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Phytochemical composition, *in vitro* antioxidant and anticancer activities of quercetin from methanol extract of *Asparagus cochinchinensis* (LOUR.) Merr. tuber

Hoang Le Son* and Nguyen Phuc Anh

School of Biotechnology, International University, Vietnam National University, HCM City, Vietnam.

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Five compounds including quercetin (AC01), asparagine (AC02), sucrose (AC03), β-sitosterol-3-O-β-D-glucopyranoside (AC04) and β-sitosterol (AC05) were isolated from the methanol extract of *Asparagus cochinchinensis* (Lour.) Merr. tuber collected in Ba Ria–Vung Tau Province of Vietnam. Their structures were elucidated by NMR (1D and 2D-NMR). Quercetin (AC01) was subjected to the assay for antioxidant and anticancer activities. The 1,1-diphenyl-2-picrylhydrazyl (DPPH) free radical scavenging assay was employed for determining the antioxidant activity, while sulforhodamine B (SRB) method was applied for evaluating the anticancer activity against four selected human cancer cell lines. Quercetin had strong antioxidant activity with IC_{50} = 14.52 ± 2.12 µg/ml (as compared to standard vitamin C with IC_{50} = 10.49 ± 2.00 µg/ml). Meanwhile, quercetin (AC01) exhibited strong cytotoxicity against the HeLa, human cervical cancer cell line with IC_{50} = 5.78 ± 0.36 µg/ml, followed by lung cancer cell line (NCI–H460), lung cancer cell line with IC_{50} = 12.57 ± 1.19 µg/ml and liver cancer cell line (Hep-G2) liver cancer cell line with IC_{50} = 20.58 ± 0.85 µg/ml. The anticancer activity of quercetin against breast cancer cell line (MCF-7), breast cancer cell line was recorded with IC_{50} = 31.04 ± 3.14 µg/ml.

Key words: *Asparagus cochinchinensis*, 1,1-diphenyl-2-picrylhydrazyl (DPPH), sulforhodamine B (SRB), human cervical cancer cell line (HeLa), lung cancer cell line (NCI-H460), breast cancer cell line (MCF-7), liver cancer cell line (Hep-G2).

INTRODUCTION

Asparagus cochinchinensis (Lour.) Merr. (*A. cochinchinensis*), belonging to Liliaceae family, has been known as a traditional medicinal herb in China over thousand years. It is a perennial tuber that usually grows in abundance in Eastern Asia including China, Japan and Korea (Bich, 2007).

The Asparageceae is a large group including *Asparagus officinalis, Asparagus racemosus, Asparagus asparagoides, Asparagus scandens, Asparagus plumosus* and *Asparagus falcatus* (Tatsuya et al., 2011). Among all, there were some deeply investigated plants: fruits of *A. officinalis* were well known because of the antifungal activity while its roots and seeds were highly toxic (Makoto et al., 1996; Shao et al., 1997; Shimoyamada et al., 1990; Velavan et al., 2007). The roots of *A. racemosus* were proved to have potential antioxidant, anticancer, antibacterial and antitussive activities based on some active components such as steroidal saponins, racemofuran, asparagamine A and racemosol (Velavan et al., 2007; Mandal et al., 2006; Wiboonpun et al., 2004; Mandal et al., 2000a; Mandal et al., 2000b).

A. cochinchinensis is often used for the treatment of fever, cough, hemoptysis, diabetes, constipation, swollen

*Corresponding author. E-mail: hlson@hcmiu.edu.vn. Fax: 08. 37244271.

