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LC-ESI-MS analysis, antitumor and antioxidant activities of methanolic extract of Egyptian *Allium kurrat*

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ABSTRACT

Human have been constantly using plants to overcome many diseases. Allium kurrat is an edible cultivated vegetable in Egypt. The aim of this survey is to determine total phenolic and flavonoid contents of the methanol extract and certainly obtained fractions from it. Antioxidant activity of these extracts was evaluated by DPPH (2,2-diphenyl-1-picrylhydrazyl radical), ABTS (2,2-azino-bis-3-ethylbenzothialozine-sulphonic acid) and total antioxidant capacity assays. Also, cytotoxic activity of these extracts was determined against human hepatocellular carcinoma (HepG2) and human colon carcinoma (Caco-2) using Neutral red assay. LC-ESI-MS analysis was carried out for the most active extracts. The results revealed that ethyl acetate fraction has the highest phenolic and flavonoid contents (127.45 ± 0.71 (mg GAE eq./g extract) and 98.55 ± 1.87 (mg RE eq./g extract)) as well as showed the highest antioxidant activity. On the other hand, the butanolic fraction had the highest antitumor activity on HepG2 and Caco-2 cell lines (36.2 µg/mL and 44.6 µg/mL) respectively. These results proved that the antitumor activity of this plant is correlated with many active ingredients in it. The LC-ESI-MS analysis identified a mixture of phenolic compounds (flavonoid glycosides and phenolic acids). Thus, Allium kurrat contains active ingredients which inhibit the risk of many chronic diseases.

INTRODUCTION

Cancer is not only a human disease but it is a serious problem affects the survival of all human beings. It has shown economic and social changes to the healthcare system (Hagag *et al.*, 2011; El-Hady *et al.*, 2014). Cancer is a set of diseases expressed as the abnormal control in cell growth. Normal cells often repair mutations in their DNA but once they cannot make the repairs, the cells become cancerous (Krishnamurthi, 2007). Cancer includes several types among them hepatic carcinoma (HCC) which represents the 5th most common cancer worldwide and the 3rd cause of cancer disease worldwide (El-Serag, 2002) while, colon cancer is the second cause of cancer death worldwide (Ricci-Vitiani *et al.*, 2007).

Oxidative stress which is an intra or extracellular disorders that lead to generating ROS (reactive oxygen species). ROS contains hydroxyl radicals, hydrogen peroxide and singlet oxygen that can cause oxidative damage to essential cellular constituents. Also, free radicals produced to contribute to several diseases as liver cirrhosis, diabetes, and cancer (El-Hady *et al.*, 2014). Compounds that have the ability to scavenge these free radicals show great potential improvement in these diseases. So, in recent years, there are excessive efforts to find natural antioxidant from plants to exchange synthetic ones that have been limited according to their carcinogenicity (Sasaki *et al.*, 2002).

Pure natural compounds, as well as different crude extracts from plants, have been reported as natural antioxidants according to their free radical scavenging ability (Chang *et al.*, 2008). Medicinal plants used widely as traditional medicine in the treatment of many diseases. There is a huge scientific confirmation that nutritive and non-nutritive plants can inhibit, reverse and prevent carcinogenesis (Krishnamurthi, 2007).

There are about 500 species of *Allium* genus, garlic, onions, and leeks are the most widely used ones (Malairajan *et al.*, 2006). *Allium* species are rich sources of phytonutrients and many pharmacological activities including cholesterol lowering, anti-hypertensive, anti-spasmodic, anti-bacterial, antiviral,

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antimicrobial and anticancer effects. They are also the richest sources of naturally occurring flavonoids that help us in intake a high level of phenolic compounds through the daily diet (Ghavam-Haghiand Dinani, 2017). The most popular species in Egypt and Eastern Mediterranean countries is *Allium kurrat*. It has been used in medicine and has been considered efficient food in several countries contains proteins, fats, carbohydrates, minerals, and vitamins. Therefore, *A. kurrat* is a dietary plant of low toxicity and therapeutic values (Sharaf *et al.*, 1969; Adao *et al.*, 2011; Abd El-Rehem & Ali, 2013).

The present study was designed to estimate the total phenolic and flavonoid contents of the methanolic extract and derived fractions from it of *Allium kurrat*. The antioxidant and cytotoxic activities of these extracts were evaluated. Also, the chemical constituents of the methanolic extract and the ethyl acetate fraction were identified using LC-ESI-MS analysis.

MATERIALS AND METHODS

Chemicals

ABTS (2-2'azinobis (3-ethylbenzthiazoline-6-sulphonic acid), DPPH (1,1diphenyl-2-picryl hydrazyl radical), and Folin-Ciocalteu reagent were purchased from Sigma-Aldrich (Germany). Sodium hydrogen phosphate, Potassium persulphate, Sodium nitrite, Sodium hydroxide, Ammonium molybdate, Sodium bicarbonate and Aluminum chloride were purchased from Merck (Germany). Gallic acid, Rutin, and Ascorbic acid were purchased from Sigma-Aldrich (USA). α-Tocopherol (Vitamin E) was purchased from Sigma-Aldrich (England).

