoids tend to be more potent than their unmethylated counterparts.  
428 **Walle et al. (2007)** also reported that extensive conjugation of the free hydroxyl groups  
429 is responsible for low oral bioavailability of unmethylated flavonoids *in vivo*. Extensive  
430 studies have shown that methylated flavonoids were important because they are  
431 metabolically stable and experienced slower hepatic metabolism compare to the  
432 unmethylated flavonoids (**Wen and Walle, 2006**) and have higher rate of intestinal  
433 permeability (**Walle et al., 2007**). Apigenin and luteolin (polyphenol standards) have



434 more potent cytotoxic effects against both HeLa and LN18 cells with lower IC<sub>50</sub> values  
435 than the other standards chosen, while quercetin showed selective potent cytotoxic  
436 activity against LN18 cells. From the results it could also be concluded that flavones  
437 (apigenin, luteolin, disomitin, isolated compounds **1-3**) have more cytotoxic activity than  
438 flavonol (quercetin). **Scambia et al. (1994)** reported that flavones turned out to be a  
439 powerful *in vitro* antiproliferative agent against human cancer cell lines such as primary  
440 colorectal, ovarian, lymphoblastoid, and breast cancer cells. This study demonstrated that  
441 isolated cytotoxic flavonoid compounds have similar efficacy compared with standard  
442 cytotoxic flavonoids, as proven by both *in vitro* cytotoxicity assay and statistical analysis.

#### 443 **4. Conclusions**

444 The employed MTT assay enabled the determination of cell viability and cell  
445 cytotoxicity after treatment with plant extract and compounds on two different cancer cell  
446 lines *i.e.*, HeLa and LN18. Present study suggested that *Coronopus didymus* contains a  
447 high amount of flavonoids, which contributed mainly to the *in vitro* cytotoxic activity in a  
448 concentration dependent-manner. The bioassay-guided fractionation of phenolic extract  
449 of *Coronopus didymus* had led to the isolation of three cytotoxic flavonoids. Cytotoxic  
450 activity of ethanolic extract, 5 out of 10 sephadex fractions and its isolated anticancer  
451 constituents strongly showed the remarkable importance of this plant in anticancer  
452 activity. Compound **3** has the highest cytotoxic activity, while flavonoid **1** possessed  
453 moderate activity against both cancer cell lines, but compound **2** is selective towards  
454 HeLa. These results supported the traditional use of the plant as anticancer agent. Further  
455 studies should be carried out for further pharmacological and clinical studies, to  
456 determine the additive effects of the active components and to elucidate their mechanism

457 of action in order to develop new anticancer agents. Isolation of compound **1** from this  
458 genus and cytotoxic activities were reported for the first time in this study.

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