and throat pain (Bich, 2007). This plant is also used to treat lung cancer, tuberculosis, heart diseases (Jiang et al., 2010), premature birth, diuretic haemoptysis and nervous diseases (Chi, 2012; Dan, 2004; Loi, 2011). Previous researches discovered that saponins, steroids, phytosterols, polysaccharides and free amino acids are the main constituents of *A. cochinchinensis* (Bich, 2007). The chemical constituents such as spirostanol saponins (asparacoside), spirosteroids (asparacosine A and B), acetylenic derivatives (3''-methoxyasparenydiol), polyphenol (3'-hydroxyl-4'-methoxy-4'-dehydroxynyasol) and phenols (aparennydiol, nyasol, 3''-methoxynyasol, 1,3-bis-di-*p*–hydroxyphenyl-4-penten-1-one and *trans*-coniferyl alcohol) showed the potential in cytotoxicities in a panel comprised of KB, Col-2, LNCaP, Lu-1, and HUVEC cells (Zhang et al., 2004). Besides, four saponins including (25S)-26-O-*β*-D-glucopyranosyl-5*β*-furost-20(22)-en-3*β*, 15*β*, 26-triol-3-O-[α-L-rhamnopyranosyl-(1–4)]-*β*-D-glucopyranoside (namely aspacochioside D), aspacochioside C, (25S)-5*β*-spirostan-3*β*-yl-O-[O-α-L-rhamnopyranosyl-(1–4)]-*β*-D-glucopyranoside) and pseudoprotoneodioscin, which were isolated from *A. cochinchinensis* (Lour.) Merr, were evaluated in a cytotoxicity assay against the human tumor cell line, A549 (Yang et al., 2011).

The aim of this research was to isolate and identify compounds from methanol extract of *A. cochinchinensis* tuber collected in Vietnam and to test bioactivities including anticancer and antioxidant activities of active compound.

MATERIALS AND METHODS

Reagents and equipment

Medium pressure liquid chromatography was performed on CombiFlash Rf machine (USA) using silica gel (E. Merck, 230-400 mesh) column (RediSep cartridge, USA). Precoated silica gel 60 F254 plates (Germany) were used for thin layer chromatography (TLC). Sulforhodamine B (SRB) solution, 1,1-diphenyl-2-picrylhydrazyl (DPPH), ascorbic acid and various solvents were purchased from Merck (Germany). All reagents and solvents were of analytical grade.

The cervical cancer HeLa, lung cancer NCI-H460, breast cancer MCF-7 and liver cancer Hep-G2 cell lines were supplied from the National Cancer Institute of the United States (NCI - Frederick, MD, USA). The cells were cultured in E'MEM environment including L-glutamine (200 mM), HEPES (1 M), amphotericin B (0.1%), penicillin-streptomycin 200X and 10% (v/v) fetal bovine serum (FBS) and incubated at 37°C with 5% CO_2.

The ^{1}H-NMR and ^{13}C-NMR spectra (500 MHz for ^{1}H-NMR and 125 MHz for ^{13}C-NMR) were recorded using DMSO as solvent. The structures of pure compounds were elucidated based on the distortionless enhancement by polarization transfer (DEPT), correlation spectroscopy (COSY), heteronuclear single quantum coherence (HSQC) and heteronuclear multiple-quantum correlation (HMQC).

Collection of plant material and preparation of extract

A. cochinchinensis tubers were collected from Ba Ria-Vung Tau Province of Vietnam. The tubers were washed with water and cut into thin slices before being heat dried at 60°C. The samples were then ground into fine powder by a mechanical grinder. The powder (1.5 kg) was extracted with MeOH at room temperature to result in a dark brown material (1.2 kg). The methanol extract was subjected to further separation by column chromatography over silica gel eluting with MeOH, $CHCl_3$ and distilled H_2O in increasing order of polarity to obtain 4 fractions (A to D).

Isolation and identification of compounds

The fraction A (100% $CHCl_3$) was subjected to medium pressure liquid chromatography (MPLC) eluting with MeOH-$CHCl_3$ in increasing order of polarity to produce 7 sub-fractions (A to G). The sub-fraction D showed a major spot on TLC yielded quercetin (AC01, 10 mg). The sub-fraction B was re-chromatographed over silica gel eluting with EtOAc-$CHCl_3$ in increasing order of polarity to obtain 5 sub-fractions (A' to E'). Asparagine (AC02, 9 mg) was precipitated from sub-fraction A'. Sub-fraction C', D' and E' were combined to subject to HPLC to obtain 4 fractions. *β*-sitosterol-3-O-*β*-D-glucopyranoside (AC04, 45 mg) and *β*-sitosterol (AC05, 30 mg) were isolated by crystallization in the 2nd and 3rd sub-fractions. The sub-fraction C was re-chromatographed over silica gel eluting with $CHCl_3$ and MeOH in increasing order of polarity. From the 4th sub-fraction, sucrose (AC03, 6 mg) was isolated by crystallization.

