Journal of Applied Sciences Research, 8(11): 5564-5571, 2012
ISSN 1819-544X
This is a refereed journal and all articles are professionally screened and reviewed

ORIGINAL ARTICLES

Activity of *Chorisia insignis* HBK. against Larynx Carcinoma and Chemical Investigation of its Polar Extracts

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ABSTRACT

The chemical composition of the ethyl acetate and *n*-butanol fractions of the 70% ethanol extract of *Chorisia insignis* HBK. leaves (Family Bombacaceae) was studied. Ten compounds were identified from the *n*-butanol fraction, 3,4,5-trihydroxy cyclohexan-1-ol (1`→1)-rhamnoside and phenyl ester of 3,5-dimethyl gallic acid were isolated by PPC for the first time from the plant, while kampferol 5,7,4`-trimethyl ether 3-*O*-*α*-L-rhamnosyl-(1```→6``)-*O*-*β*-D-glucuronide, dihydroquercetin 5,7,3`,4`-tetramethyl ether 3-*O*-glucuronide, quercetin, 5,4`,5`-trihydroxy-7,3`-dimethoxy 2,3-dihydroflavonol, quercetagetin-5,6,7,3`,4`-pentamethyl ether 3-*O*-*β*-D-glucuronide, 5,8,5`-trihydroxy-6,7,3`,4`-tetramethoxy 3-*O*-glucuronyl dulcitol, dihydroquercetin 3-*O*-*α*-L-rhamnopyranosyl-(1```→6``)-*O*-*β*-D-glucopyranoside and quercetin 5,7,3`-trimethyl ether 3-*O*-*α*-L-rhamnopyranosyl-(1```→6``)-*O*-*β*-D-glucopyranoside analyzed using HPLC/MS. HPLC/MS-MS technique was applied to investigate the ethyl acetate fraction and seven compounds were identified as: 5,6,7,3`,4`,5`-hexahydroxy-dihydroflavonol-3-*O*-glucuronide, 5,6,7,3`,4`,5`-hexahydroxy-dihydroflavonol-3-*O*-*β*-D-$\Delta^{1,3}$-octadienyl-glucuronide, kampferol 5,7,4`-trimethyl ether 3-*O*-ethylene glycol, 3,5,4`-trimethoxy-7-isobutyl flavone, 3,5,4`-trimethoxy-7-isobutyl dihydroflavone, dihydroquercetin-4`-methyl ether 3-*O*-*β*-D-*n*-hexyl-diglucuronide and quercetin-4`-methyl ether 3-*O*-*β*-D-*n*-hexyl-diglucuronide. The potential cytotoxicity of the total alcohol extract and the successive fractions was determined against different human cell lines. All showed significant cytotoxic activity against the larynx cell line except the chloroform fraction.

Key words: *Chorisia insignis*, Cytotoxicity, HPLC/MS, Phenyl ester of 3,5-dimethyl gallic acid, 3,4,5-trihydroxy cyclohexan-1-ol (1`→1)-rhamnoside.

Introduction

Chorisia insignis HBK. (white floss silk tree) belongs to family Bombacaceae and is native to South America, Peru, Brazil and Argentina (Huxley, 1992; Bailey, 1976; Barwick, 2004).

Previously, three flavonoids were isolated from the *n*-butanol fraction of the 70% ethanol extract (El Alfy *et al.*, 2010). The anti-inflammatory, antihyperglycemic, antioxidant and hepatoprotective activities of the plant were also studied (El Alfy *et al.*, 2010).

The present work continues studying the chemical composition of the *n*-butanol fraction. It also includes investigation of the ethyl acetate fraction and determination of the potential cytotoxicity of the total alcohol extract and the successive fractions of the 70% ethanol extract of the leaves.

Experimental:

Plant Material:

Samples of the leaves of *C. insignis* HBK. were collected from National Research Centre (NRC) garden, Dokki, Egypt in June, and were kindly authenticated by Dr. Mohamed Gibali, senior botanist and by Agr. Eng. Therese Labib, consultant of plant taxonomy at the Ministry of Agriculture and ex. director of Orman Botanical Garden, Giza, Egypt. A voucher specimen (no. 23569) is kept at National Research Centre Herbarium.

