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Phytochemical and Biological Investigations of *Onopordum alexandrinum* Seeds.

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ABSTRACT

The investigation of the ethyl acetate fraction from *Onopordum alexandrinum* seeds resulted in the isolation of β-sitosterol compound and arctiin; a lignan glucoside compound. Whereas, the study of flavonoidal constituents of the alcoholic extract from *Onopordum alexandrinum* seeds resulted in the isolation of caffeic acid and three flavonoidal compounds, which were identified as 7- methoxy luteolin, Quercetin 7- *O*- glucoside and kampferol 7- *O*- glucoside. These compounds were isolated and identified for the first time from the genus *Onopordum*. Fractionation of *Onopordum alexandrinum* constituents was performed by applying column chromatography. Moreover, further purification was achieved using successive chromatographic techniques, *viz*: preparative thin layer chromatography (PTLC), paper chromatography (PPC), as well as sephadex LH-20 column chromatography. Identification of the isolated compounds was carried out through chromatographic means and spectroscopic analyses (*viz*: UV, MS and NMR). Different extracts of *Onopordum alexandrinum* seeds (ethyl acetate, methanol and the isolated compounds) were studied for their hepatoprotective activity using hepatocytes monolayer culture from rat. The cells were exposed to certain hepatotoxic doses of paracetamol, which was capable of inducing the IC_{50} of cultured isolated cells. The results showed that the ethyl acetate fraction of *Onopordum alexandrinum* showed hepatoprotective activity against paracetamol cytotoxicity at concentration 12.5 µg/mL, while its IC_{50} were >1000 µg/mL. Consequently, these fractions were assumed to be promising with regard to their hepatoprotective and hepatotoxic activities. The antioxidant activity of the seed extracts showed that the ethyl acetate fraction of *Onopordum* seeds exhibited the highest antioxidant activity followed by the alcoholic extract. Also, Compound 3 (caffeic acid) and compound 5 **(**quercetin-7- O- glucoside) showed high antioxidant activity as well as hepatoprotective activity and very low toxicity on liver cells.

Keywords: Onopordum alexandrinum, Asteraceae, Flavonoids, hepatoprotection, Antioxidant.

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INTRODUCTION

Onopordum alexandrinum is one of the two species of the Genus *Onopordum* (*Onopordum alexandrinum and O. ambiguum*), which are represented in the wild flora of Egypt (Tackholm, 1974; Zahran, 1989 and Kawashty *et al.,* 1996). It is belonging to family Asteraceae (Compositae) native to Europe (mainly the Mediterranean region), Northern Africa, and many regions of the world. The plant grows on disturbed land, roadsides, arable land and pastures (Boulos, 2002), so that it is fitting cultivation in desert areas. As many members of the compositae, *Onopordum alexandrinum* is of certain significance on the bases of being used in the folk medicine of various countries (Zareh, 2005).

Plants as *Onopordum alexandrinum* may be also promising for potential exploitation in biotechnology as utilization in pharmacology as molecular probes, in medicine as therapeutic agents or even in agriculture as bio rational pesticides (Wink, 1999). In these instances, detailed information is required to detect the most suitable habitats and ecological circumstances of the plant, growth and productivity in space and time, to be connected with qualitative and quantitative productivity of respective products (Youssif, 1988).

The study of the nitrogenous base of *Onopordum alexandrinum* Boiss. resulted on the isolation of stachydrine and choline. The flavonoid components detected are: luteolin-7monoglucoside, apigenin-7-glucoside and quercetin (Wassel, 1975). Previous investigation of *Onopordum alexandrinum* resulted in the isolation of two flavone rhamnosides (Khafagy et al ., 1977a), three lactones, onopordopicrin and two other lactonic substances designated as substance C and substance D were isolated from the aqueous extract of the fresh plant (Khafagy et al ., 1977b), arctiin lignan glucoside (Omar, 1978).

Constituents of *Onopordum alexandrinum* were extracted, defatted and analyzed using column chromatography to give taraxasterol, lupeol, ß-sitosterol, stigmasterol, sutellarein 4' –methyl ether and takakin. The structure of the hitherto isolated products were inferred by high–field 1H-NMR spectroscopy (Ayyad *et al.,* 1994).

From the leaves and stems of *Onopordum alexandrinum* eleven flavonoid compounds were isolated and identified as apigenin, luteolin, chrysoeriol and their 7-galactosides and 7glucosides, together with the 7-diglucosides of apigenin and chrysoeriol (Kawashty *et al.,* 1996).