The characteristics of isolated components, AC01 (Quercetin), AC02 (Asparagine), AC03 (Sucrose), AC04 (*β*-sitosterol-3-O-*β*-D-glucopyranoside) and AC05 (*β*-sitosterol), were evaluated by standard methods and the data obtained must be in agreement with those reported in literature (Aarti et al., 2012; Lide, 1997; Sucrose, 2003; Oja et al., 2009; Vauquelin et al., 1806).

Biological activity

Antioxidant assay

The antioxidant activity was determined using DPPH free radical scavenging assay with slight modifications (Amin et al., 2006). The initial absorbance of 150 µl of ethanolic DPPH was measured at 517 nm without sample. An aliquot (50 µl) of samples was mixed with ethanolic DPPH solution. With different prepared concentrations, 200 µl of each solution was loaded into wells in the same row, 3 replicates were loaded into 3 adjacent wells. Each test took 30 min to complete (not including the negative control test). The change in absorbance at 517 nm was measured after 30 min of incubation at room temperature. Ascorbic acid was served as positive control with the concentration between 5 and 100 µg/ml. The experiment was replicated three times. The percentage of DPPH free radical scavenging activity was evaluated using the following equation:

$$\text{Inhibition (\%)} = [1- (OD_s - OD_c)] \times 100\%$$

where OD_c is the OD value of the well which is control; OD_s is OD value of the well which contains sample.

Anticancer assay

The anticancer activity was determined using SRB assay with minor modifications (Skehen et al., 1990; Vichai and Kanyawim, 2006). First, the cells were inoculated and incubated in the 96-well plates at room temperature with 5% CO_2, 95% air and 100% relative humidity for 24 h. The plates were then fixed with TCA, to represent a measurement of the cell population for each cell line at the time drug was added. The samples were prepared to double concentration of initial sample concentration and loaded into wells

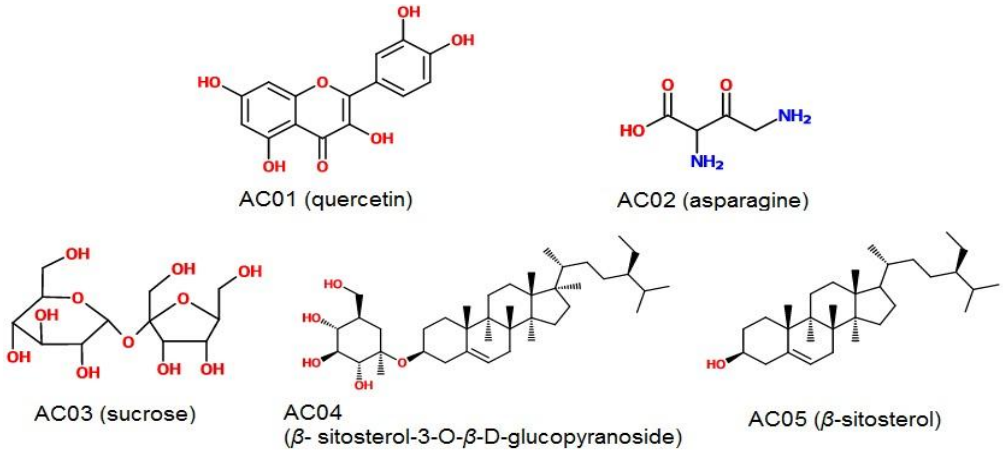

Figure 1. Structure of five pure compounds.

before being incubated for further 48 h at room temperature with 5% CO_2, 95% air and 100% relative humidity. The cells were then fixed by the gentle addition of 50 µl of cold 50% (w/v) TCA (final concentration of TCA per well was 10%) and incubated for 60 min at 4°C. The supernatant was discarded and the plates were washed five times with tap water and air dried. Finally, SRB solution (100 µL) at 0.4% (w/v) in 1% acetic acid was added to each well and plates were incubated for 10 min at room temperature. After staining, unbound dye was removed by washing five times with 1% acetic acid and the plates were air dried. Bound stain was subsequently solubilized with 10 mM trizma base, and the absorbance was read on an automated plate reader. DMSO was used as negative control while camptothecin was served as positive control with different concentrations for each cancer cell line: HeLa (1 µg/ml); NCI-H460 and MCF-7 (0.01 µg/ml); Hep-G2 (0.07 µg/ml).