Samples of the plant under investigation were separately air-dried, powdered and kept in tightly closed amber coloured glass containers.

Solvents:

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The solvents used in this work, viz: petroleum ether (40-60°C), diethyl ether, chloroform, ethyl acetate and ethanol, were purified as described by Vogel (1966). Absolute ethanol, 95% ethanol and *n*-butanol were analytically pure grade. Methanol used for spectrophotometric analyses was supplied by E Merck, Darmstadt, Germany.

Adsorbents for Chromatography:

a. Sheets of Whatmann filter paper No.3 for PPC.
b. Sephadex LH-20 for CC (Fluka Chemie AG, Switzerland).

Solvent Systems (v/v):

- S_1: *n*-BuOH: Acetic acid: H_2O (4: 1: 5)
- S_2: MeOH (100%)
- S_3: Acetic acid: H_2O (15: 85)
- S_4: Formic acid: H_2O (1: 99)

In vitro Cytotoxic Activity:

The following cancer cell lines available at National Cancer Institute, Cairo, Egypt were used: U25l (Brain tumor cell line), HEPG2 (Liver carcinoma cell line), MCF7 (Breast carcinoma cell line), HELA (Cervix carcinoma cell line), HCT116 (Colon carcinoma cell line) and HEP2 (Larynx carcinoma cell line).

Chemicals and Kits:

- Sulforhodamine B stain (Sigma Co, Egypt).
- Tris EDTA buffer (Sigma Co, Egypt).
- Cisplatin (Glaxo-Wellcome, Egypt): used as a reference cytotoxic agent.
- Doxorubicin (DOX) (Pharmacia, Belgicum): used as a reference cytotoxic agent.
- 5-fluorouracil (5-FU) (India): used as a reference cytotoxic agent.

Apparatus:

1. UV-Visible Spectrophotometer: UV-VIS double beam UVD-3500 spectrophotometer, Labomed, Inc.
2. Electrospray Ionization Mass Spectrometer: ESI-MS, Thermo Finnigan (ion trap).
3. NMR: Joel ECA 500 (run ^{1}H-NMR at 500 MHz and ^{13}C-NMR at 125 MHz).
4. LC-MS-MS and LC-MS: were conducted using an Agilent LC-MSD ion trap mass spectrometer (Bruker Daltonics, Bremen, Germany) equipped with 1100 series HPLC and a Cosmosil Water's 5 C18 (150 mm × 4.6 mm, 5 μm) column. The UV detection was set at 280 nm.
5. ELISA Reader: for cytotoxic activity test.

Techniques:

Preparation of Crude Extracts:

A. Successive Extracts: 550 gm of air-dried powdered leaves were exhaustively defatted using petroleum ether (40-60°C) (E1) in a Soxhlet apparatus, then extracted by refluxing with 70% ethanol. The ethanol extract was combined and evaporated under reduced pressure to dryness to give 145 gm. The dry residue was then suspended in water (600 ml) and partitioned successively with ether (E2) (10×100 ml) followed by chloroform (E3) (15×100 ml), ethyl acetate (E4) (15×100 ml) and *n*-butanol (E5) (12×100 ml). The solvents were evaporated to dryness under reduced pressure at 40°C.

B. Total Alcohol Extract (E6): 100 gm of air-dried powdered leaves were exhaustively extracted by refluxing with 95% ethanol. The combined extract was evaporated under reduced pressure at 40°C to give 14 gm total alcohol extract.

C. Aqueous Decoction (E7): 100 gm of air-dried powdered leaves were exhaustively extracted by refluxing three times with distilled water. The combined extract was evaporated under reduced pressure at 40°C to give 30 gm aqueous extract.

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Isolation and Identification of Some Constituents of Fraction E5:

17 gm of E5 were separated by ascending PPC using Whatmann No. 3 sheets with S_1 as the developing system. Band 3 was subjected to PPC with S_3 as the developing system then purified on Sephadex LH-20 column using S_2 as eluant to give compounds D1 and D2.

