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Chemical constituents of the different parts of *Colchicum micranthum* and *Colchicum chalcedonicum* and their cytotoxic activities

Gizem Gulsoy-Toplan[a]*, Fatih Goger[b], Ayca Yildiz-Pekoz[c], Simon Gibbons[d], Gunay Sariyar[e], Afife Mat[a]

[a]*Department of Pharmacognosy, Faculty of Pharmacy, Istanbul University, Istanbul, Turkey*
[b]Department of Pharmacognosy, Faculty of Pharmacy, Anadolu University, Eskisehir, Turkey
[c]*Department of Pharmaceutic Technology, Faculty of Pharmacy, Istanbul University, Istanbul, Turkey*
[d]*Research Department of Pharmaceutical and Biological Chemistry, UCL School of Pharmacy, London, UK*
[e]*Faculty of Pharmacy, Cyprus International University, Lefkosa, Turkish Republic of Northern Cyprus*

eczgizemgulsoy@gmail.com

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Colchicum species, which have been widely used as a medication for years, still remain important in treatment of Familial Mediterranean Fever, gout and Behcet's Disease. In the present work, alkaloids, phenolic compounds and cytotoxic activities from different parts of two *Colchicum* species, namely *C. chalcedonicum* and *C. micranthum* were investigated for the first time. From different parts of two species, alkaloids were isolated and colchicine concentrations were also determined by HPLC. The methanol extracts were investigated for their cytotoxic activity against the A549 cell line using the MTT and LDH methods. Additionally, the phenolic compounds of each extract were investigated by LC–MS/MS. Six alkaloids, namely colchicine, colchifoline, 2-demethylcolchicine, demecolcine, 4-hydroxycolchicine and *N*-deacetyl-*N*-formylcolchicine were isolated from different parts of two species. The autumn corm of *C. micranthum* possessed the highest amount of colchicine among all extracts. All extracts showed high cytotoxicity, while the highest toxicity was determined in the seed extract. According to the LC–MS/MS analysis, 19 phenolic compounds were shown to be present. This is the first study which highlights that the seeds of *C. chalcedonicum* and autumn corms of *C. micranthum* could be valuable for the pharmaceutical industry to obtain colchicine and other tropolone alkaloids.

Keywords: *Colchicum* species, alkaloids, cytotoxic activity, colchicine, LC–MS/MS.

Turkey is one of the richest regions for the number of *Colchicum* species. The genus is represented by 100 species all around the world and 50 grow in Turkey [1]. Turkey is considered as a major center for *Colchicum* species, not only because of numbers but also because of the high rates of endemism. Alkaloids of the species have been investigated by many researchers [2-5]. Tropolone alkaloid content, and in particular the major alkaloid colchicine, have provided medicinal significance from past to present [6]. Several studies demonstrated that colchicine possesses antitumor and anti-inflammatory activities [7-9]. Colchicine and its natural analogues are used clinically for the treatment of several disorders such as FMF (Familial Mediterranean Fever), gout, amyloidosis and Behcet's Disease [10-11]. However, the narrow therapeutic index of this alkaloid limits its use in therapy. Demecolcine, the other major alkaloid, possesses low toxicity and has been used in treatment, particularly for myeloid leukemia and Hodgkin's syndrome [6].

The distribution of tropolone alkaloids in the plant kingdom is limited. They are sensitive compounds, generally affected by light and high temperature and decompose to inactive lumi derivatives [12]. An economic and efficient chemical synthesis method for colchicine and its derivatives has not been found to date. *C. autumnale*, which is used for the isolation of colchicine and demecolcine in the pharmaceutical industry, does not grow in Turkey. The presence of a wide variety of *Colchicum* species in Turkey has driven researchers to find new sources of colchicine-rich species. Studies on several *Colchicum* species of Turkish origin revealed the existence of colchicine-rich species and prompted us to continue looking for further colchine-abundant producers [13-17].

In the present work, *C. micranthum* (endemic to Turkey) and *C. chalcedonicum* were collected at different growing stages and evaluated for their chemical composition and biological activities by analyzing their aerial and underground parts separately. The extract yield percentage of each parts and dry weight of two *Colchicum* species are given in Table 1.