By measuring the absorbance at 492 and 620 nm, the percentage of growth inhibition was calculated using the following formulas:

$$OD_{492} \text{ (or } OD_{620}) = OD_{cell} - OD_{blank} \quad (1)$$

$$OD = OD_{492} - OD_{620} \quad (2)$$

$$\text{Growth inhibition (\%)} = [1- (OD_s - OD_c)] \times 100\%$$

where OD_{cell} is the OD value of the well which contains cells; OD_{blank} is OD value of the well which is blank; OD_s is OD value of the well which contains samples calculated from Equations 1 and 2; OD_c is OD value of the well which contains the control solution calculated from Equations 1 and 2.

RESULTS

Five pure compounds were isolated from methanol extract of *A. cochinchinensis* including quercetin (AC01), asparagine (AC02), sucrose (AC03), *β*-sitosterol-3-O-*β*-D-glucopyranoside (AC04) and *β*-sitosterol (AC05) as shown in Figure 1. The characteristics of these compounds were summarized in Table 1.

Antioxidant defense mechanisms include enzymatic and nonenzymatic strategies. Common antioxidants include the vitamins A, C and E, glutathione, and the enzymes superoxide dismutase, catalase, glutathione peroxidase and glutathione reductase while the others involve α-lipoic acid, mixed carotenoids, coenzyme Q_{10}, biofavonoids, antioxidant minerals and cofactors (Maritim et al., 2003). Among five isolated compounds, quercetin shows the potent antioxidant activity, because it belongs to flavonoid group and had been studied previously (Seyed et al., 2012; Jin-feng et al., 2012; Nimmi and George, 2012; Vasagam et al., 2011). Grzegorz (2013) reported the low antioxidant activity of asparagine compared to butylhydroxytoluene (BHT) which was six times weaker than ascorbic acid (Samruan et al., 2012). Sucrose and other sugars were proved to reduce hydroxyl radical (Nishizawa et al., 2008). The antioxidant activity of sucrose was evaluated with IC_{50} value of 2.7 mM compared to ascorbic acid (IC_{50} value was 1.9 mM) (Wim and Ravi, 2009; Mahakunakorn et al., 2004). Phytosterols such as *β*-sitosterol are responsible to revert the impairment of the glutathione/oxidized glutathione ratio induced by phorbol esters in RAW 264.7 macrophage cultures so that *β*-sitosterol can affect antioxidant enzymes on the estrogen/phosphatidylinositol 3-kinase pathway (Viviancos and Moreno, 2005). *In vitro*, it was reported that *β*-sitosterol reduced the level of free radicals in mice by up to 78% (Panigua et al., 2008). In this research, besides quercetin, the antioxidant activity of asparagine were also tested, sucrose, *β*-sitosterol-3-O-*β*-D-glucopyranoside and *β*-sitosterol have no significant result (data not shown). The antioxidant activity of quercetin (AC01) was compared to that of ascorbic acid as positive control. As shown in Table 2 and Figure 2, the percentage of DPPH free radical scavenging activity was increased in a concentration-dependent manner in quercetin and ascorbic acid. The antioxidant activity of quercetin and ascorbic acid was comparable. However, quercetin showed the IC_{50} = 14.52 ± 2.12 µg/ml, higher than that of ascorbic acid with IC_{50} = 10.49 ± 2.00 µg/ml.

Antioxidants reduce free radical reactions and free radicals are chemical entities with one or more unpaired electrons. Therefore, free radicals can cause DNA

Table 1. Characterization of pure compounds.

Characterization	Component				
	Quercetin	**Asparagine**	**Sucrose**	***β*-sitosterol-3-O-*β*-D-glucopyranoside**	***β*-sitosterol**
Chemical formula	$C_{15}H_{10}O_7$	$C_4H_8N_2O_3$	$C_{12}H_{22}O_{11}$	$C_{35}H_{60}O_6$	$C_{29}H_{50}O$
Molecular weight	302.3 g/mol	132.2 g/mol	342.2 g/mol	576.8 g/mol	414.8 g/mol
Description	Yellow needles	Colorless crystal	Colorless crystal	Colorless powder	Colorless needles
Boiling point	Sublimes	438.2°C	170°C	501.7°C	-
Melting point	316.4°C	235.5°C	185.2°C	276°C	134°C
Solubility	Soluble in ethanol and acetone, slightly soluble in water and diethyl ether	Soluble in acid, ethanol, ether and benzene	Soluble in water and methanol; slightly soluble in ethanol; insoluble in ethyl ether.	Soluble in alcohol	Soluble in alcohol
Density	1.82 g/cm^3	1.53 g/cm^3	1.58 g/cm^3	-	-
R_f (solvent system)	0.22 ($CHCl_3$:MeOH=9:1)	-	0.54 ($CHCl_3$:MeOH=7:3)	0.3 ($CHCl_3$:MeOH=9:1)	0.86 ($CHCl_3$:MeOH=9:1)