HPLC Investigation of Fractions E4 and E5:

LC-MS-MS and LC-MS were conducted using an Agilent LC-MSD ion trap mass spectrometer equipped with 1100 series HPLC and a Cosmosil Water's 5 C18 reversed phase (RP) column. The UV detection was set at 280 nm and the chromatographic separation was carried out using a gradient elution of solvent S_2 and S_4 at a flow rate of 0.8 ml/min as follows: 0–30 min, 15–35% S_2; 31–45 min, 35% S_2; 46–55 min, 35–70% S_2; 56-70 min, 70-15% S_2.

In vitro Cytotoxic Activity:

Potential cytotoxicity of E6, against human tumor cell lines previously mentioned, was tested using the method of Skehan *et al.* (1990) as follows:

Cells were plated in 96-multi-well plate (10^4 cells/ well) for 24 hrs before treatment with E6. Different concentrations of E6 (0, 1, 2.5, 5 and 10 µg/ml DMSO) were added to the cell monolayer, triplicate wells being prepared for each individual dose. Monolayer cells were incubated with E6 for 48 hrs at 37°C and in atmosphere of 5% CO_2. After 48 hrs, cells were fixed, washed and stained with sulforhodamine B (SRB) stain. Excess stain was washed with acetic acid and attached stain was recovered with tris-EDTA buffer.

Colour intensity was measured in an ELISA reader. The relation between surviving fraction and the plant extract concentration was plotted to get the survival curve of each tumor cell line after treatment. The potency was compared with reference (Cisplatin, DOX, and /or 5-FU).

Results And Discussion

Isolation and Identification of Some Constituents of Fraction E5:

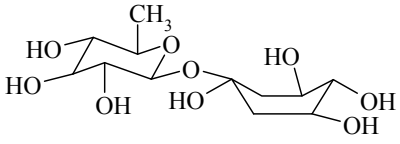
Compound I isolated as yellowish white amorphous powder (10 mg), R_f= 0.41 and 0.46 in solvent systems S_1 and S_3, respectively. Under UV light, it appeared as a rose fluorescent spot changed to blue fluorescent on exposure to ammonia vapour or spraying with $AlCl_3$. UV spectral data in MeOH was 256, 298sh, 351, 364. The ^{1}H-NMR spectrum exhibited a multiplet at δ 1.84 for H-2 and H-6, suggesting a cyclohexanol moiety in the molecule. An anomeric proton signal at δ 5.36 (d, *J*= 2.5 Hz, 1H) together with a singlet of three protons at δ 1.20 for Me-6` indicating the presence of rhamnose. The structure was confirmed by determination of positive electrospray ionization mass spectrometry (ESI-MS): *m/z* 312 [M^++2H].

Therefore compound I was identified as 3,4,5-trihydroxy cyclohexan-1-ol (1`→1)-rhamnoside.

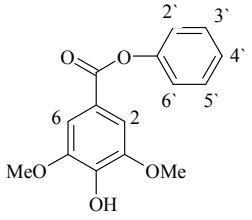
Compound II isolated as yellowish white amorphous powder (40 mg), R_f= 0.76 and 0.73 in solvent systems S_2 and S_8, respectively. Under UV light, it appeared as a blue fluorescent spot unchanged on exposure to ammonia vapour or spraying with $AlCl_3$. UV spectral data in MeOH showed one main band at 282 which does not give shift by addition of NaOMe. The ^{1}H-NMR spectrum exhibited a singlet at δ 7.20 assigned to H-2 and H-6 and a singlet at δ 5.42 assigned to a hydroxyl group at C-4, suggesting a gallic acid moiety in the molecule. A singlet at δ 3.80 assigned to a methoxy group at C-3 and C-5. A multiplet of 5 protons at δ 7.67-8.14, suggesting a benzene ring in the molecule. The structure was confirmed by determination of positive electrospray ionization mass spectrometry (ESI-MS): *m/z* 275 [M^++H].

From the above data, compound II was identified as phenyl ester of 3,5-dimethyl gallic acid.

This is the first report concerning the isolation of compounds I and II from the plant.



