ABSTRACT

The present study aimed to find out cytotoxic agent from Allium cepa L. (ssp. red onion) family Alliaceae as well as chromatographic isolation of its active constituents.

Methods: The defatted methanol extract of Allium cepa (ssp. red onion) was fractionated with chloroform, ethyl acetate and n-butanol. Each of ethyl acetate and butanolic fraction was separately submitted to chromatographic isolation using different chromatographic techniques such as column chromatography (CC), thin layer chromatography (TLC) and paper chromatography (PC). The structures of the isolated compounds were elucidated through spectroscopic analysis.

Results: Four compounds were isolated from ethyl acetate fraction and identified as kaempferol (1), quercetin (2) isorhamnetin (3) andisorhamnetin-4'-O-β-D-glucopyranoside (4) whereas two compounds were isolated and identified from the butanolic fraction as a D-glucopyranose (5), β-D-glucopyranosyl-α-D-glucopyranoside (6). The cytotoxic activity of defatted methanolic extract of A. cepa (ssp red onion), ethyl acetate and butanolic fractions against human liver carcinoma cell line (HepG2) was IC50 = 10.9, 6.08 and 9.95 µg/ml respectively. Also, compounds 4 isolated from ethyl acetate fraction and compound 6 isolated from butanolic fraction showed cytotoxic activity (IC50 = 11.90 and 22.0 µg/ml) respectively.

Conclusion: It was appeared that the ethyl acetate fraction was the most active due to it contain high amount of phenolic compounds.

Keywords: Allium cepa, Cytotoxic, Human liver carcinoma cell lines HepG2, Chromatographic isolation.

INTRODUCTION

Cancer is a complex disease involving numerous tempospatial changes in cell physiology, which ultimately lead to malignant tumors. Abnormal cell growth is the biological endpoint of the disease. Tumor cell invasion of surrounding tissues and distant organs is the primary cause of morbidity and mortality for most cancer patients [1]. Hepatocellular cancer (HCC) is the fifth most common cause of cancer and the third leading cause of cancer-related deaths worldwide [2]. The main risk factors for HCC, chronic infections of hepatitis B virus (HBV), hepatitis C virus (HCV), aflatoxin, alcohol, smoking as well as other dietary agents are the most important in humans [3].

The plant kingdom represents an enormous reservoir of biologically active phytochemicals with various chemical structures and protective properties. These phytochemicals are often secondary metabolites in the plants and including alkaloids, steroids, flavonoids, terpenoids, tannins, and other groups. Many of these groups have scavenging activity for free radicals therefore; they can be reducing the tissue injury [4-6]. Now several drugs used in medicine are of plant origin, therefore much current research devoted to phytochemicals of the plants [7].

The Allium (family Alliaceae) has over 700 members each of them has special tastes, forms and colors. They have several pharmacological and biological properties such as anti-bacterial, anti-fungal and anti-inflammatory activities [8]. Many of these biological effects are related to the volatile sulfur compounds which are responsible of their characteristic pungent aroma and taste. However, these compounds are unstable and give rise to transformation products. For this reason, recent attention has been focused on polar compounds that are more stable to cooking and to the storage. Among these compounds, sapogenins, saponins, and flavonoids are the main classes found in Allium family [9]. In this study the defatted methanolic extract of A. cepa (ssp. red anion) was fractionated using different organic solvent as CHCl3, EtOAc and n-BuOH. Each of the ethyl acetate and n-butanol fractions was submitted to chromatographic isolation and evaluated as cytotoxic agent against Hep-G2 cell lines.

MATERIALS AND METHODS

General experimental procedures

Melting points were determined on an electronic thermostatic apparatus (Electrothermal 9200). The nuclear magnetic resonance as 1H-NMR and 13CNMR spectra were recorded on a Brucker Avance- 500 and JEOL GX-spectrometer (500 MHz for 1H and 125 MHz for 13C). The chemical shifts were expressed in δ (ppm) with TMS as reference. The coupling constant (J) in Hertz. UV spectra (λmax) of the isolated compounds were measured in methanol before and after addition of different reagents on Camspec M550 UV/Vis spectrophotometer (UK). Silica gel 60 (70-230 mesh, Merck) and Sephadex LH-20 (25-100 µm, Sigma) were used for column chromatography. Analytical and preparative thin layer chromatographies (TLC) were performed on silica gel GF254 pre-coated plates (Merck). Paper chromatography (PC) was carried out on Whatmann No. 1 and No. 3 paper sheets. Spots were visualized by absorption of UV radiation and spraying with ethanolic AlCl3 (2%), FeCl3 (1%) and 20% H2SO4 followed by heating for flavonoids compounds and sugars.