Onopordum alexandrinum Boiss. (Compositae) has been highly reputed as a curing and useful plant. The aerial parts are used by the native as expectorant, for healing wounds, as a treatment of skin cancers and leprosy (Wassel, 1975; El-Moghazy *et al.,* 2002). Several plants of the genus *Onopordum* have been reported to have antibacterial and anti tumor (Omar, 1978). Also *Onopordum* species have been reported as folk medicine for the treatment of skin cancers (Madaus, 1979). The extract of *Onopordum acanthium*, which also grows in Turkey is being used as folk medicine for treatment of face cancers since early times (Mericli and Tuzlaci, 1989). *Onopordum* extract having antioxidant properties and was used as an excellent protective agent in formulation of drugs caring the skin (Joudi and Habibi, 2010).

MATERIALS & METHODS

Plant material: *Onopordum alexandrinum* Seeds, family Asteraceae (Compositae) was collected from Borg El-Arab near Alexandria during April 2012, 2013. The plant was identified by Prof. Dr. Kamal M. Zayed and Dr. Ibrahim Elgarf, Taxonomists, Cairo Univ., Faculty of Science, Botany Dept., Cairo, Egypt, to whom the authors are deeply indebted. The seeds of the plant were ground into fine powder. A voucher specimen was kept in the herbarium of Cairo University.

Apparatus and Techniques:

- Shimadzu UV. Pc. 2401 spectrophotometer.
- Mass spectrophotometer GC-MS finnigan mat SSQ 7000 Mass spectroscopy 70 ev.
- $^{1-}$H-NMR spectra were recorded in (DMSO-do) Jeol-Ex-270 MHz spectrometer.

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Chromatography:

- **Paper Chromatography:** Paper chromatographic investigations were carried out on Whatman 1MM sheets using n-butanol: acetic acid: water (4: 1: 5) and 15 % acetic acid as a solvent systems.
- **Column chromatography:** was performed on Silica gel 60 (70-230 mesh, Merck); polyamide 250 g column (120 x 3 cm) and sephadex LH-20 column (40x 2 cm) eluted with methanol/water (90:10).
- **Thin Layer Chromatography: Thin Layer Chromatography** was carried out on precoated 20x20 silica gel G plates (Merck).
- **Solvent systems:** chloroform: acetone: formic acid (9: 2: 1); hexane: ethyl acetate (1:3); methanol-water (90:10); 15% acetic acid in water; n-butanol-acetic acid- water (4:1:5).

Extraction and Fractionation of *Onopordum alexandrinum* Seeds:

About 300 g dried rinced seeds of *Onopordum alexandrium* was defatted by n-hexane (in a soxhlet) till exhaustion. The dried powder was subjected to extraction with methanol. The extract was evaporated under vacuum at 40^{o}C to obtain a residue of 9 g. The residue dissolved in hot ethyl acetate (3x 500 ml). After stripping off the solvent, the methanol residue (5g) and ethyl acetate fraction (4g) were subjected to column chromatographic fractionation.

Column Chromatographic Fractionation of the Ethyl Acetate Fraction:

About 4 g of the residue of ethyl acetate fraction was dissolved in 50 ml methanol mixed with 5 g silica gel. The solvent was evaporated in vacuum at 40^{o}C leaving a dry powdered matter which was applied on the top of a glass column (120 x 4 cm) packed with 200g silica gel (70-230 mesh). Elution was first carried out using $CHCl_3$ followed by increasing polarity with methanol by increment of 5% of methanol up to 70% MeOH-$CHCl_3$ mixture.

Fractions (100ml each) were collected. The course of the chromatographic fractionation was monitored by TLC on precoated 20x20 silica gel plates developed with chloroform: acetone: formic acid (9: 2: 1) as a solvent system. Table (1) shows the course of chromatographic fractionation. TLC examination of the fraction eluted with chloroform- methanol (90:10) revealed the presence of one major spot with R_f 0.83, using solvent system hexane: ethyl acetate (1:3). Repeated crystallization from methanol afforded white granules (compound 1, 100 mg).

Fraction eluted with chloroform-methanol (85:15) was subjected for further purification by applying a silica gel column (50x 2 cm) eluted with chloroform and chloroform-methanol gradient. Subfraction was further purified by sephadex LH-20 column (40x 2 cm) eluted with 95% methanol to afforded (compound 2, 25 mg).

Table 1: Column Chromatography of seeds ethyl acetate fraction

Eluent	Composition (%)	R_f value	Isolated Material
$CHCL_3$	100%	--	--
$CHCL_3$:MeOH	95-5	--	--
$CHCL_3$:MeOH	90-10	0.83	comp. 1
$CHCL_3$:MeOH	85-15	0.19*	comp. 2
$CHCL_3$:MeOH	80-20	--	--
$CHCL_3$:MeOH	75:25	--	--
$CHCL_3$:MeOH	65-35	--	--
$CHCL_3$:MeOH	50-50	--	--
MeOH	100%	--	--

*R_f Corresponding to the isolated compound with Ethyl acetate: formic acid: acetic acid: water (30: 0.8: 1.2: 8).