Table 1: The extract yield percentage of *Colchicum* species

Species	Plant parts	Dry Weight (g)	A extract (g)
C.micranthum	Autumn corm	120	1.69
	Flower	95	0.60
C.chalcedonicum	Autumn corm	285	2.6
	Flower	20	0.9
	Seed	20	0.261

Colchicine (1), colchifoline (2), 2-demethylcolchicine (3), *N*-deacetyl-*N*-formylcolchicine (4) were isolated from *C. micranthum* while colchicine (1), colchifoline (2), 2-demethylcolchicine (3), *N*-deacetyl-*N*-formylcolchicine (4), 4-hydroxycolchicine (5), demecolcine (6) were isolated from *C. chalcedonicum* and their structures were elucidated by ^{1}HNMR and ESI/MS methods and comparison with reference physical data [12,18]. The isolated alkaloids of different parts from two species are given in Table 2.

Table 2: The isolated alkaloids from different parts of two *Colchicum* species

Colchicum chalcedonicum			*Colchicum micranthum*	
Seed	**Autumn Corm**	**Flower**	**Autumn Corm**	**Flower**
Colchicine	Colchicine	Colchicine	Colchicine	Colchicine
Demecolcine	Colchifoline	4-hydroxycolchicine	Colchifoline	*N*-deacetyl-*N*-formylcolchicine
N-deacetyl-*N*-formylcolchicine	2-demethylcolchicine	Colchifoline	2-demethylcolchicine	Colchifoline
Colchifoline				

The seed part of *C.micranthum* did not worked.

The results showed that the seed of *C. chalcedonicum* possess the richest diversity in tropolone alkaloids when compared with other parts of the plants. Colchicine and colchifoline were found to be the main alkaloids in all plant parts studied. Demecolcine, an another significant alkaloid in *Colchicum* species, was found only in the seed extracts of *C. chalcedonicum*. Besides, 4-hydroxycolchicine was isolated from the flowers which is a rare compound in the tropolone alkaloid group.

Colchicine amounts in the methanol extracts from each part of the plants during different growth stages were examined by HPLC. The results of the HPLC analyses are summarized in Table 3. Additionally, the HPLC method was validated according to the experimental results of colchicine: linear range (0.02–0.0015 mg/ml); recovery (99.74±4.94 %); LOD (0.0007 mg/ml); LOQ (0.0023 mg/ml) and RSD (5.91 %). As one would expect, there are differences in colchicine concentrations among different plant parts of the species. Among the extracts of different parts from *C. micranthum*, the autumn corms contained the highest amount of colchicine (0.183 ± 0.021 %). Additionally, when comparing the amounts of colchicine in the two species, the highest colchicine content was found in the autumn corm extracts of *C. micranthum*. The corms of both species possessed higher colchicine content than the flower parts. According to previous studies, the colchicine level was found to be in the range of 0.039 % to 0.3 % in an Anatolian *Colchicum* species [20].

The corm extracts of *C. micranthum* contained a moderate level of colchicine while *C. chalcedonicum* possessed a low amount. Previous studies demonstrated that the chemical composition and colchicine concentrations of *Colchicum* species showed variety in different plant parts and varies during the autumn and spring season. Our results confirmed the presence of significant alkaloid colchicine with different concentrations in the different parts in two species.

Table 3: Colchicine concentrations of the methanol extracts

Plants	Plant parts	Sample name	% Value of colchicine
C. micranthum	Autumn corm	MAC.	0.183 ± 0.021
	Spring corm	MSC.	0.103 ± 0.018
	Flower	MF.	0.040 ± 0.005
C. chalcedonicum	Flower	CF.	0.063 ± 0.003
	Autumn corm	CAC.	0.041 ± 0.006
	Spring corm	CSC.	0.045± 0.002
	Seed	CS.	0.124 ± 0.016

The content of the phenolic compounds of each methanol extract was also studied by LC/MS-MS and 19 phenolic compounds were identified. The results are given in Table 4. LC chromatogram of *C. micranthum* autumn corm is shown in Figure 2.