mutations, change in enzymatic activity, lipid peroxidation of cellular membrane and cause death (Birangane et al., 2011). Ashok et al. (2012) also demonstrated that free radicals can damage DNA and cause mutagenicity and cytotoxicity. This can cause carcinogenesis. The reactive oxygen species can induce mutations and inhibits DNA repair process that results in inactivation of certain tumor repressor genes to lead to cancer. With those studies, quercetin, the most active compound, was subjected to anticancer activity tests. The anticancer activity of quercetin (AC01) was assayed against four selected human cancer cell lines, HeLa, NCI-H460, MCF-7 and Hep-G2 cells. As shown from Table 3 and Figure 3, quercetin exhibited a potent anticancer activity against all four selected human cancer cell lines. Quercetin showed the strongest anticancer activity against HeLa cancer cell line with IC_{50} = 5.78 ± 0.36 µg/ml, followed by NCI-H460 and Hep-G2 with IC_{50} = 12.57 ± 1.19 and 20.58 ± 0.85 µg/ml, respectively. The weakest growth inhibition was found on MCF-7 cancer cell line with IC_{50} = 31.04 ± 3.14 µg/ml.

DISCUSSION

A. cochinchinensis is widely distributed in the North and Central of Vietnam and has been traditionally used by local people to cure various diseases including cancer, so it is necessary to examine the pharmacological properties of this plant. This is the first research focusing on the elucidation of components from methanol extract of *A. cochinchinensis* tuber growing in Vietnam and evaluation of biological activities of active compound including antioxidant and anticancer activities. Quercetin (3,3',4',5,7-pentahydroxyflavone), the most represented polyphenolic derivative of flavonols, was isolated from methanol extract of *A. cochinchinensis* tuber and subjected to the assay for antioxidant and anticancer activities. Quercetin is also found in plant food sources such as apples, broccoli, berries, and onions as well as in various medicinal plants such as *Dorema aitchisonii*, *Lysimachia clethroides* and *Doplotaxis harra*.

Quercentin has been evaluated for antioxidant activity by DPPH assay reported from many plant species and the IC_{50} values were found differently. Examples are quercetin from *Dorema aitchisonii* with IC_{50} = 5.28 ± 0.2 µg/ml (Seyed et al., 2012) and *Lysimachia clethroides* with IC_{50} = 6.94 ± 0.03 µg/ml (Jin-feng et al., 2012). Quercetin was even used as a standard in evaluation of antioxidant potential of compounds from various medicinal plants and plant food sources, and the IC_{50} values were also varied from 10.25 ± 1.45 µg/ml (Nimmi and George, 2012) to 60 µg/ml (Vasagam et al., 2011). In this work, quercetin was found to have IC_{50} value of 14.52 ± 2.12 µg/ml, indicating that our finding is consistent with the previous results. It should be noted, however, that the different values of IC_{50} of quercetin resulting from DPPH assay using different plant sources are due to the

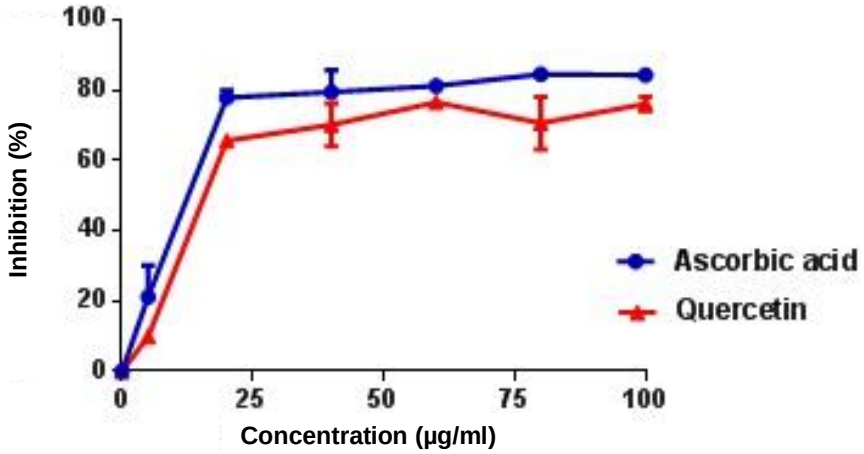

Figure 2. Antioxidant activity of quercetin and ascorbic acid.