Chromatographic Fractionation of the Methanol Fraction:

Residue of the methanol fraction (5 g) was chromatographed on a column (120 x 3 cm) packed with

250 g polyamide. Elution was carried out using distillated water followed by the addition of methanol with increasing concentrations till 100 % methanol. Fractions of 500 ml were collected and concentrated under reduced pressure, then subjected to paper chromatographic investigation on Whatman 1MM using BAW and 15 % acetic acid as a solvent systems. The chromatograms were examined under UV light, before and after spraying with $AlCl_3$ and NA reagents. Fractions exhibiting the same chromatographic pattern were pooled together, and screened by paper chromatography using BAW (4: 1: 5) as shown in **Table (2)**.

Fraction eluted with H_2O – MeOH (70 – 30) was chromatographed on silica gel column (60x 2 cm), eluted with chloroform and chloroform-methanol mixtures in a gradient manner. The main fraction obtained was rechromatographed on Sephadex LH-20 column (40x 3 cm), eluted with ethanol (100%) to yield 45 mg of (compound 3).

The concentrated eluant obtained from fraction eluted with H_2O–MeOH (60–40) was applied on a small column of Sephadex LH-20 (40 x 1.5cm) and elution was carried out using methanol/water (90:10). Fractions each of 20 ml were collected. The course of chromatographic fractionation was followed up using paper chromatography on Whatman No. 1 developed with n-butanol: acetic acid: water (4: 1: 5, upper layer). The fractions containing (compound 4, 25 mg) in pure form were collected and concentrated in vacuo till dryness. The purity of compound 4 was checked by TLC and PC using different adsorbent and solvent systems.
Fraction eluted with H_2O–MeOH (50 – 50) and H_2O–MeOH (40 – 60), was chromatographed on silica gel column (40x 2 cm) eluted with chloroform: methanol (90:10). The eluant was further purified using Sephadex LH-20 column eluted with 90% methanol to afford the compound in a pure form using PC and TLC in different solvent systems. The similar fractions were combined to yield (**compound 5**, 20 mg).

Fractions eluted with H_2O–MeOH (30–70) was rechromatographed on silica gel column (60x 2cm) eluted with chloroform: methanol (90: 10) to give (**compound 6**, 30 mg). The compound was subjected for further purification on sephadex LH-20 column using 90 % methanol.

Table 2: Column chromatographic fractionation of the methanol fraction of *Onopordum alexandrinum* seeds.

Solvent	R_f	Color under U. V.			Isolated Compound
			NH_3	NA	
H_2O	0.11	F.y.	F.Y.	Y.	Traces
	0..25	R.	R.Y.	Y.	Traces
H_2O–MeOH (90–10)	0.30	F. y.	Y.	Y.	Traces
	0.35	F.y.	Y.	Y.	Traces
H_2O–MeOH (80–20)	0.35		F.y.	F.y.	Traces
	0.25		F.y	Y.	Traces
H_2O–MeOH (70–30)	0.17		F. y	F.y.	Traces
	0.65		S.b	F.b	**C3**
H_2O–MeOH (60–40)	0.59	Br.	Y.g.	Y.g.	**C4**
	0.42	-	Y.g..	Y.	Traces
	0.44	-	Y.	Y.	Traces
H_2O–MeOH (50–50)	0.44	-	Y.	Y.	Traces
	0.52	-	Y. g.	Y.g.	**C5**
H_2O–MeOH (40–60)	0.52	F.Y.	Y.g	Y.g.	**C5**
	0.49	-	F.y.	Y.	Traces
	0.59	F.y.	Y.	Y.	Traces
H_2O–MeOH (30–70)	0.33	F.y.	Y.	Y.	**C6**
	0.57	-	Y.g.	Y.g.	Traces
H_2O–MeOH (20–80)	0.38	-	F.Y.	F.Y.	Traces
	0.62	-	F.y.g.	Y.g.	Traces
H_2O–MeOH (10-90)	0.37	-	F.y.	Y.	Traces
	0.52	Br.	Y.	Y.	Traces

Paper chromatography : Whatman No. 1
Solvent system : B. A. W (4: 1: 5, upper layer)
Spray reagent : NA
Br. : Brown
Y . : Yellow
F.y. : Faint yellow
R. : Red
S.b. : Sky Blue

Acid Hydrolysis

About 5 mg of the compound was dissolved in 10 ml methanol, mixed with 10% HCl and refluxed on a boiling water bath for 3 hours. The solution was diluted with distilled water and extracted with ether (4x 25ml). The ether extract was washed with distilled water and evaporated in vacuo at 40^{o}C till dryness.