Compound I



Compound II

Fig. 1: Structure of the compounds isolated from the *n*-butanol fraction of *C. insignis* (Compound I: 3,4,5-trihydroxy cyclohexan-1-ol (1`→1)-rhamnoside and Compound II: phenyl ester of 3,5-dimethyl gallic acid)

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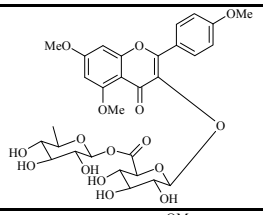
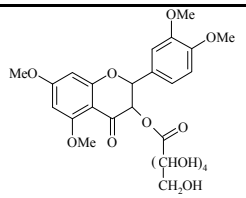
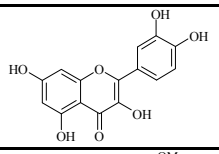
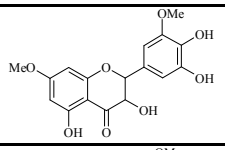
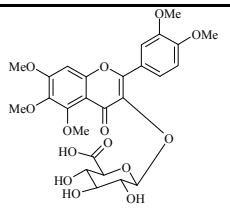
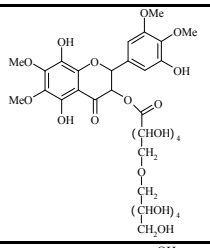
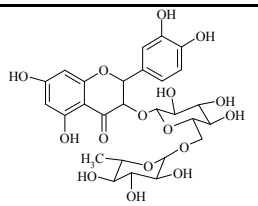
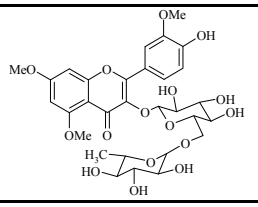
HPLC Investigation of Fraction E5:

The compounds expected to be present in E5 analyzed using HPLC/MS technique are placed in Table (1). These compounds were 74.12% and detected for the first time in the genus *Chorisia*.

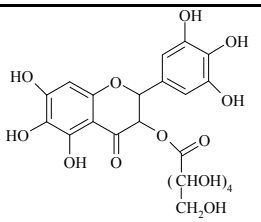
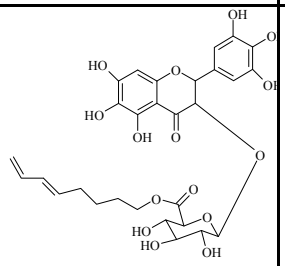
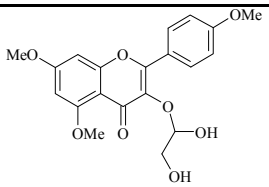
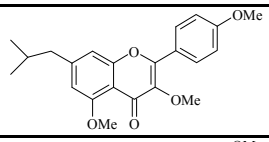
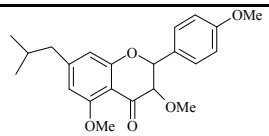
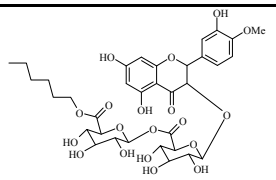
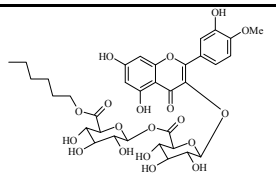
Table 1: The compounds expected to be present in fraction E5 analyzed using HPLC/MS technique:

Peak No.	Rt. (min.)	Area %	M.S.		Molecular Formula	Proposed Structure	Name
			M^+	M^+-H			
B1	2.9	2.46	650	649	$C_{30}H_{34}O_{16}$		Kampferol 5,7,4`-trimethyl ether 3-*O*-*α*-L-rhamnosyl-(1```→6``)-*O*-*β*-D-glucuronide
B2	4.7	46.52	538	537	$C_{25}H_{30}O_{13}$		Dihydroquercetin 5,7,3`,4`-tetramethyl ether 3-*O*-glucuronide
B3	25.5	10.69	302	301	$C_{15}H_{10}O_7$		Quercetin
B4	27.1	2.29	348	347	$C_{17}H_{16}O_8$		5,4`,5`-trihydroxy-7,3`-dimethoxy 2,3-dihydroflavonol
B5	30.8	2.33	564	563	$C_{26}H_{28}O_{14}$		Quercetagetin-5,6,7,3`,4`-pentamethyl ether 3-*O*-*β*-D-glucuronide
B6	31.2	1.64	750	749	$C_{31}H_{42}O_{21}$		5,8,5`-trihydroxy-6,7,3`,4`-tetramethoxy 3-*O*-glucuronyl dulcitol
B7	33.1	7.21	612	611	$C_{27}H_{32}O_{16}$		Dihydroquercetin 3-*O*-*α*-L-rhamnopyranosyl-(1```→6``)-*O*-*β*-D-glucopyranoside
B8	36.0	0.98	652	651	$C_{30}H_{36}O_{16}$		Quercetin 5,7,3`-trimethyl ether 3-*O*-*α*-L-rhamnopyranosyl-(1```→6``)-*O*-*β*-D-glucopyranoside