Chemicals

Aluminum chloride, Sodium acetate, Boric acid and Sodium methoxide were purchased from Merck (Darmstadt, Germany), Quercetin, kaempferol and isorhamnetin, were purchased from Sigma Aldrich (Milan, Italy) and all solvents are analytical grade.

Plant Materials

The bulbs of Allium cepa (ssp. red onion) were collected from El-Sharkia governorate, Egypt in March 2012. The collected plant was kindly identified by Prof. Dr. Waffa Amer, Professor of plant
taxonomy, Faculty of Science, Cairo University. Voucher specimen of
the plant was deposited at laboratory of medicinal chemistry, Theodor Bilharz Research Institute, Giza, Egypt. Then the plant
materials were submitted to extraction process.

Extraction and Fractionation

Fresh bulbs of Allium cepa (ssp. red onion) (4 Kg) were cut to small
pieces then grinded with electric mixer to be ready for extraction
process. The pieces of plant bulbs were extracted with pure
methanol on cold several times till exhaustion. The solvent was
distilled using rotary evaporator till dryness to give dried extract
290 g. The dried methanolic extract (250 g) was defatted with
petroleum ether. The defatted methanolic extract was successively
extracted with aqueous chloroform, ethyl acetate and n-butanol. The
discovered fractions were evaporated under reduced pressure
to dryness using rotary evaporator. The ethyl acetate and
butanolic extracts were kept for chromatographic separation
process.

Chromatographic Separation of EtOAc Extract

Ethyl acetate extract of A. cepa (ssp. red onion) (5.50 g) was
subjected to column chromatography (3×60 cm) packed with silica
gel 60 (70-230 mesh, Merck) as stationary phase. Elution started
with petroleum ether, petroleum ether/ CHCl3 mixture, CHCl3 and
then CHCl3/ MeOH gradient and ending with pure methanol.
Fractions 250 ml each were collected, concentrated and examined
by TLC silica gel using solvent systems; Benzene; MeOH (9:1 and 8:2);
CHCl3: MeOH: H2O (1: 5, PC). UV λ
values= 0.036 (15% Acetic acid, PC), 0.68 (n-butanol, acetic acid,
266, 302; (MeOH + AlCl3) 256, 268, 304; (MeOH + NaOAc) 275, 320,
392; (MeOH + NaOAc + H2O) 257, 275, 368. The H-NMR
δ; 12.39 (1H, s, 5-OH), 9.52 (1H, s, 3-OH), 8.99 for (1H, sh), 7.66
(1H, d, J= 2.0 Hz, H-2), 7.59 (1H, dd, J= 8.6 and 2.1 Hz, H-6), 7.21
(1H, d, J= 8.6 Hz, H-5), 6.41 (1H, d, J= 2.0 Hz, H-8), 6.16 (1H, d, J= 2.0
Hz, H-6), 5.15 (1H, d, J= 6.7 Hz, H-1''), Glc and 3.70 (3H, s, OCH3).

C-TLC: NMR 8: 176.57 (C-4’), 146.84 (C-3’), 146.40 (C-4’), 136.95 (C-3’), 125.61 (C-1’), 120.04 (C-6’), 116.27 (C-5’), 115.64 (C-2’), 103.59 (C-10’), 101.82 (C-1’), 98.78 (C-6), 98.04 (C-8), 77.99 (C-3’), 76.44 (C-5’), 73.77 (C-2’), 70.40 (C-4’), 69.72 (C-6’), 61.19 (3’-OCH3).