Isolation and Preparation of Rat Hepatocytes Monolayer Culture:

A primary culture of rat hepatocytes was prepared according to (Seglen, 1976) method, which was modified by **(Kiso *et al*., 1983)** using a waster male rat (250-300 gm) The rat was obtained from the animal house of the NRC (National Research Center, Cairo). Animal procedures were performed in accordance with the Ethics Committee of the National Research Centre and followed the recommendations of the National Institutes of Health Guide for Care and Use of Laboratory Animals **(NIH Guide, 1985).**

IC_{50} Determination on Rat Hepatocytes Monolayer Culture:

After 22-24 hours, the rat hepatocyte monolayer was washed twice with Phosphate Buffer Saline (PBS). In order to determine IC_{50}, different concentrations were prepared for each sample (100 – 1000 µg /mL). After two hours of cells incubation with the extract, cell viability was determined using the MTT assay. The assay was performed according to the method of **(Mosmann, 1983)** modified by **Carmichael *et al*., (1987)**. Absorbance of formasan crystals produced by viable cells was read at 540 and 630 nm dual wave-length using the Automatic Kinetic Microplate Reader (Labsystems Multiskan RC reader). Each experiment was repeated three times and the mean absorption of each concentration was calculated. A graph plotted with X-axis showing the different concentrations of the extract used and the y-axis showing the absorbance percentage of viable cells. The IC_{50} was graphically determined from the concentration that yielded an absorption coinciding with the 50% of cells that received no extract.

Evaluation of Hepatoprotective Activity:

The primary rat hepatocyte monolayer was prepared as above. Different concentrations were prepared from the tested compounds (1.25-10 µg /mL) using serial dilutions technique by dissolving in DMSO (1% maximum concentration). For each concentration, three replicates were carried out; in addition to positive control that was 50 µg/ mL Silymarin. The plate was incubated for 2 hrs at 37 ºC and 5% CO_2, then washed twice with PBS. A 200 µL of 25 mM paracetamol was added to each well. After one hour of cells incubation with the paracetamol, cell viability was determined using the MTT assay. The concentration of the extract that was able to protect the cells from the hepatotoxic effect of paracetamol by hundred percent was considered hepatoprotective.

Lactate dehydrogenate (LDH)

LDH leakage from cell cytosol to the culture medium has been generally used as an indicator of cell integrity. Primary culture of rat hepatocytes were incubated for 2 hours with ascending concentrations (12.5, 25, 50, 100 µg) of different tested samples/mL culture medium. LDH was measured in culture medium to reflect cell viability. The LDH activity was measured by an optimized standard method according to the recommendations of the Deutsche Gesellschaft fur Klinische Chemie **(Rec. GSCC DGKC) (1970)**.

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Determination of Nitric Oxide (NO) production

Nitric oxide is very specific to liver injury and is almost always produced during liver inflammation. To test for the possible hepatoprotective effects of tested silymarin samples, different concentrations (1.25, 2.5, 5, 10 µg/mL) of tested samples were added to culture medium of primary hepatocyte culture that has been subjected to paracetamol injury and incubated for 2 hours. Culture medium was used for measurements of nitrite (a stable metabolite of NO) to reflect NO production by Griess method **(Montgomery and Dymock, 1961)**. Decrease levels of NO production are indicative of hepatoprotection.

Glutathione Reductase (GR)

Reduced glutathione is the main non enzymatic antioxidant within the cell and plays an important role in the defense against oxidative stress. It is assumed that glutathione reductase depletion reflects intracellular oxidation. On the contrary, an increase in glutathione reductase concentrations could be expected to prepare the cell against a potential oxidative insult.

The content of reduced glutathione was quantified by the fluorometric assay of **Goldberg and Spooner (1983)**. The method takes advantage of the reaction of reduced glutathione with N-(1-naphthyl) ethyenediamine. Addition to the cell culture evoke a dramatic decrease of cytosolic GR which have been pretreated for 2 hours with different concentrations (1.25, 2.5, 5, 10 µg/mL) of tested samples.

NB: Quercetin was used as a positive control; paracetamol (25 mM/mL) was used to induce liver injury. Each concentrations was placed in three wells so that mean of triplicates and standard deviation was used for statistical analysis.