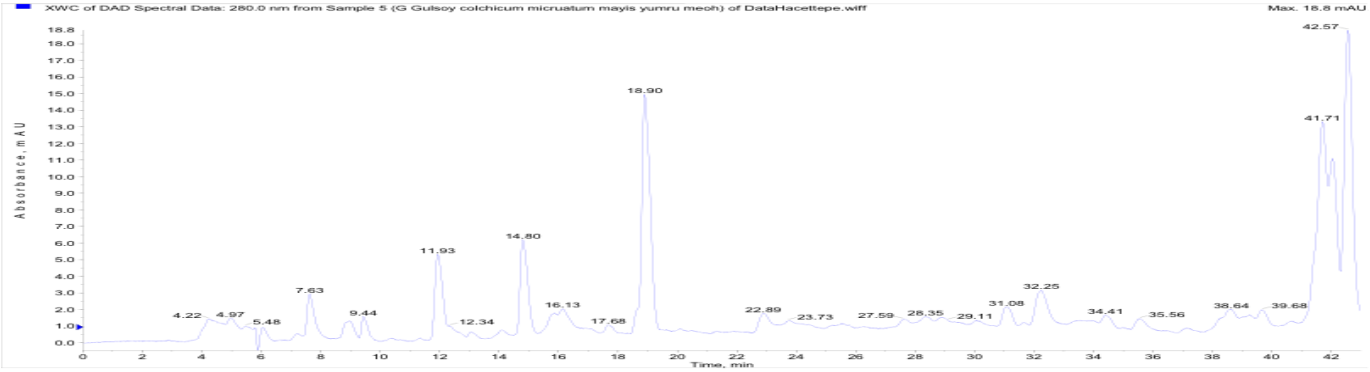
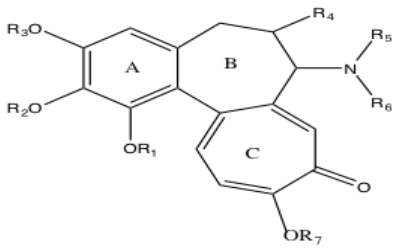


Figure 1: LC-DAD chromatogram of *Colchicum micranthum* autumn corm (MAC)



1: R_1: CH_3, R_2: CH_3, R_3: CH3, R4: H, R_5:H, R_6: $COCH_3$, R_7: CH_3
2: R_1: CH_3, R_2: CH_3, R_3: CH_3, R_4: H, R_5:H, R_6: $COCH_2OH$, R_7: CH_3
3: R_1: CH_3, R_2: H, R_3: CH_3, R_4: H, R_5:H, R_6: $COCH_3$, R_7: CH_3
4: R_1: CH_3, R_2: CH_3, R_3: CH_3, R_4: H, R_5:H, R_6: CHO, R_7: CH_3
5: R_1: CH_3, R_2: CH_3, R3: CH_3, R_4: OH, R_5:H, R_6: $COCH_3$, R_7: CH_3
6: R_1: CH_3, R_2: CH_3, R_3: CH_3, R_4: H, R_5:H, R_6: CH_3, R_7: CH_3

Figure 2: The structures of isolated alkaloids: colchicine (1), colchifoline (2), 2-demethylcolchicine (3), *N*-deacetyl-*N*-formylcolchicine (4), 4-hydroxycolchicine (5), demecolcine (6).

Luteolin and caffeic acid were determined by comparing retention time and mass spectra of standards. Other compounds were tentatively identified by comparison to the retention times and mass fragmentation pattern with references.

Peak 1 was determined as gluconic acid which showed a characteristic $[M-H]^-$ molecular ion peak at m /z 195 and fragment ions at 177 and 129 [19]. Peak 2 was presented a deprotonated molecular ion peak at m/z 315 and showed a base peak ion at m/z 153, formed after loss of a glucose unit (-162 amu). A further product ion peak at m/z 108 was also characteristic for dihydroxybenzoic acid. According to previous reports 2, 5-dihydroxybenzoic acid (gentisic acid) is a known molecule from *Colchicum* species [14, 20]. This compound was identified as gentisic acid glucoside due to its fragmentation pattern. Peak 3 showed an $[M-H]^-$ at m/z 593 and fragmented giving several product ions at m/z 473 (-120 amu), 353 (-240 amu) due to the characteristic 120 amu losses of C-glucosides from the deprotonated molecular ion. Such losses are indicative of two sugar units so the aglycone of peak 3 must be apigenin at m/z 269. Based on this data peak 3 was identified as apigenin C-hexoside C-hexoside.

Peak 4 showed an $[M-H]^-$ molecular ion peak at m/z 401 and a base peak ion at m/z 269 (apigenin) due to a loss of a pentose residue. Further product ions were observed at m/z 161 and m/z 113. According to these results peak 4 was identified as apigenin pentoside.