Plant material

Arial parts of *Allium kurrat* were collected from a regional market in Giza, Egypt. The plant was identified by Dr. Rim Samir Hamdy, Professor of Plant Taxonomy, Faculty of Science, Cairo University. The voucher specimen of the plant under investigation was stored in Medicinal Chemistry Department, Theodor Bilharz Research Institute.

Extraction and fractionation

500 gram of the plant dry powder was extracted with 85% methanol and then concentrated under vacuum by using a rotatory evaporator (BUCHI, Switzerland). The dried methanolic extract was defatted by petroleum ether. The aqueous defatted methanolic extract was fractionated by dichloromethane (CH_2Cl_2), ethyl acetate (EtOAc), and n-butanol (n-BuOH). The methanolic extract and the fractions were kept away from moisture for the present study.

Determination of total phenolic content

The phenolic content of the tested extracts was evaluated using the spectrophotometric method described by El-Sayed *et al.* (2017). 0.5 mL of the extract (250 μg/mL), 2.5 mL of Folin-Ciocalteus reagent mixed with H_2O (10:90) and 2.5 mL$NaHCO_3$ (7.5%). The blank sample contains 0.5 mL of MeOH, 2.5 mL of 10% Folin-Ciocalteus reagent and 2.5 mL 7.5% $NaHCO_3$ in H_2O All mixtures were shaken and incubated at 45°C for 45 min. the absorbance was recorded at 765 nm versus a blank sample and gallic acid as the standard. The experiment was achieved in triplicate. The total phenolic content was expressed in terms of mg GAE eq./g extract.

Determination of total flavonoid content

The content of flavonoid was determined using a colorimetric assay reported by El-Sayed *et al.* (2017). 0.5 mL of each extract was dissolved in 2 ml H_2O and 150 μL of 5% $NaNO_2$ for 6 min, then 150 μL of 10% $AlCl_3$ was added and left to stand for 5 min then added of 2mL of 4% NaOH and the mix was completed up to 5 mL with distilled water. The mix was incubated at 25-27°C for 15 min. the absorbance was measured at 510nm versus a blank sample, and rutin was used as the standard. The total flavonoid content was expressed as mg RE eq./g extract. The experiment was achieved 3 times and all chemicals ($NaNO_2$, $AlCl_3$, and NaOH were dissolved in water).

Antioxidant assays

DPPH radical scavenging activity

The antioxidant activity of the tested extract was evaluated using DPPH free radical scavenging method (Akroum *et al.*, 2010). 2 mL of each plant extract dissolved in methanol was mixed with 2 mL of DPPH in MeOH (0.1 mmol/L). The control only contained solvent and DPPH. The mixtures were shaken and kept in dark for 30 min at 37°C. The absorbance was measured at 517 nm. The experiment was achieved in triplicates. The DPPH scavenging activity was calculated from this equation.

$$\% \text{ DPPH scavenging activity} = [(\text{control absorbance} - \text{sample absorbance})/(\text{control absorbance})] \times 100$$

ABTS radical scavenging activity

The plant extract can able to quench $ABTS^{\cdot+}$ (2-2'azino-bis (3-ethylbenzthiazoline-6-sulphonic acid). The concentrated reagent solution was designed by dissolving 9.6 mg ABTS in 2.5 mL water and then adding 110 μLof potassium persulphate (37.5 mg/mL) dissolved in water. The stock solution was kept in the darkroom for 12-16 hours to produce $ABTS^{\cdot+}$ radical cation. The $ABTS^{\cdot+}$ solution was diluted by MeOH till the absorbance reach value between 0.7 and 0.8 at wavelength 734 nm. Subsequently, 100 μL of aqueous or alcoholic plant extract (according to solubility) was added to 1 mL of work solution, and it was measured exactly after 2.5 min. Also, an appropriate solvent blank was measured. Calibration curve for $ABTS^{\cdot+}$ was obtained using Trolox® (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid) as standard. The experiment was carried out in triplicates. Results were expressed in terms of mmol Trolox® eq./100 g dry weight of plant extract (El-Sayed *et al.*, 2015).

Total antioxidant capacity

The total antioxidant capacity of the tested extract was determined by Saeed *et al.* (2012) and Abdel-Hady *et al.* (2014). An aliquot of 0.1 mL of sample (200 μg/mL) solution was mixed with 1 mL of reagent solution (0.6 M sulphuric acid, 28 mM sodium phosphate and 4 mM ammonium molybdate). Blank was contained 1 mL of the reagent solution and nearly volume of the same solvent used for the samples. The tubes were capped and

incubated at 95°C for 90 min. After the samples had cooled to 25-27°C, the absorbance of the mix was measured at 695 nm versus the blank and ascorbic acid was used as the standard. The experiment was repeated for 3 times. The antioxidant activity of the extracts was expressed as mg AAE eq./g extract.