Statistical analysis:

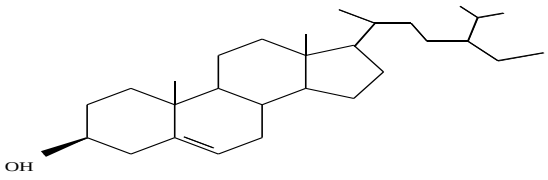
All data presented are based on means of triplicate absorbance determinations. Experiments were repeated twice for all samples.

RESULTS AND DISCUSSION

Identification of Compound 1

Compound **1** (100 mg) occurred as white needles (chloroform- methanol), R_f 0.83 solvent system (Hexane: EtOAc 1: 3). It was freely soluble in chloroform but insoluble in methanol. It gave a positive Lieberman's and Salkowski's tests for sterols and/ or triterpene. Compund (**1**) was isolated as white needles by repeated crystallization of the residue obtained from the fractions eluted with $CHCl_3$: MeOH (90:10.).
The mass spectrum of compound **1** showed a molecular ion peak at m/z 414, suggesting a molecular formula $C_{29}H_{50}O$. The mass fragmentation pattern showed fragments at *m/z* 396 (M^+-H_2O), 381(M^+-H_2O-CH3), 330, 303, 275, 255, 231 and 228 indicating a compound with Δ5-steroidal skeleton.

The ^{1}H-NMR spectrum showed six methyl signals at δH 0.69, 0.81, 0.82, 0.83, 0.92 and 0.99 ppm for six methyl groups (18, 27, 26, 29, 21 and 19). A well defined multiplet signals at δH 3.54 ppm was typical for axial H-3α of a 3β-hydroxy 5α-steriod. The presence of unsaturation was confirmed by the presence of one proton signal at δH 5.35 ppm. The above mentioned data suggested the presence of β-sitosterol. This was confirmed by direct comparison of ^{1}H-NMR, MS and TLC with published data (**Kamboj and Saluja 2011**) and authentic sample of β-sitosterol.



β-sitosterol

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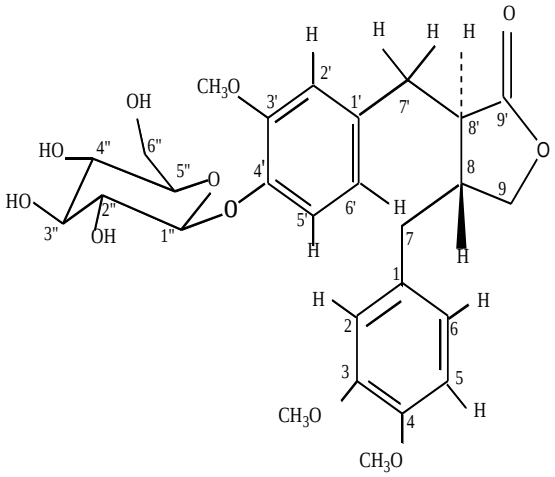
Identification of Compound 2

Compound **2** (25 mg) was isolated as a white amorphous powder, R_f 0.19 solvent system (ethyl acetate: formic acid: acetic acid : water 30: 0.8: 1.2: 8). It gave positive test of lignans by using Molish's, Benedict's and Fehling's tests (**Al- Jumaily *et al.,* 2012**).
On acid hydrolysis, compound **2** afforded glucose that was identified by authentic samples of glucose and lignan.

The ^{1}H NMR spectrums showed six signals due to six aromatic protons, the two doublets at 7.03 and 6.81; each coupled to another proton with an ortho coupling constant 8.4 Hz. The two double doublets at δ 6.64 and δ 6.60 ortho and meta coupling constants 8.2 and 1.5 Hz, respectively. The other two meta coupling at δ 6.81 and 6.59, respectively. The ^{1}H NMR also showed three methoxyl groups at δ 3.78, 3.77 and 3.73. The ^{1}H NMR spectrum also showed one signal for anomeric proton, one hexose anomeric proton resonance at δ 4.86 (1H, d, J = 7.28 Hz), indicating the presence of one sugar with β-configuration.

The ^{13}C NMR spectrum of compound **2** showed 18 carbon signals of lignans, one carbonyl carbon at δ 180.1. The ^{13}C-NMR spectra revealed that compound **2** contained two aromatic ring carbons, including four oxygen-carbons at [δC 145.4 (C-3), 147.8 (C-3), 149.1 (C-4), 149.3 (C-4)] and eight olefinic carbons [δC 111.7 (C-5), 112.2 (C-2),113.4 (C-2), 116.5 (C-5), 120.7 (C-6), 121.6 (C-6), 131.4 (C-1) and 132.9 (C-1)] as well as three methoxy groups at [δC 55.1, 55.2 and 55.3].