Table 4: LC-MS/MS analysis results for *Colchicum* methanol extracts

No	R_t	$[M-H]^-$	MS^2	Compound	Sample name
1	5.4	5	177, 129	Gluconic acid	CS.
2	8.0	315	165, **153**, 108	Gentisic acid glucoside	CF.
3	9.1	593	473, 383, 3**53**	Apigenin C-hexoside C-hexoside	CF.
4	9.6	401	**269**, 161, 113	Apigenin pentoside	CF.
5	10.7	463	301, 257	Unknown glucoside	CF.
6	10.9	609	447, **285**	Luteolin diglucoside	MF., CF.
7	11.9	179	135	Caffeic acid	MAC., MSC.
8	12.1	579	447, 327, 2**85**	Luteolin apiosyl-glucoside	CF.
9	12.1	331	313, 211, 168, 125	Gallic acid hexoside	CF., CAC.
10	12.5	593	431, 385, 311, **269**	Apigenin diglucoside	MF.,CF.
11	13.1	447	327, **285**, 256	Luteolin glucoside	MF., CF.,CSC
12	14.1	593	299, **284**, 255	Luteolin methyl ether apiosyl-glucoside	CF., CSC.,
13	14.9	384	**369**, 354, 339, 280	Unknown	MSC., MAC.,CS
14	15.1	431	311, **269**	Apigenin glucoside	MF., CF.
15	15.1	153	135, 109	Dihydroxybenzoic acid	MSC.
16	15.8	299	284, 255, 227, 151	Luteolin methyl ether (diosmetin, chrysoeriol)	CF.
17	17.6	187	125	Benzoic acid derivative	MSC.
18	18.9	-	-	Not ionised	MSC., MAC.
19	20.7	285	241, 217, 175 133	Luteolin	CF.

Peak 5 presented a deprotonated molecular ion at m/z 463 and a base peak ion at m/z 301 due to the loss of a glucose moiety. A further fragment for the aglycone was also observed at m/z 257 which indicated that the aglycone was most probably quercetin or an ellagic acid. Because some of the aglycons fragmentation was absent (for example m/z 179 and 161 for quercetin and m/z 229 for ellagic acid), we were unable to unequivocably identify the compound as quercetin/elagic acid glucoside. So peak 5 was proposed as a similar unknown glucoside. The peak 7 showed a molecular ion peak at m/z 179 and a product ion at m/z 135 which was characteristic for caffeic acid. Caffeic acid has previously been identified in *C. baytopiorum* [14]. This peak was confirmed with standard caffeic acid. Peak 19 was identified as luteolin which was previously determined in *Colchicum* species several times [14, 22]. This spectrum was also confirmed by using a luteolin standard. Peak 6 was determined as a diglucoside of luteolin due to the loss of two glucose moieties (162 + 162) from the molecular ion peak at m/z 609. Similar identification was achieved for peak 8 which showed a deprotonated molecular ion peak at m/z 579 and a product ion at m/z 447 (-132 / apiose) and luteolin ion as an aglycon at m/z 285 (-132 + -162). Peak 8 was identified as luteolin apiosyl-glucoside. Peak 12 was identified as methyl ether derivative of peak 8 (diosmetin or chrysoeriol apiosyl-glucoside). The aglycone of peak 16 was also determined with an Rt 15.8 to have a molecular ion peak at m/z 299 which yielded an ion at m/z 284 due to the loss of a 15 amu methyl moiety and other fragments at m/z 227 and 151 matched with the standard luteolin fragments. Peaks 15 and 17 showed similar fragmentation behaviour with benzoic acid derivatives which were previously identified in several *Colchicum* species [19]. Peak 9 was also determined to be a glucoside of trihydroxybenzoic acid (Gallic acid glucoside), with a molecular ion $[M-H]^-$ at m/z 331. Peak 10 at an Rt of 12.5 min was identified as apigenin diglucoside. The molecular ion $[M-H]^-$ at m/z 593, with the other fagment ions were m/z 431, 385, 311, 269. Peak 14 was determined as apigenin glucoside based on the loss of a sugar moiety (-162 amu) and a characteristic aglycone ion at m/z 269 confirming this identification.

The methanol extracts were tested for general toxicity against the A549 (human lung adenocarcinoma) cell line. The IC_{50} values of the extracts are given in Table 5. All extracts were found to have moderate bioactivity but the seed extract showed high toxicity against the A549 cell line.

Table 5: IC_{50} values of the extracts of the *Colchicum* samples and colchicine

Extracts Name	MTT (IC_{50} µg/ml)	LDH (IC_{50} µg/ml)
MAC.	424 ± 20.1	51 ± 12.5
MSC.	> 500	83 ± 27.06
MF.	> 500	95 ± 16.0
CAC	126 ± 11.8	51 ± 8.8
CSC	99 ± 4.5	113 ± 22.6
CF.	> 500	> 500
CS.	56 ± 3.2	89 ± 13.7
Colchicine	108 ± 5.3	64 ± 9.9

This is the first report of the chemical composition and cytotoxic activity of *C. micranthum* and *C. chalcedonicum*. According to this study, these two *Colchicum* species, which grow in Istanbul, may have potential as a source of colchinoids. Further studies are needed to be conducted on other *Colchicum* species for pharmaceutical industry sources of tropolone alkaloids.