Investigation of Fraction E4:

The compounds expected to be present in E4 analyzed using HPLC/MS-MS technique are placed in Table (2). These compounds were 73.90% and detected for the first time in the genus *Chorisia*.

Table 2: The compounds expected to be present in fraction E4 analyzed using HPLC/MS-MS technique:

Peak No.	Rt. (min.)	Area %	M.S.		Molecular Formula	Proposed Structure	Name
			M^+	Daughter ions			
A1	4.3	29.95	514	334 (100%) [M^+-side chain-H]	$C_{21}H_{22}O_{15}$		5,6,7,3`,4`,5`-hexahydroxy-dihydroflavonol-3-*O*-glucuronide
A2	4.7	10.14	620	334 (100%) [M^+-side chain-H], 583 [M^+-2H_2O-H] and 308 [M^+-CO+H]	$C_{29}H_{32}O_{15}$		5,6,7,3`,4`,5`-hexahydroxy-dihydroflavonol-3-*O*-*β*-D-$\Delta^{1,3}$-octadienyl-glucuronide
A3	5.4	5.12	388	311 (100%) [M^+-side chain] and 387 [M^+-H]	$C_{20}H_{20}O_8$		Kampferol 5,7,4`-trimethyl ether 3-*O*-ethylene glycol
A4	5.5	4.83	368	365 (100%) [M^+-3H] and 321 [M^+-OCH_3-CH_3-H]	$C_{22}H_{24}O_5$		3,5,4`-trimethoxy-7-isobutyl flavone
A5	5.8	4.44	370	333 (100%) [M^+-2H-OCH_3-4H]	$C_{22}H_{26}O_5$		3,5,4`-trimethoxy-7-isobutyl dihydroflavone
A6	6.1	6.23	754	735 (100%) [M^+-H_2O-H] and 709 [M^+-OCH_3-CH_3+H]	$C_{34}H_{42}O_{17}$		Dihydroquercetin-4`-methyl ether 3-*O*-*β*-D-*n*-hexyl-diglucuronide
A7	6.3	13.19	752	653 (100%) [M^+-$(CH_2)_5CH_3$+H] and 705 [M^+-OCH_3-OH+H]	$C_{34}H_{40}O_{17}$		Quercetin-4`-methyl ether 3-*O*-*β*-D-*n*-hexyl-diglucuronide

In vitro Cytotoxic Activity:

As the plant has high median lethal dose (LD_{50} of E6= 7.8 g/kg b.wt.), indicating its low toxicity (El Alfy *et al.*, 2010), it was deemed of interest to investigate its cytotoxic activity. Table (3) illustrates the potential

cytotoxicity of E6 of *C. insignis* leaves, against human tumor cell lines. It showed a significant cytotoxic activity against the larynx cell line (IC50= 2.21 µg) as compared with Cisplatin (IC50= 0.66 µg), DOX (IC50= 0.74 µg) and 5-FU (IC50= 2.20 µg).

Table 3: Potential cytotoxicity of the total alcohol extract of *C. insignis* leaves:

Cell line	Conc. µg/mL	Total alcohol extract		Cisplatin		Doxorubicin (DOX)		5-fluorouracil (5-FU)	
		SF	MSE	SF	MSE	SF	MSE	SF	MSE
Brain (U25l)	0.00	1.00	0.07	1.00	0.07	Nt	Nt	Nt	Nt
	1.00	0.87	0.03	0.61	0.07	Nt	Nt	Nt	Nt
	2.50	0.83	0.01	0.56	0.05	Nt	Nt	Nt	Nt
	5.00	0.82	0.01	0.56	0.07	Nt	Nt	Nt	Nt
	10.00	0.80	0.01	0.49	0.05	Nt	Nt	Nt	Nt
Liver (HEPG2)	0.00	1.00	0.07	1.00	0.10	1.00	0.00	1.00	0.02
	1.00	0.98	0.02	0.60	0.04	0.39	0.04	0.65	0.05
	2.50	0.92	0.02	0.59	0.06	0.25	0.03	0.53	0.04
	5.00	0.91	0.01	0.52	0.04	0.22	0.02	0.51	0.03
	10.00	0.77	0.01	0.52	0.05	0.26	0.02	0.46	0.01
Breast (MCF7)	0.00	1.00	0.07	1.00	0.00	1.00	0.00	1.00	0.03
	1.00	0.90	0.02	0.11	0.01	0.48	0.03	0.66	0.03
	2.50	0.83	0.01	0.13	0.00	0.41	0.03	0.47	0.02
	5.00	0.78	0.01	0.13	0.00	0.26	0.02	0.40	0.03
	10.00	0.75	0.02	0.14	0.01	0.30	0.02	0.40	0.05
Cervix (HELA)	0.00	1.00	0.05	1.00	0.08	1.00	0.00	1.00	0.00
	1.00	0.96	0.01	0.90	0.06	0.42	0.02	0.47	0.03
	2.50	0.90	0.01	0.42	0.02	0.37	0.02	0.37	0.02
	5.00	0.85	0.01	0.17	0.04	0.28	0.02	0.29	0.01
	10.00	0.83	0.01	0.06	0.02	0.19	0.01	0.20	0.02
Colon (HCT116)	0.00	1.00	0.03	1.00	0.00	1.00	0.00	1.00	0.04
	1.00	0.99	0.01	0.25	0.02	0.45	0.06	0.67	0.10
	2.50	0.92	0.01	0.30	0.04	0.34	0.04	0.69	0.11
	5.00	0.86	0.01	0.33	0.02	0.30	0.03	0.46	0.03
	10.00	0.79	0.01	0.37	0.02	0.28	0.02	0.44	0.05
Larynx (HEP2)	0.00	1.00	0.08	1.00	0.00	1.00	0.06	1.00	0.00
	1.00	0.80	0.12	0.20	0.02	0.27	0.03	0.56	0.05
	2.50	0.47	0.09	0.23	0.02	0.30	0.03	0.49	0.05
	5.00	0.55	0.03	0.31	0.04	0.23	0.03	0.38	0.02
	10.00	0.60	0.02	0.27	0.02	0.24	0.01	0.66	0.14

SF: Survival fraction, MSE: Mean standard error, Nt: Not tested.

E6 showed slight effects towards the breast cell line (surviving fraction= 0.753), the liver cell line (surviving fraction= 0.770), the brain cell line (surviving fraction= 0.799) and the cervix cell line (surviving fraction= 0.826).

As E6 showed a significant cytotoxic activity against the larynx cell line (HEP2), potential cytotoxicity of the successive extracts of *C. insignis* leaves was carried out against this cell line; the results are shown in Fig. (2).

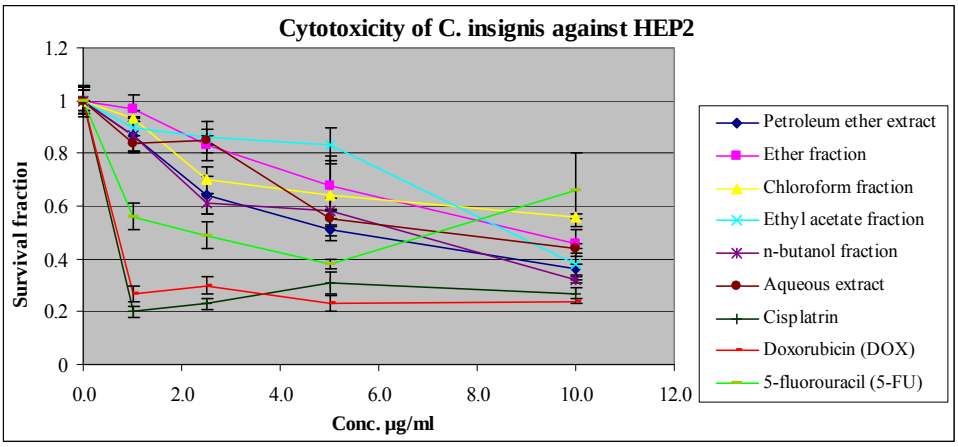


Fig. 2: Cytotoxicity of the aqueous and petroleum ether extracts and fractions of the 70% ethanol extract of *C. insignis* against larynx cell line (HEP2)