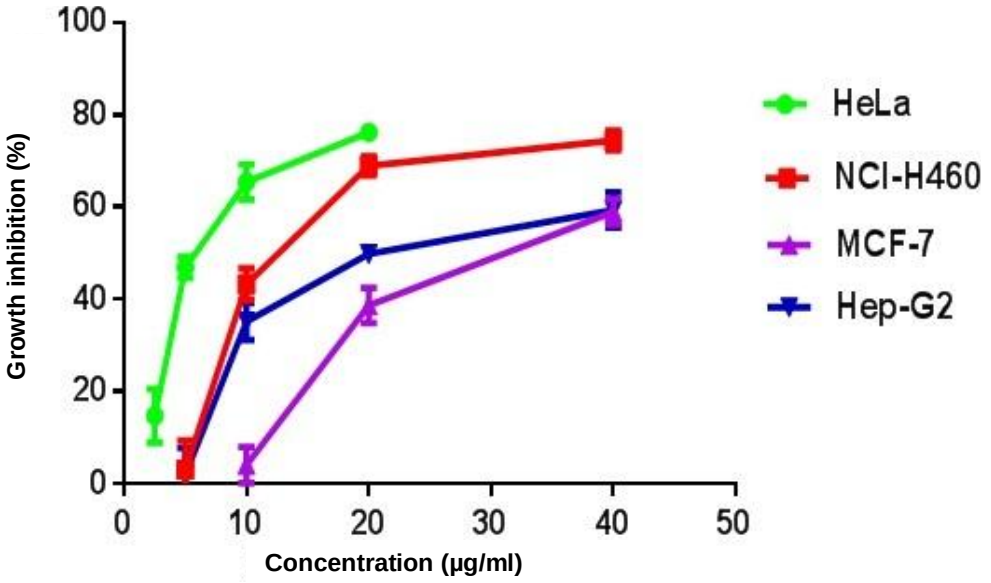

Figure 3. Percent growth inhibition of quercetin against four selected human cancer cell lines: HeLa, NCI-H460, MCF-7 and Hep-G2.

Table 2. Antioxidant activity of quercetin compared to that of ascorbic acid.

Concentration (µg/ml)	Inhibition (%)	
	Quercetin	Ascorbic acid
0	0	0
5	9.99 ± 1.22	21.24 ± 8.93
20	65.74 ± 1.46	78.00 ± 2.18
40	70.17 ± 6.24	79.56 ± 6.24
60	76.65 ± 1.34	81.31 ± 1.93
80	70.67 ± 7.51	84.63 ± 1.11
100	76.21 ± 2.03	84.38 ± 1.86
IC_{50} (µg/ml)	14.52 ± 2.12	10.49 ± 2.00

Data obtained from triplicate results of three experiments and shown as mean ± standard deviation (SD).

distribution of several factors such as differences in used solvents, concentration of DPPH working solutions, ratio between volumes of sample/reagent, duration of reaction, and equations for calculation of the results (Molyneux, 2004). To other components including asparagine, sucrose, *β*-sitosterol-3-O-*β*-D-glucopyranoside and *β*-sitosterol, the antioxidant activities were found insignificant.

Quercetin, isolated from different medicinal plants, has been reported to possess cytotoxicity against various human cancer cell lines. Quercetin was found to inhibit the growth of MCF-7 breast cancer cell line with IC_{50} = 5.2 µg/ml, HT-29 colon cancer cell line with IC_{50} = 81.2 mM (Jorge et al., 2010); HeLa cancer cell line with IC_{50} = 546.2 ± 1.87 µg/ml (MTT assay) (Murat et al., 2011); and HCT116 human colon carcinoma with IC_{50} = 20.1 µg/ ml

Table 3. Anticancer activity of quercetin against four selected cancer cell lines.