Experimental

Plant material: *C. micranthum* was collected from Ömerli-Istanbul on the 05.10.2014 (ISTE 103638) and the 10.05.2015 (ISTE 106891) whereas *C. chalcedonicum* was collected from Süreyyapaşa-Istanbul on the 05.04.2014 (ISTE 103636) and 26.09.2014 (ISTE 103637). Voucher specimens (voucher numbers above) were deposited at the Herbarium of the Pharmacy Faculty of Istanbul University (ISTE). All plant parts were separated and dried at room temparature, except for the corms which were separated and sliced then left to dry in a drying oven at 60°C.

Extraction and isolation: All dried plant materials (corms and flowers) were extracted separately with methanol using a Soxhlet apparatus. The seeds were extracted firstly with petroleum ether to defat and then with methanol using a Soxhlet apparatus. The solvents were evaporated in a rotavapor at 40^0C. The obtained methanol extracts were dissolved in water using an ultrasonic bath, and then filtered. The filtrate was made acidic with 3% H_2SO_4 (pH 3–4) and extracted with chloroform. The chloroform extracts were combined and dried over anhydrous Na_2SO_4, filtered to yield neutral-phenolic extract (extract A). During the study, all extracts were protected from direct light to reduce compound degradation.

Preliminary separation of the alkaloids of the autumn corms (extract A) from *C. micranthum* was achieved by SPE with a normal phase cartridge, eluting with a gradient elution of hexane:chloroform:methanol. Similar fractions were combined and monitored with Dragendorff and Carr-Price reagents. Fractions 12-13 (100 mg) were purified by preparative HPLC (Waters 2555 Quaternary Gradient Module) using Phenomenex C18 column (5 µm, 21.20 mm x 250 mm) with an isocratic mobile phase system (acetonitrile:water, 25:75) with a 10 mL/min flow rate, monitoring at 254 nm and injecting 100 mg of fraction. Compounds **1** (8,5 mg), **2** (1.5 mg) and **3** (5mg) were isolated and monitored by TLC. The alkaloids of the flowers from *C. micranthum* (extract A) were subjected to an SPE normal phase cartridge followed by preparative TLC. The combined fractions 12-14 (50 mg) were purified with preparative-TLC on silica gel using a

chloroform:acetone:diethylamine (5:4:1) solvent system to yield compound **1** (3.5 mg). Fractions 15-16 (170 mg) were purified by preparative-TLC on silica gel using a chloroform:acetone:diethylamine:methanol (11:8:2:3) solvent system to yield compounds **2** (1.5 mg) and **4** (1.4 mg).

The alkaloids of *C. chalcedonicum* were isolated in three different parts. The seed (A) extract (200 mg) was purified by preparative-TLC on silica gel using toluene:ethylacetate:diethyamine:methanol (50:40:10:8) as solvent system. Further purification of the mixture used TLC on silica gel using a chloroform:acetone:diethylamine (5:4:1) solvent system. Compounds **1** (5,5 mg), **2** (5 mg), **4** (3 mg) and **6** (3 mg) were isolated and monitored by TLC. The separation of the autumn corm (A) extract was achieved by SPE with a normal phase cartridge, eluting with a gradient elution of hexane:chloroform:methanol. Similar fractions were combined and then again monitored by TLC with Dragendorff and Carr-Price reagents. The separation of fractions 11-13 (165 mg) was achieved by preparative-TLC on silica gel with chloroform:acetone: diethylamine:methanol (11:8:2:3) to yield compounds **1** (3.8 mg), **2** (3.8 mg) and **3** (1.8 mg). The flower extract was separated by silica gel column chromatography eluting with chloroform and methanol. Fractions 44-45 were combined and purified by preparative-TLC on silica gel using chloroform:acetone:diethylamine (5:4:1) to yield compound **1** (2.2 mg). Fractions 47-50 were combined and purified with preparative-TLC on silica gel with chloroform:acetone:diethylamine:methanol (11:8:2:3) to yield compounds **2** (1.6 mg) and **5** (1.2 mg). All of the pure alkaloids were eluted with chloroform:methanol (8:2).