Petroleum ether extract IC50= 5.12 µg, Ether fraction IC50= 9.06 µg, Chloroform fraction IC50> 10.00 µg, Ethyl acetate fraction IC50= 8.61 µg, *n*-butanol fraction IC50= 6.58 µg, Aqueous extract IC50= 7.11 µg, Cisplatin IC50= 0.66 µg, DOX IC50= 0.74 µg, 5-FU IC50= 2.20 µg.

All the extracts showed significant cytotoxic activities against the larynx cell line except E3. E1 has the highest activity (IC50= 5.12 µg) followed by E5 (IC50= 6.58 µg) and E7 (IC50= 7.11 µg) then E4 (IC50= 8.61 µg), in comparison with Cisplatin (IC50= 0.66 µg), DOX (IC50= 0.74 µg) and 5-FU (IC50= 2.20 µg). The least cytotoxic activity was exhibited by E2 which showed IC50= 9.06 µg.

The cytotoxic activity of E1 could be attributed to its hydrocarbon and sterol contents (Hirose *et al.*, 1991; Hwang, 1992; Hahismoto *et al.*, 2008).

The significant cytotoxic activity of the *n*-butanol fraction could possibly be attributed to its flavonoidal content. Oxidative DNA damage is a known risk factor of cancer. Antioxidants, such as quercetin, apigenin and luteolin, are thought to play an important role in protecting cells from oxidative stress induced by reactive oxygen species.

Flavonoids, and in particular flavonols, are inversely related to laryngeal cancer risk (Garavello *et al.*, 2007). *In vitro* and animal model systems showed that they influence signal transduction pathways, stimulate apoptosis and inhibit inflammation and proliferation in human cancer cell lines (Neuhouser, 2004).

Low levels of flavonols may be associated with an increased risk of laryngeal cancer. This association could be partially responsible for the well-established inverse association between intake of fruits and vegetables and the occurrence of laryngeal cancer (Garavello *et al.*, 2007).

Quercetin aglycone has been shown to interact with some receptors, particularly an aryl hydrocarbon receptor, which is involved in the development of cancers induced by certain chemicals. Quercetin aglycone has also been shown to modulate several signal transduction pathways, which are associated with the processes of inflammation and carcinogenesis. Rodent studies have demonstrated that dietary administration of this flavonol prevents chemically induced carcinogenesis. Dietary quercetin is, therefore, a promising agent for cancer prevention (Murakami *et al.*, 2009).

Luteolin completely inhibits the catalytic activity of eukaryotic DNA topoisomerase I, similar to camptothecin, a cytotoxic quinoline alkaloid, which supports its therapeutic potential as a lead anti-cancer compound that poisons topoisomerases (Chowdhury *et al.*, 2002). Luteolin induces apoptosis in various cancer cells (Horinaka *et al.*, 2005).

In vitro effects of quercetin and luteolin against human carcinoma of larynx (HEP-2) and sarcoma-l 80 (S-l 80) cell lines were studied by Elangovan *et al.* (1994). They found that luteolin inhibited the proliferation of these cells and the inhibitory concentration (IC50) was found to be closely equal to plumbagin, an anticancer drug, quercetin showed less inhibition.

Priming HEP-2 cells with quercetin increased the cisplatin-induced apoptosis by 16.3% through the mitochondrial pathway which improve the efficacy of chemotherapy for head and neck cancer (Kuhar *et al.*, 2007).

Conclusion:

From this study it can be concluded that *C. insignis* extracts possess significant cytotoxic activities against the larynx cell line which could be attributed to their hydrocarbon, sterol and flavonoidal contents, so they could be used in pharmaceutical formulations after carrying out the clinical trials.

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