Cytotoxic assay

Cell culture

Colon cancer (Caco-2) and Liver cancer (HepG2) cell lines were obtained, and cultured in the cell culture lab (CURP), Faculty of Agriculture, Cairo University. Also, the *in-vitro* cytotoxic assay was performed there.

Neutral Red (NR) assay

The NR assay is one of common sense used cytotoxic assays. It depends on measuring the lysosomal activity of the cells (Repetto *et al.*, 2008). The methanol extract, Ethyl acetate and n-butanol fractions of *Allium kurrat* were tested at concentrations ranged from 10 to 200 μg/mL for HepG2 while their concentrations for Coca-2 were ranged from 50 to 600 μg/mL. HepG2 and Coca-2 cells were seeded in 96-well plates at concentrations of 1×10^4 cells/well. 100 μL of PBS was dispensed into the peripheral wells and the plate incubated at 37°C, CO_2 and humidified atmosphere in a sterile environment until half-confluent monolayer formed. The 200 μL of treatment medium (serum-free medium) was added in an independent manner to the cells. 100 μL of neutral red (NR) dye was added to each well then was incubated for 2 hrs. Cells were washed by 100 μL of PBS and 150 μL of neutral red destain solution was added on microtiter shaker for at least 10 min or until all neutral red dye that has been extracted formed the homogeneous solution. Measure the optical density (OD) of NR extract by spectrophotometer at 530 nm and 640 nm respectively. The treatment medium acts as a negative control. The experiment was achieved 3 times. The results represent IC_{50} is the percentage of inhibitory concentration of cell viability.

HPLC-ESI-MS analysis

(5 mg/mL) solution of the methanolic extract and ethyl acetate fraction of *A. kurrat* was prepared and filtered using a membrane disc filter (0.45 µm) then analyzed by liquid chromatography coupled with electrospray ionization mass spectrometry (LC-ESI-MS) according to the method described by Abdel-Lateef *et al.* (2016) by using HPLC analytical grade solvent mixture of $CH_3CN/MeOH/H_2O$ (1: 1: 2; v/v/v), Analysis was performed using HPLC system (Waters Alliance 2695, Waters, USA) and mass analyzer (Waters 3100). 20 μL of a sample was injected into the HPLC instrument equipped with reverse phase C-18 column (Phenomenex 250 mm, 5 μm particle sizes). The flow rate of 0.4 mL/min using gradient mobile phase comprising two solvents: Solvent A contains water acidified with 0.1% formic acid and solvent B is $CH_3CN/MeOH$ (1:1; v/v) acidified with 0.1% formic acid. Elution was performed using the following gradient: 5% B; 0-5 min, 5-10% B; 5-10 min, 10-50 % B; 10-55 min, 50-95 % B; 55-65 min, and 5% B; 65-70 min. The parameters for analysis were carried out using a negative ion mode as follows; source temperature 150°C, cone voltage 50 eV, capillary voltage 3 kV, desolvation temperature 350°C, cone gas flow 50 L/h, and desolvation gas flow 600 L/h. Mass spectra were detected in the ESI negative ion mode between *m/z* 50-1000. The peaks and spectra were processed using the Maslynx 4.1 software and tentatively identified by comparing its retention time (Rt) and mass spectrum with reported data.

RESULTS AND DISCUSSION

Total phenolic and flavonoid contents

The flavonoid and phenolic compounds are the main components of many plants. It has been reported that they have therapeutic and protective properties to cure many diseases because of these compounds possess major of hydroxyls group which responsible for the free radical scavenging activity. The obtained total phenolic and flavonoid contents from any plant depend on the ability of the used solvent in the extraction process (Marinova *et al.*, 2005; Deepa *et al.*, 2009; Atanassova *et al.*, 2011; Safaeian *et al.*, 2017).

In this study, the results showed in Table 1 that the methanolic extract and derived fractions from it exhibit high phenolic and flavonoid contents**.** The ethyl acetate and n-butanol fractions of *A. kurrat* have the highest phenolic and flavonoid contents (127.45 ± 0.71 and 116.43 ± 2.05 (mg GAE eq./g extract) and 98.55 ± 1.87 and 91.73 ± 0.091 (mg RE eq./g extract)) respectively. On the contrary, the water residue had the lowest phenolic and flavonoid contents (30.45 ± 0.76 (mg GAE eq./g extract) and 15.21 ± 0.60 (mg RE eq./g extract). The order of other extracts was methanol extract < dichloromethane fraction (58.43 ± 0.68 and 83.05 ± 1.07 (mg GAE eq./g extract) respectively. These results are agreed with previous studies on *Allium* species which reported that the different *Allium* extracts have a high content of phenolic and flavonoid (Škerget *et al.*, 2009; Lu *et al.*, 2011; Abdel-Gawad *et al.*, 2014; Safaeian *et al.*, 2017).