Quantification of colchicine by HPLC: Colchicine concentrations in the methanol extracts were determined using HPLC. The HPLC analysis was performed on an Agilent 1200 series instrument. The analytical HPLC column was Sepax C18 column of 5 µm particle size and 4.6mm x 250mm dimensions and the column was used with mobile phase acetonitrile (solvent A) and 1 % acetic acid in water (solvent B). The column was run with gradient elution at 1 mL/min (0–25 min 10–60% A, 25–30 min 60% B, 30-35 min 60-10 % and 35-40 min 10% A). Detection was carried out with a UV/VIS detector. The methods described by Alali et al., were adopted with some modifications [16]. A reference standard of colchicine was prepared from the USP. The flow rate was set to 1 mL/min; injection volume and column temperature were adjusted to 10 µL and 40°C, respectively. The detection was performed at 352 nm. The HPLC method was validated based on the experimental results; LOD, LOQ, RSD and recovery. The colchicine concentration in each extract was calculated by the following regression equation y= 69716x – 24,23 with good linearity (r2 = 0.9992).

Sample preparation: The six point calibration curve for colchicine was prepared with the external standard solution within the concentration range of 0.02–0.0015 mg/mL, in methanol. The dried methanol extracts were redissolved in methanol. The methanol extracts were at a concentration of 5 mg/mL concentration for the corm and flower samples and at a 2 mg/mL concentration for seed samples. All samples and solvents were filtered using a 0.45 µm membrane filter before HPLC analysis. The analysis were performed in triplicate for each extract.

LC-MS/MS analysis of extracts: LC-MS/MS analysis was carried out using an Absciex 3200 Q trap MS/MS detector. Experiments were performed with a Shimadzu 20A HPLC system coupled to an Applied Biosystems 3200 Q-Trap LC-MS/MS instrument equipped with an ESI source operating in the negative ion mode. For the chromatographic separation, a GL Science Intersil ODS 250 × 4.6 mm, i.d., 5 µm particle size, octadecyl silica gel analytical column operating at 40ºC was used. The solvent flow rate was maintained at 0.5 mL/min. Detection was carried out with a PDA detector. The elution gradient consisted of mobile phases (A) acetonitrile:water:formic acid (10:89:1, v/v/v) and (B) acetonitrile:water:formic acid (89:10:1, v/v/v). The composition of B was increased from 10% to 100% over 40 min. The LC-ESI-MS/MS data were collected and processed by the Analyst 1.6 software.

Cytotoxicity activities of extracts: The MTT and LDH tests were used to evaluate the cytotoxic potential of the methanol extracts that were prepared from different plant parts against the A549 cell line. IC_{50} value of each extract were calculated according to cytotoxicity index. The A549 cell line was obtained from the American Type Culture Collection (ATCC, USA). Five concentrations of each extract and colchicine was prepared (500, 100, 50, 10, 1 µg/mL). After cell adhesion, the solution was removed and new cell culture solution with various concentrations of the methanol extracts was added. After incubation with the extracts for 24 h, the cytotoxicity of each extract was determined using the MTT and LDH assays. All assay were repeated three times for each concentration of all methanol extracts.

The cytotoxic effects of the extracts were studied by using in part Plumb's method [22]. In brief, after 24 h incubation with the extracts, an MTT solution (50 µL) (2 mg/mL in PBS) was added to each well. The cells were incubated at 37°C for 4 h. Following incubation, the supernatant containing the MTT was removed and then 200 µL of DMSO and 25 µL of Sorensen's glycine buffer were added. The absorbance (A) of each well was then read at 570 nm (against the reference wavelength of 670 nm) using an ELISA reader. A cytotoxicity index of each extract was calculated using the formula below:

Cytotoxicity Index (%) = 1 - [(Atreated wells/A control cells] x 100

A Biovision (K311-400) cytotoxicity detection kit was used for the LDH assay. Folllowing 24 h treatment with extracts, the cell culture solution was removed separately from the cultures. Afterwards, this solution was mixed with the assay reagent prepared by mixing two separate solutions. This was incubated for 30 minutes, protected from light, and the absorbance was measured at 490 nm with an ELISA reader. 10% triton-X100 was used as the positive control (100% cell lysis). The results were compared with the absorbance values of the triton X-100 (10%) (positive control), which give the maximum activity. The cytotoxicity index was calculated according to the formula below;

Cytotoxicity Index (%) = 1 - [(Atreated wells-A negative control) / (Apositive control-Acontrol cells)] x 100

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