Chromatographic Separation of n-BuOH Extract

Butanolic extract of Allium cepa (ssp. red onion) (30 g) was
subjected to column chromatography (6×120 cm) packed with silica
gel 60 (70-230 mesh, Merck) as stationary phase. Elution started
with pure CHCl3 and then CHCl3/ MeOH gradient and ending with
pure methanol. Fractions 250 ml were collected, concentrated and
examined by TLC silica gel using solvent systems; Benzene: MeOH
(9:1 and 8:2). The defatted methanolic extract was successively
extracted with aqueous chloroform, ethyl acetate and n-butanol. The
discovered fractions were evaporated under reduced pressure
to dryness using rotary evaporator. The ethyl acetate and
butanolic extracts were kept for chromatographic separation
process.

Cytotoxic Assay

The defatted methanolic extract of A. cepa (ssp. red onion) and their
fractions (EtOAc and n-BuOH) were investigated toward cytototoxic
assay of human liver carcinoma cell line (HepG2). This part was
carried out at National Cancer Institute, Cairo, Egypt, according to
protocol of Skehan et al., 1990 [10]. This is a colorimetric assay
that estimates cell number indirectly by staining total cellular
dna. Cells were seeded in 96-well microtiter plates at a concentration of 5×104-105 cell/well in a fresh medium and left to attach to the plates for 24 h. For each sample, different concentrations (0, 5, 12.5, 25, and 50 µg/mL) were
added to 100 µL wells. Wells were completed to total of 200 µL
volume/ well using fresh medium and incubated for 48 h at 37°C in
5% CO2. Following 48th treatment, the cells were mixed with 50 µL
cold 50% trichloroacetic acid for 1 h at 4°C. Wells were washed 5
times with distilled water and stained for 30 min at room
temperature with 50uL of 1% SRB solution in 1% acetic acid.
The plates were air-dried, and the dye was solubilized with 100
µL/well of 10mM tris base [pH 10.5] for 5min on a shaker and optical density (O.D.) of each well was measured spectrophotometrically at 564 nm
with an ELIZA microplate reader (Meter tech. Σ 960, USA). The experiment was repeated 3 times. The percentage of cell survival
was calculated according to the following equation:

Survival fraction (%) = [O.D. of treated cell/O.D. of control cells] ×

Statistical analysis

All experimental were carried out in triplicate, and statistical
analysis were performed using SPSS (13) software and Microsoft
Excel program.

RESULTS AND DISCUSSION

Compound 1 was obtained as yellow powder, its m.p 273-278 °C, Rf values 0.036 (15% Acetic acid, PC), 0.68 (n-butanol, acetic acid, water, 4:1:5, PC). UV spectrum of methanolic solution of the compound 1 showed characteristic bands of flavonoid structure at 255, 268 and 368 nm. Bathochromic shift of band I (52 nm) was observed with NaOMe indicating the presence of free OH at 4. A, also bathochromic shift of band II (20 nm) was observed indicating the presence of free 5 and 7 OH. Bathochromic shift of band II with AlCl3 (10 nm) reflected the presence of free OH at position 5. A, bathochromic shift of band II (13 nm) after addition of NaOAc indicated the presence of OH group in position 7. Addition of NaOAc/H3BO3 led to small shift of band I (5 nm) indicating the absence of OH group in position 3. Compound 1 showed no depression in m.p. when mixed with authentic kaempferol. Its UV data was in good agreement with that of kaempferol [11, 12]. By comparing its m.p., Co-PC behavior with authentic sample of kaempferol, thus compound 1 was identified as kaempferol.

Compound 2 was obtained as yellow powder, m.p. 315-317 °C, Rf values 0.051 (15% AcOH, and 0.60 (n-BuOH: AcOH: H2O: 4: 1: 5, PC). UV spectrum of the compound in methanol showed the presence of major absorption bands at 256, 268, 372 which confirmed the flavonoid structure. Observation of bathochromic shift in both compound 1 and 2 with NaOMe indicated the presence of free 7-OH, 5-OH, 3’OH and 4’OH groups. There are strong bathochromic shift in band I with AlCl3 (73 nm) reflected the presence of O-dihydroxy group in B-ring at 3’ and 4’ positions bathochromic shift of band II with (19 nm) indicated of free 5-OH group Hypsochromic shift in band I with AlCl3/HCl (28 nm) indicated the presence of 3’, 4’OH groups. Bathochromic shift (16 nm) in band I with NaOAc/H3BO3 also indicated the presence of O-dihydroxy groups in B-ring at 3’ and 4’ positions [13, 14]. By comparing its m.p., Co-PC behavior with authentic sample of quercetin. Thus compound 2 was identified as quercetin.