Table 1: Total phenolic and flavonoid contents of the methanolic extract and derived fractions from it of *Allium kurrat*.

Extract/Fraction	Total phenols (mg GAE/g of ext.)	Total flavonoids (mg RE/g of ext.)
MeOH extract	96.43 ± 0.68	55.51 ± 2.80
CH_2Cl_2 fraction	80.05 ± 1.07	45.14 ± 0.09
EtOAc fraction	127.45 ± 0.071	98.55 ± 1.87
BuOH fraction	116.43 ± 2.05	91.73 ± 0.091
H_2O residue	53.45 ± 0.76	27.21 ± 0.60

The data represents as mean ± standard deviation.

Antioxidant activity of plant extract

Different free radicals as hydroxyl groups, peroxyl radicals, and single oxygen, etc. have a harmful effect due to their ability to oxidize cell components and causing different diseases (Aqil *et al.*, 2006; Bozin *et al.*, 2008). So, the antioxidant property plays an important role in reducing chronic diseases like cancer and cardiovascular diseases by scavenging the free radicals. Plants are primary and the main sources of antioxidants as polyphenolic compounds and saponins (Aqil *et al.*, 2006; Bozin *et al.*, 2008).

In this study, evaluation the antioxidant activity of the methanolic extract and the tested fractions of *A. kurrat* by using three methods including DPPH, ABTS radical scavenging activity, and total antioxidant capacity assays.

DPPH radical scavenging assay

The DPPH is a stable radical. It was commonly used to exam the ability of compounds as free-radical scavengers or hydrogen donors and also, to evaluate the antioxidant activity of plant extracts and foods (Akroum *et al.*, 2010; Kaur *et al.*, 2016; Abdel-Hady *et al.*, 2017). The results of this study in Table 2 proved that the methanolic extract and different fractions derived from it have antioxidant property. The ethyl acetate fraction has the highest activity than other extracts (41.56 ± 1.06 µg/mL) followed by butanolic fraction (56.13 ± 1.64 µg/mL). The order of the activity of other extracts is MeOH extract > CH_2Cl_2 fraction > H_2O residue respectively. These results proved there is a correlation between the antioxidant of the tested extracts and their phenolic and flavonoid contents. Also, these results are agreed well with previous studies on other *Allium* species (Benkeblia, 2005; Kaur *et al.*, 2016).

Table 2: DPPH, ABTS scavenging activity, and total antioxidant capacity of the methanolic extract and certain fraction derived of *A. kurrat*.

Extract/ Fraction	DPPH radical-scavenging activity SC50 [µg/ml]	ABTS assay [mmol Trolox® eq./100 g extract]	Total antioxidant capacity [mg AAE/g extract]
MeOH extract	57.29 ± 0.72	31.15 ± 0.94	170.59 ± 1.03
CH_2Cl_2 fraction	66.15 ± 1.083	38.05 ± 1.08	160.88 ± 0.20
EtOAc fraction	41.56 ± 1.06	20.32 ± 0.19	203.56 ± 1.07
BuOH fraction	56.13 ± 1.64	25.98 ± 0.16	175.45 ± 0.09
H_2O residue	95.57 ± 1.44	59.35 ± 1.49	140.46 ± 1.09

The data were expressed as mean ± standard deviation.

ABTS radical-scavenging activity

$ABTS^+$ is used by several researchers to estimate the scavenging activity of plant extracts by decolorization of ABTS radical by accept the hydrogen atom or electrons from the active compounds in plant extracts (Awika *et al.*, 2003; Abdel-Gawad *et al.*, 2014; El-Sayed *et al.*, 2015; Kaur *et al.*, 2016; Abdel-Hady *et al.*, 2017). The results in Table 2 exhibited that the ethyl acetate fraction has the highest activity (20.32 ± 0.19 mmol Trolox® eq./100 g extract) followed by butanolic fraction (25.98 ± 0.16 mmol Trolox® eq./100 g extract) and methanolic extract (31.15 ± 0.94 mmol Trolox® eq./100 g extract). The lowest activity was the water residue (59.35 ± 1.49 mmol Trolox® eq./100 g extract). These results proved that there is a correlation between the phenolic and flavonoid contents of the tested extracts and ABTS radical-scavenging activity. Also, these results are agreed with Najjaa *et al.* (2011); Fidrianny (2016) and Safaeian *et al.* (2017) studies on some *Allium* species.