Spectra revealed that compound **2** contained also five aliphatic carbons including three methylene carbons, two methine carbons and one oxymethine together with six carbons of one glucose moiety. From the previous data and on comparison with the published spectra (**Harraz *et al.,* 1988**), it was concluded that compound **2** was identified as arctiin (arctigenin β-D- glucopyranoside).



Arctiin

Identification of Compound 3

Compound **3** (45 mg) appeared as sky blue fluorescent spot under UV, and after spraying with NA reagent with R_f value 0.65 in BAW 4:1:5.

The UV spectrum, table (3), showed absorption peaks at λmax 268 and 274 nm in MeOH, and a bathochromic shift with high intensity at 270 and 286 nm upon addition NaOMe, indicating a phenolic nature of the compound.

The mass spectrum showed a molecular ion peak at m/z 180 for (M^+) which correspond to the molecular formula $C_9H_8O_4$, the peak at m/z 135 for (M^+-COOH).

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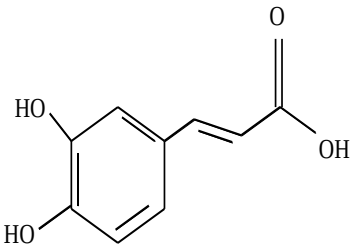
The ^{1}H-NMR spectrum displayed signals at δ 8.5 and 7.5 ppm (d,d, 2-H olefinic protons), 7.78 (s, 1H, H-2'), 7.69 (d, 1H, H-5), and (d, 1H,H-6).

From the above data and on comparison with the published spectra **(Mabry *et al.,* 1970**), it was concluded that compound **3** is caffeic acid.



Caffeic acid

Identification of Compound 4

This compound was isolated as yellowish white powder (25mg) and it appeared as a dark brown spot changed to yellow with AlCl3 under UV light.

It is an aglycone in nature according to its chromatographic behavior on PC in different solvent systems (Rf = 0.59, in BAW 4:1:5 and Rf = 0.17 in 15 % acetic acid (Mabry et al., 1970).

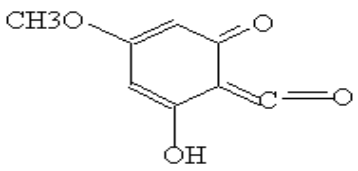
The UV absorption spectrum, table (3), in methanol showed band–I at 325 nm and displayed a bathochromic shift (62 nm) with high intensity on addition of NaOMe, which indicated the presence of a free OH group at C4'. The presence of free OH group at C-5 was also confirmed through the high bathochromic shift value of band-I from 346 in methanol to 426 in AlCl3 (Mabry et al., 1970).

The presence of an ortho-dihydroxy system was confirmed through AlCl3/HCl spectrum, where band–I was hypsochromically shifted from 426 nm in AlCl3 spectrum to 364 nm in AlCl3/ HCl , Band-I in NaOAc/ H3BO3 spectrum was also bathochromic shifted from 346 in methanol to 377 nm (31nm) .

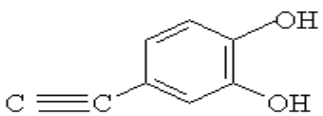
It was found that there was no bathochromic shift in band–II of NaOAc spectrum relative to methanol, which confirmed the absence of a free OH group at C-7 that might be occupied by a mehoxy group.

The mass spectrum showed a molecular ion peak at m/z=301 corresponding to (M++1). Another peak also occured at m/z =271 due to (M+-HCHO), m/z =252 (M+- (OCH3+OH). Two peaks at m/z =166 and 134 resulted from retero Diel's Alder fragmentation. By reviewing the literature for formula index, it agreed with the molecular formula C16H12O6 (Markham, 1982).

The compound undergoes retero Diel's Alder rearrangement followed by fragmentation giving rise to A1+ fragment ion at m/z=166 and B1+-1 fragment ion at m/z= 133 which confirmed the presence of the methoxy group at ring A not at ring B and the ortho dihydroxy system at ring B not at ring A.



A1(166)



B1-1(134)

The ^{1}H- NMR of the compound showed signals at δ in 7.8 ppm (d,d ,2H, H- 2', H-6'), 7.93 (d,1H, H-5'), 6.96 (s,1H, H- 3), 6.94 (d,1H, H- 6), 6.7 (d, H, H-8-) and finally 3.9 (s, 3H, C-7-OCH_3). From the previous data, the compound **4** could be identified as 7- methoxy luteolin.