Compound 3 was obtained as yellowish brown powder, m.p. 306-308 °C, Rf values 0.10 (15% AcOH, and 0.56 (n-BuOH: AcOH: H2O: 4: 1: 5, PC). UV spectrum of compound 3 in methanol showed the presence of major absorption bands at 254, 268, 372 which confirmed the flavonoid structure. Observation of bathochromic shift in both compound 1 and 2 with NaOMe indicated the presence of free 7-OH and 5-OH group. Bathochromic shift in band I with AlCl3 (61 nm) reflected the presence of free OH group in B-ring at 3’ and 4’ positions and band II with (14 nm) indicated of free 5-OH group. A very small hypsochromic shift in band I with AlCl3/HCl (1 nm) indicated the presence only free OH at 3’ or 4’ position. There is a limited Bathochromic shift (8 nm) in band I with NaOAc/H3BO3 also indicated the absence of O-dihydroxy groups in B-ring at 3’ and 4’ positions [13, 14]. By comparing their m.p., Co-PC behavior with authentic sample of isorhamnetin. Thus compound 3 was identified as isorhamnetin.

Compound 4 was isolated as yellow powder, UV spectrum exhibited characteristic absorption bands of flavonoid structure at 254, 303 and 365 nm [13, 16]. Bathochromic shift in band II (10 nm) was clear by addition NaOMe. This reflected the presence of free OH at C-7. Moderate bathochromic shift (58 nm) in band I by addition of AlCl3 reflected the absence of free OH groups at position 3’ and 4’. Also no hypsochromic shift with AlCl3/HCl was observed which means that 3’ and 4’ positions are substituted. Bathochromic shift was obtained by band I (21 nm) with NaOAc indicating the presence of substituted groups at position 3’ and 4’ [13]. The 1H-NMR spectrum showed sharp singlet was appeared at δ 12.39 for (1H, s, 5-OH), 9.52 for (1H, s, OH) and 8.99 for (1H, s, OH). Other protons of the aglycone moiety was appeared at δ 7.66 (1H, d, J= 2 Hz), 7.59 (1H, d, J= 3.6 Hz), 7.21 (1H, d, J= 8 Hz) and 6.41 (1H, d, J= 2.1 Hz) and 6.16 (1H, d, J= 2 Hz) corresponding to H-2’, H-6’, H-5’ and H-8 and H-6 respectively. The anomeric proton signal was appeared at δ 5.15 (1H, d, J= 6.7 Hz, H-1’ Glc) and methoxy group signal at δ 3.70 (s, 3H) [13, 17, 18]. 13C-NMR showed characteristic peak of (C=4) at δ 176.57 and 101.82 for (C=1’) of aglycone carbon of glucose unit and 61.19 for 3’OCH3 group. This confirmed compound 4 was identified as isorhamnetin - O-β-D-glucopyranoside.

Compound 5 was obtained as a white amorphous powder. The 1H-NMR spectrum showed the presence of six peaks in region δ 4.3-6.18 this area of sugar moiety. There are a sharp doublet peak was appeared at δ 6.68 for (1H, d, J= 3.8 Hz, H1 anomeric proton). Other protons were appeared at δ 4.85 (1H, d, H6), 4.75 (1H, d, H5), 4.63 (1H, d, H3), 4.44 (1H, d, H2) and 4.36 (1H, d, H4) these signals of D-glucose unit. In the carbon spectra of glucoligosaccharides three chemical shift regions for the signals are usually seen resulting from the anomeric (90-105 ppm), methane (69-83 ppm), and methylene carbons (61-69 ppm) [19, 20]. 13C-NMR of compound 5 showed the presence a single peak of anomeric carbon (C1) at δ 92.70 of D-glucose and other five carbon atoms at δ 73.55, 72.82, 72.44, 71.01 and 61.67 for C3, C2, C5, C4 and C6 respectively. The conformational space of α and β -D-glucose has been widely studied with experimental and theoretical methods [19]. In vacuo the α - anomeric is more stable than the β-anomer because of the anomeric effect, but in aqueous solution the α / β ratio is reversed due to solvent effects and hydrogen bonding [21]. Therefore from 1H, 13C NMR data and co-TLC compound 5 was identified as α -D - glucopyranosyl. This is the first time to isolate this free glucose from A. cepa (ssp. red onion).