Total antioxidant capacity

It is a simple assay depends on the reduction of Mo (VI) into a green color of phosphate/Mo (V) complex by the antioxidant agents (Kumaran and Karunakaran, 2006). The results demonstrated in Table 2 that the ethyl acetate fraction has higher antioxidant activity (203.56 ± 1.07 mg ascorbic acid/g plant extract) followed by the butanolic fraction (175.59 ± 1.03 mg AAE/g extract) followed by the methanolic extract (170.59 ± 1.03 mg AAE/g extract) and the dichloromethane fraction (160.88 ± 0.20 mg AAE/g extract) while the lowest antioxidant activity was recorded by water residue (140.46 ± 1.09 mg AAE/g extract). These results proved that the antioxidant activity of extract and its fractions is correlated with their phenolic and flavonoid contents. Also, these results are agreed with previous studied on other Allium species (Lu *et al.*, 2011; Safaeian *et al.*, 2017).

In this study, the antioxidant activity evaluation of the methanolic extract and the tested fractions of A. *kurrat* by using DPPH, ABTS radical-scavenging activity and total antioxidant capacity assays .the results revealed that the antioxidant of the tested extracts is correlated with the contents of phenolics and flavonoids. These results are agreed well with other previous studies of *Allium* species (Safaeian *et al.*, 2017; Fidrianny, 2016).

Cytotoxicity assay

Life threating diseases are related mainly to cancer (Abdel-Hady *et al.*, 2016). These diseases including cancers are due to reactive oxygen species, lipid peroxidation, and free radicals but their activities can be halted by antioxidants that can delay or inhibit the oxidation process and lipid peroxidation (Singh *et al.* 2009; Kumar and Santhi, 2012). Polyphenols have been applied for treating cancer patients in traditional Chinese medicine (Mukhtar *et al.*, 2000). And also, saponins have shown effective anticancer potential in various cancer cell lines because they are diverse and complex in the structure which can be shown by inhibiting cell growth and by inducing apoptosis (Xu *et al.*, 2016).

In the present study, preliminary phytochemical screening of the plant extracts revealed the presence of flavonoids, steroids, terpenoids, and saponins in high quantities. Also, the results of the cytotoxic activity of the crude methanol extract of *A. kurrat* and their fractions versus human hepatocellular carcinoma (HepG2) and human colon carcinoma (Caco-2) cell lines using Neutral red assay method was carried out in triplicates. Results in Figure 1 revealed that the butanolic fraction has high activity against the HePG2 and Coca-2 cell lines (IC_{50} = 36.2 and 44.6 µg/ml, respectively) than ethyl acetate fraction (IC_{50} = 48.7 and 53.7 µg/ml, respectively) and methanol extract (IC_{50} = 58.5 and 68.4 µg/ml, respectively). These results proved that the antitumour activity of the methanolic extract of *A. kurrat* do not only depend on the phenolic and flavonoid contents but also on other chemical constituents of this plant especially the saponins (Singh *et al.*, 2009; Kumer *et al.*, 2012; Mukhtar *et al.*, 2009; Xu *et al.*, 2016).

HPLC-ESI-MS analysis

In the present study, the negative ionization mode of the methanolic extract of *A. kurrat* and the ethyl acetate fraction of was carried out by using HPLC-ESI-MS. The identification of the major chemical compounds of the two extracts was based on their fragmentation pattern and the standards data which that widely reported in the literature. The Neutral losses of 132 (pentose), 162 (hexose), 146 (deoxyhexose), 176 (hexuronic acid), 86 (malonyl

residue) and 100 (succinyl residue) were identified according to Guarnerio *et al.* (2012). The results exhibited that the major chemical constituents as shown in Table 3 and 4 and Figure 2 were flavonoids and phenolic acid derivatives.

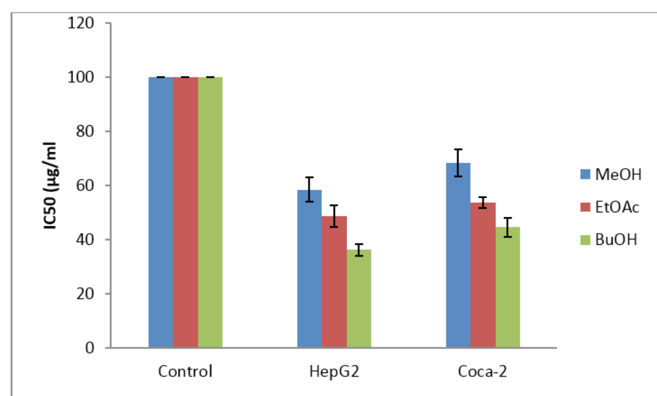

Fig. 1: Cytotoxic inhibition concentration (IC50) of *A. kurrat* MeOH ext., EtOAc and *n*-BuOH fractions against HepG2 and Caco-2 cell lines.