HeLa cancer cell line		NCI-H460 cancer cell line	
Concentration (µg/ml)	**Growth inhibition (%I)**	**Concentration (µg/ml)**	**Growth inhibition (%)**
2.5	14.78 ± 5.87	5	3.11 ± 6.34
5	46.99 ± 2.28	10	43.31 ± 3.46
10	65.45 ± 3.83	20	68.97 ± 2.13
20	76.22 ± 1.89	40	74.47 ± 2.09
-	IC_{50} = 5.78 ± 0.36 µg/ml	-	IC_{50} = 12.57 ± 1.19 µg/ml
MCF-7 cancer cell line		**Hep-G2 cancer cell line**	
Concentration (µg/ml)	**Growth inhibition (%)**	**Concentration (µg/ml)**	**Growth inhibition (%)**
10	4.16 ± 3.97	5	2.08 ± 5.89
20	38.73 ± 3.85	10	35.19 ± 4.02
40	59.00 ± 2.76	20	49.88 ± 0.82
-	-	40	59.40 ± 3.89
-	IC_{50} = 31.04 ± 3.14 µg/ml	-	IC_{50} = 20.58 ± 0.85 µg/ml

Data obtained from triplicate results of three experiments and shown as mean ± standard deviation (SD).

(Magdy et al., 2013). Statistically, the quercetin concentration at which cancer cell was inhibited by 50% inhibitory concentration (IC_{50}) ranged from 7 nM to just over 100 µM (Satyendra et al., 2012). These data support our findings in considering this medicinal plant as an alternative therapy of certain cancer. Furthermore, our findings were even supported by recent studies concerning with the mechanism of quercetin in inhibition of different cancer cell lines.

It has been suggested that quercetin may induce apoptosis by direct activation of the caspase cascade through the mitochondrial pathway in MCF-7 cells (Chou et al., 2010; Mozhgan et al., 2011). Quercetin can also modulate the estrogen receptor activity to inhibit the growth of breast cancer cell lines, MCF-7 (Urmila et al., 2011). Quercetin could suppress the viability of Hela cells in a dose-dependent manner by inducing G2/M phase cell cycle arrest and mitochondrial apoptosis through p53-dependent mechanism (Vidya et al., 2010); and quercetin may induce apoptosis by direct activation of caspase cascade through mitochondrial pathway and by inhibiting survival signaling in Hep-G2 (Granado-Serrano et al., 2006).

Conclusion

Five compounds isolated from the methanol extract of *A. cochinchinensis* tuber were identified with their structures including quercetin (AC01), asparagine (AC02), sucrose (AC03), *β*-sitosterol-3-O-*β*-D-glucopyranoside (AC04), *β*-sitosterol (AC05). Quercetin (AC01) has been proven to be a potent component in antioxidant and anticancer against all four selected human cancer cell lines, MCF-7, Hep-G2, NCI-H460 and HeLa cells. These findings reveal that *A. cochinchinensis* can be considered to be a potent medicinal plant in therapy of certain cancer.

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ABBREVIATIONS

KB, hela contaminated carcinoma/papilloma cells; **Col-2,** human colon carcinoma cells; **LNCaP,** androgen-sensitive prostate adenocarcinoma cells; **Lu-1,** lung adenocarcinoma cells; **HUVEC,** umbilical vein endothelial cells; **A549,** carcinomic human alveolar basal epithelial cells; **MeOH,** methanol; **EtOAc,** ethyl acetate, **$CHCl_3$,** chloroform; **MPLC,** medium pressure liquid chromatography; **NMR,** nuclear magnetic resonance spectroscopy; **DMSO,** dimethyl sulfoxide; **DPPH,** 1,1-diphenyl-2-picrylhydrazyl; **HEPES,** buffering agent in cell culture media; **FBS,** fetal bovine serum; **OD,** absorbance (optical density); **%I,** inhibition percentage; **SRB,** sulforhodamine B; **TCA,** trichloacetic acid; **SD,** standard deviation; **HeLa,** human cervical cancer cell line; **NCI-H460,** lung cancer cell line; **MCF-7,** breast cancer cell line; **Hep-G2,** liver cancer cell line; **IC_{50},** half maximal inhibitory concentration.

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