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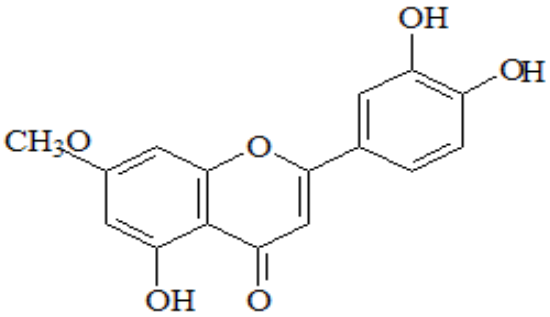

7-methoxy luteolin

Identification of Compound 5:

This compound was isolated as yellowish powder (20 mg) and it appeared as a faint yellow spot changed to yellowish green with NA reagent under UV light (R_f = 0.52, in BAW 4:1:5).

The UV absorption spectrum, table (3), of compound 5 in methanol exhibited band–I at 364 nm, which indicated a flavonol type structure.

It showed a bathochromic shift with fast decomposition in band-I (66 nm with NaOMe indicates the presence of free OH groups at C- 4 and C- 3). The batochromic shift in band-I (67nm) with $AlCl_3$ indicated the presence of free OH group at C-5 and the hypsochromic shift in band –I (31nm) with $AlCl_3$ / HCl relative to $AlCl_3$ indicated the presence of an ortho– dihydroxy group in ring B at C- 3', C- 4'.

The NaOAc spectrum showed no bathochromic shift in band- II, indicating the absence of free OH group at C-7. The bathochromic shift in band-I (12 nm) with NaOAc/ H_3BO_3 was indicative of the presence of an ortho- dihydroxy system in ring B.

The EI-Mass spectrum of compound **5** showed a molecular ion peak at m/z 463 (M^+- 1), corresponding to the molecular formula $C_{21}H_{20}O_{13}$-1.

The fragment ion at m/z 301 corresponding to M^+- 1- 162 [M^+- 1– hexose moiety]. The fragment at m/z 283 (M^+- 1– hexose- H_2O) and the fragment at m/z 273 (M^+- 1– hexose– CO). The fragmentation pathway undergoes Retero– Diels Alder reaction giving rise to fragment ion at m/z 152($A1^+$) and fragment ion at m/z 150 ($B1^-$).

The results obtained from the ultra- violet spectrum and the fragmentation pathways of the flavonoidal compound **5** are identical with that reported for quercetin-7- O- glucoside (**Mabry *et al.*, 1970**).
Compound **5** was subjected to acid hydrolysis. The obtained residue was purified by passing through Sephadex LH-20 column and its purity was checked by two dimentional paper chromatography using different solvents (B: A: W, 3: 1 :1) (R_f: 0.91) and 15% AcOH (R_f: 0.03). The isolated aglycone possessed the same R_f values as authentic quercetin, using different solvent systems.

The UV absorption spectra of the isolated aglycone were found to be identical with those of quercetin as previously reported by (**Mabry *et al*. 1970**).

The position of the attachment of the sugar moiety at C- 7 in compound **5** was confirmed as the NaOAc spectrum of the obtained aglycone showed bathochromic shift in band-II (7nm), indicating the presence of free OH group at C-7, i.e the sugar moiety was attatched at C-7.

The aqueous acidic solution after separation of the aglycone was neutralized with barium carbonate, and then filtered through a centered glass funnel. The clear filtrate was evaporated till dryness.The residue was dissolved in redistilled pyridine, filtered and evaporated in vacuo at 70ºC.The residue was dissolved in 10% isopropanol and subjected to paper chromatography, using EtOAc: Pyridine: H_2O (12: 5: 4) as a developing solvent.

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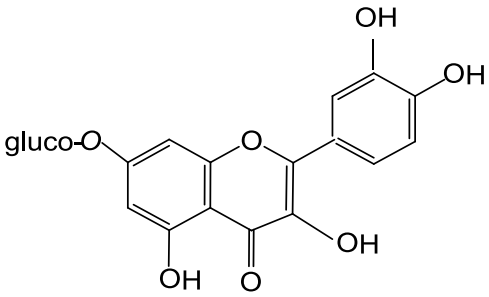
The chromatogram was visualized by spraying with aniline phthalate (Herbert *et al.,* 1964), and heated at 105°C for few minutes. Glucose was the only sugar detected in the hydrolyzate.

From the data obtained above for TLC, PC, UV, MS and acid hydrolysis, the flavonidal compound **5** was identified as quercetin-7-*O*-glucoside.



Quercetin-7-*O*-glucoside

Identification of Compound 6

Compound 6 (20 mg) showed dull spot under UV turning to yellow with NA reagent (Rf 0.33 and 0.83 in BAW and 15% acetic acid, respectively), which were indicative of a glycoside nature.