Flavonoids

Compound 1 in MeOH extract with RT = 21.79 min exhibited deprotonated molecular ion *m/z* 609 [M-H]⁻. The fragment ions at *m/z* 447 [M-H-162]⁻ and 315 [M-H-162-132]⁻ means the liberation of hexose and pentoside units. Therefore this compound was tentatively identified as isorhamnetin-*O*-hexoside-pentoside (Bonaccorsi *et al.*, 2008). Compound 2 in MeOH extract (RT = 24.79 min) showed precursor ion peak at *m/z* 787 [M-H]⁻, and fragment peaks at *m/z* 625 [M-H-162]⁻ , *m/z* 463 [M-H-(2×162)]⁻, *m/z* 301 [M-H-(3 × 162)]⁻ this means that these liberations of three hexoside units. So, the compound 2 was characterized as Quercetin-tri-*O*-hexoside. Compound 8 in MeOH extract with RT = 36.32 min as well as compounds 5 and 7 in EtOAc fraction with RT = 31.61 and 36.40 min, respectively which exhibited the same molecular ion peak at *m/z* 463 [M-H]⁻ and base peak at *m/z* 301 [M-H-162]⁻, it was indicating the presence of hexoside unit attached with quercetin. Therefore, the compound was identified as Quercetin-*O*-hexoside. These compounds were previously reported in other *Allium* species (Bonaccorsi *et al.*, 2008; Lee and Mitchell, 2011; Soininen *et al.*, 2012; Farag *et al.*, 2017).

Table 3: Tentative identification of chemical constituents of methanolic extract of A. Kurrat by LC-ESI−MS/MS.

Compound No.	RT (Min)	MW	[M-H]⁻ *m/z*	MS fragments	Identified compounds
1	21.79	610	609	447, 315	Isorhamntin-*O*-hexoside-pentoside
2	24.79	788	787	625, 463, 301	Quercetin-tri-*O*-hexoside
3	25.63	772	771	609, 447, 285	Kaempferol-tri-*O*-hexoside
4	27.38	772	771	609, 447, 285	Kaempferol-tri-*O*-hexoside isomer
5	31.22	610	609	447, 285	Kaempferol-di-*O*-hexoside
6	35.23	948	947	785, 623, 447, 285	Kaempferol-*O*-trihexoside-hexuronoide
7	35.82	610	609	285	Kaempferol-di-*O*-hexoside isomer
8	36.32	464	463	301, 179, 62	Quercetin-*O*-hexoside
9	38.99	610	609	447, 285	Kaempferol-di-*O*-hexoside isomer
10	39.74	448	447	285, 151	Kaempferol-*O*-hexoside
11	42.91	460	459	185, 179	Kaempferol-*O*-hexuronoide
12	45.25	948	947	771, 623, 447, 285	Kaempferol-*O*-trihexoside-hexuronoide isomer
13	47.51	786	785	609, 285	Kaempferol-*O*-dihexoside-hexuronoide
14	52.10	532	531	283	Acacetin-7-*O*-malonoyl hexoside
15	55.61	518	517	455, 293,179	Unidentified

Each compound 3 and 4 in MeOH extract with RT = 25.63 and 27.38 min, respectively showed the same deprotonated molecular ion at *m/z* 771 [M-H]⁻, and other signals at *m/z* 609 [M-H-162]⁻, *m/z* 447 [M-H-(2×162)]⁻, and *m/z* 285 [M-H-(3 × 162)]⁻. Therefore each of these compounds was tentatively identified as Kaempferol-tri-*O*-hexoside. Each of Compounds 5, 7 and 9 in MeOH extract (RT = 31.22, 35.82, and 38.99 min, respectively) showed molecular ion peak at *m/z* 609 [M-H]⁻. And also, the other signals at *m/z* 447 [M-H-162)]⁻, and *m/z* 285 [M-H-(2 × 162)]⁻. This means that liberation of two hexoside unit. So, each of these compounds was characterized as Kaempferol-di-*O*-hexoside. Compound 10 in MeOH extract with RT = 39.74 min as well as compounds 8, 9, 14, and 16 in EtOAc fraction (RT = 39.74, 39.74, and 52.93 min, respectively) exhibited gave the molecular ion peak *m/z* 447 [M-H]⁻ and base peak at *m/z* 285 [M-H-162]⁻. Therefore, these compounds were identified as Kaempferol-*O*-hexoside (Pobłocka-Olech *et al.*, 2016; Farag *et al.*, 2017). Compounds 6 and 12 in the methanolic extract (RT = 35.23, 45.25 min, respectively) exhibited the same molecular ion at *m/z* 947 [M-H]⁻, and other fragments at *m/z* 785 [M-H-162]⁻, *m/z* 623 [M-H-(2 × 162)]⁻, *m/z* 447 [M-H-(2 × 162)-176]⁻, and *m/z* 285 [M-H-(3 × 162)-176]. This means that the loss of