Compound 6 was obtained as white crystals. The 1H-NMR spectrum showed the presence of peaks in region δ 3.34-5.185 this area of sugar moiety. There are a doublet peak was appeared at δ 5.185 for (1H, d, J= 3.6 Hz, H1 anomeric proton of α-D-glucose unit) and another peak appeared at δ 4.803 for (1H, d, J= 8 Hz, H1 anomeric proton of another β-D-glucose unit). The signals of the anomeric protons in the non-reducing unit of the α - or β -disaccharides were close to each other whereas those of the reducing unit at α - and β -equilibrium were well separated [19]. The 1HNMR signals indicate the presence of two D- glucose units. The 13C-NMR data of compound 6 showed the presence of peak at δ 104.48 of anomeric carbon (C1) of β-D-Glucose, carbon atom at δ 92.22 of anomeric carbon (C1) of α-D-Glucose. It was observed that the α-linked anomeric carbon of the non-reducing end resonates in the region 97.0-104.6 ppm, while the β-linked non-reducing anomeric carbon resonates at 98.4-103.5 ppm [19, 20]. From reported data, 1H and 13C NMR data compound 6 was identified as β-D-glucopyranosyl (1→2) - α - D - glucopyranoside. This is the first time to isolate this dimer of glucose from A. cepa (ssp. red onion).
Cytotoxic Assay

Many investigations indicate that phenolic compounds are of great value in preventing the onset and progression of cancer. They are toxic to cancer cells, but are not toxic or are less toxic to normal cells. So it plays an important role in the prevention of cancer [22, 23]. In present study, ethyl acetate fraction which derived from the defatted methanolic extracts of *A. cepa* (ssp. red onion) is the most active extract (IC₅₀ = 6.06 µg/ml) against human liver carcinoma cell lines HepG2 as shown in figure (1) and its activity is nearly doxorubicin (IC₅₀ = 4.0 µg/ml) which used as cytotoxic standard. The butanolic fraction and defatted methanolic extract have IC₅₀ = 9.95 and 10.9 µg/ml respectively lower than ethyl acetate fraction. Compound 4 (isorhamnetin-4'-O-D-D-glucopyranoside) which isolated from EtOAc fraction has IC₅₀ = 11.9 µg/ml and compound 6 (β-D-glucopyranosyl-α-D-glucopyranoside) which isolated from n-BuOH fraction has IC₅₀ = 22.0 µg/ml. According to the National Cancer Institute guideline, an extract and/or a compound with IC₅₀ values < 20 µg/ml is considered active on hepatocellular carcinoma cell line [24, 25]. These results indicated and confirmed that phenolic compounds especially flavonoids have high cytotoxic activity and it depend on the number of hydroxylation and glycosylation of phenolic compounds as reported by Kathrin et al., 2007 [26] who stated that there is inverse correlation between the cytotoxicity and the number of hydroxyl phenolic groups of flavonoids.

CONCLUSION

The present study showed the cytotoxic activity of defatted methanolic extract of *Allium cepa* (ssp. red onion) and is derived fractions (EtOAc and n-BuOH) as well as chromatographic isolation of their active chemical constituents. The results indicated that the two fractions contain phenolic and free sugars which have cytotoxic properties against HepG2 cell line.

CONFLICT OF INTERESTS

Declared None

ACKNOWLEDGMENT

The authors are thankful and grateful to Science and Technology Development Fund (STDF), Ministry of Scientific Research, Cairo, Egypt for financial support.

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