tri-hexoside units and one hexuronoid unit. So, each of these compounds was tentatively identified as Kaempferol-*O*-trihexoside-hexuronoide. Compound 13 in MeOH extract and compound 15 in EtOAc fraction (RT = 47.51, 47.59 min, respectively) exhibited pseudo molecular ion at *m/z* 785 $[M-H]^-$, and three fragment ions at *m/z* 609 $[M-H-176]^-$, *m/z* 447 $[M-H-176-162]^-$ and *m/z* 285 $[M-H-176-(2 \times 162)]^-$, which represented the liberation of one hexuronoid unit and two hexose units. Therefore, each of these compounds was identified as Kaempferol-*O*-dihexoside-hexuronoide. Compound 11 in MeOH extract (RT = 42.91 min) presented pseudomolecular ion at *m/z* 459 $[M-H]^-$, and another fragment ion at *m/z* 285 $[M-H-174]^-$, therefore this compound was identified as Kaempferol-*O*-hexuronoide (Zhu *et al.*, 2017). Compound 14 in MeOH extract (RT = 52.10 min) showed pseudo molecular ion at *m/z* 531 $[M-H]^-$, as well as fragment ion at *m/z* 283 $[M-H-86+162]^-$. This indicated the release of malonoylhexoside unit. So, this compound was identified as Acacetin-7-*O*-malonoyl hexoside (Lin and Harnly, 2010). Compound 11 in EtOAc fraction (RT = 43.25 min) presented a molecular ion at *m/z* 547 $[M-H]^-$, and another fragment at *m/z* 447 $[M-H-100]^-$ this means loss of the succinic acid unit. Also, fragment ion at *m/z* 285 $[M-H-100- 162]^-$ means loss of hexoside unit. Therefore, this compound was tentatively identified as Kaempferol-*O*-succinyl hexoside. Compound 12 in EtOAc fraction (RT = 44.25 min) exhibited molecular ion at *m/z* 489 $[M-H]^-$, and other fragment at *m/z* 447 $[M-H-42]^-$. This means the loss of acetyl group, and the fragment at *m/z* 285 $[M-H-42-162]^-$ indicated further losing of hexoside unit. Therefore, this compound was tentatively identified as Kaempferol-*O*-acetylhexoside (Navarro-González *et al.*, 2014). Compound 13 in EtOAc fraction with RT = 45.42 min displayed a molecular ion at *m/z* 641 $[M-H]^-$, and other fragments at *m/z* 478 $[M-H-163]^-$ which indicating the losing of hexoside unit. Also, a fragment at *m/z* 285 $[M-H-163-193]^-$ indicated further losing of hydroxyferulic acid unit. Therefore, this compound was tentatively identified as Kaempferol-*O*-hexosyl-hydroxyferulic acid.

Phenolic acid cerivatives

Compound 1 in EtOAc fraction (RT = 20.87 min) presented a molecular ion at *m/z* 137 $[M-H]^-$, and another fragment at *m/z* 93 $[M-H-44]^-$ that means losing of carboxylic group. So, this compound was identified as *p*-Hydroxybenzoic acid. Compound 2 in EtOAc fraction (RT = 24.79 min) showed a molecular ion at *m/z* 355 $[M-H]^-$, and another fragment at *m/z* 193 $[M-H-162]^-$ means losing hexose unit but the fragments at *m/z* 175 and 161 are characteristic for ferulic acid. So, this compound was identified as Ferulic acid-*O*-hexoside (Prakash *et al.*, 2007; Barros *et al.*, 2012). Compounds 3 in EtOAc fraction (RT = 30.89 min) presented a molecular ion at *m/z* 279 $[M-H]^-$, and the other fragments at *m/z* 163, 119) corresponded to the p-coumaric acid residue and a small signal for malic acid. Therefore, this compound was assigned as *p*-Coumaroylmalic acid (Regos *et al.*, 2009).

Overall, LC-ESI-MS analysis of *A. kurrat* extracts reflected the presence of high amount of kaempferol glycosides and phenolic acid derivatives**.** These compounds linked to decreasing the risks of many chronic disorders by depicting antioxidant and anticancer (Regos *et al.*, 2009, Zhu *et al.*, 2017; Farag *et al.*, 2017).

Table 4: Tentative identification of chemical constituents of ethyl acetate fraction derived from the methanolic extract of A. Kurrat by using LC-ESI−MS/MS.

Compound No.	RT (Min)	MW	$[M-H]^-$ *m/z*	MS fragments	Identified compounds
1	20.87	138	137	93 (100%), 65	p-hydroxybenzoic acid
2	24.79	356	355	193, 175, 161	Ferulic acid-*O*-hexoside
3	30.89	280	279	119 (100%), 93	p-coumaroylmalic acid
4	35.07	584	583	402, 193	Ferulic acid derivatives
5	31.61	464	463	301 (100%), 179	Quercetin-*O*-hexoside
6	35.73	584	583	493, 279, 173	Unidentified
7	36.40	464	463	301 (100%), 151	Quercetin-*O*-hexoside isomer
8	39.74	448	447	285, 151	Kaempferol-*O*-hexoside
9	40.99	448	447	285, 151	Kaempferol-*O*-hexoside isomer
10	42.08	578	577	447, 285, 162	Kaempferol-*O*-pentosyl-hexoside
11	43.25	548	547	447, 285 (100%)	Kaempferol-*O*-succinoylhexoside
12	44.25	490	489	447, 327, 285	Kaempferol-*O*-acetyl hexoside
13	45.42	642	641	447, 285	Kaempferol-*O*-hexosyl-hydroxyferulic acid
14	46.84	448	447	285, 151	Kaempferol-*O*-hexoside isomer
15	47.59	786	785	609, 447, 285	Kaempferol-*O*-dihexoside-hexuronoide
16	52.93	448	447	285 (100%)	Kaempferol-*O*-hexoside isomer

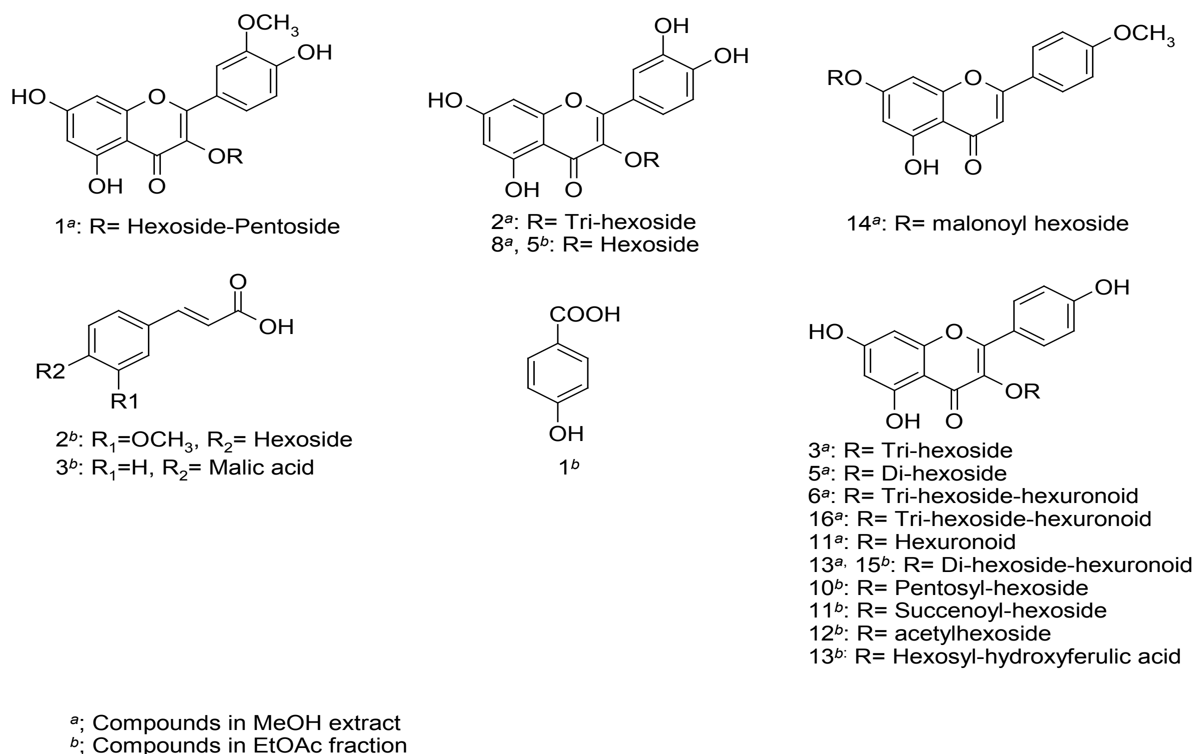

Fig. 2: Chemical structures of some compounds of *A. kurrat* methanolic extract and EtOAc fraction.

CONCLUSION

This investigation exhibited that the methanolic extract of *Allium kurrat* has antioxidant properties, so the consumption of this plant should be encouraged by making the people aware of beneficial effects to the system. Also, the methanolic extract of *Allium kurrat* and its fractions can act as a potential alternative cytotoxic effect against human hepatocellular carcinoma (HepG2) and human colon carcinoma (Caco-2) Cell lines. Therefore, the major constituents of the ethyl acetate fraction and methanolic extract were identified by using HPLC-ESI-MS analysis but the data will be more reliable if the extract will be evaluated by HPLC-DAD (UV-Visible spectrum) together with HPLC-MS.

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