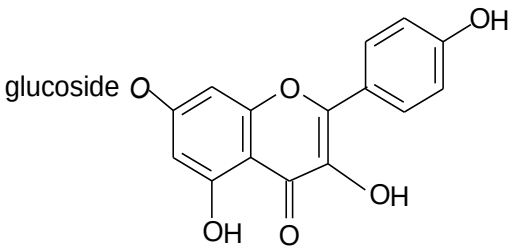
The UV absorption spectrum, table (3), showed band- I at 357 nm proving the flavonol nature of this compound. A bathochromic shift in NaOMe in band I (from 357 to 400 nm) with high intensity proved the presence of a free OH group at C4`. AlCl3 and AlCl3 ⁄ HCl spectra in band I and band II confirmed the presence of free OH groups at C-3 and C-5. Low hypsochromic shift in band–I in AlCl3/ HCl spectra, relative to AlCl3 spectrum indicated the absence of an orthodilydroxy system which was also, confirmed through band -1 with NaOAc / H3BO3 relative to methanol spectrum.

No bathocromic shift was observed in the band-II with NaOAc (256- 258) relative to MeOH spectra, which proved the absence of a free OH group at C-7, i.e the sugar moiety was attatched at C-7.

The mass spectrum showed a molecular ion peak at m/z = 448 (M+), which correspond to the molecular formula C21H19O11. The peak at m/z = 286 was due to M+- hexose moiety (162), m/z = 256 (M+ -1 (162-HCHO).

The 1H- NMR of the compound gave signals at δ = 7.72, 7.6 ppm (d, d, 2-H, H-2' , H- 6'), 6.9 (d, 1H, H-6), 6. 8 (d, 1H, H-8), 6. 6 (d, 1H, H-5'), 6.4(d, H-1, H-5') and 5.32(d, 1H, H-1'') anomeric proton of glucose (Bonilla et al., 1999).

After acid hydrolysis, glucose was identified as sugar while Kampferol was identified as an aglycone. From all the chromatographic and spectroscopic data, the compound could be identified as Kampferol -7-O-glucoside.



Kampferol 7-*O*-glucoside

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Table 3: Ultra-violet spectral data (nm) of the isolated flavonoides and their aglycones

Addition to MeOH	MeOH	NaOMe	$AlCl_3$	$AlCl_3$+Hcl	NaOAc	NaOAc+ H_3BO_3
Compound 3	268, 274	270, 286				
Compound 4	254_{sh}, 271, 346	275, 408	251, 275, 338, 426	277, 298, 364	273, 406	264, 377
Compound 5	254sh., 270, 364	268, 390, 430	260sh., 270, 361, 431	270,305, 365, 400	271,378, 430 sh.	263,290,322,376
Compound 6	203, 258, 357	203, 267, 400	203, 272, 412	203, 269, 388	261, 408	256, 367

Hepatotoxic, Hepatoprotective and Antioxidant activities:

The hepatoprotective, hepatotoxic and antioxidant capacity results of tested compounds are shown in table 4. Antioxidant capacity of silymarin (0.1 mg/ml) was compared with 10µg/ml Qurcetin. Antioxidants neutralize the effect of free radicals through different ways and prevent the body from various diseases. Antioxidants may be synthetic or natural. Synthetic antioxidants have recently been reported dangerous for human health. Thus the search for effective, non-toxic, natural compounds with antioxidant activity have been intensified in recent years. Hepatotoxic agents include not only some chemicals but also some drugs that are used in clinical practice. It has great capacity to detoxicate toxic substances and synthesize useful ones. Therefore, the damage which is caused by hepatotoxic agents is of grave consequence to the body as it deprives the liver of its principal functions. With the immense advancement of the synthetic drug there is not much progress in this discipline due to less potency and toxicity profile. (Pachpute & Deshmukh, 2012)

LDH leakage into the culture media of hepatocyte was used to assess the hepatotoxicity of different compounds (table 4, figure 1). The LDH results were correlated with the MTT hepatotoxicity experiment. Most samples showed higher therapeutic index of silymarin purchased from Sigma Aldrich or even better. Antioxidant activity of the compunds were tested using in vitro evaluation of nitric oxide (NO) (figure 2) and glutathione reductase (GR) (figure 3) enzymes in the culture media in comparing with qurcetin as a positive control. A broad number of samples induced endogenous intracellular production of GR into culture medium which reflects antioxidant capacity of tested samples. Also results revealed dose dependant decrease in the levels of NO in culture medium of hepatocytes exposed to injury. The maximal effect was achieved at the maximum tested concentration (10µg/mL).

Samples (4 and 6) showed low hepatoprotective and therapeutic index. At the same time; pretreatment of hepatocytes with different concentrations of 4 and 6 samples for two hours prior to the addition of paracetamol to the cells reduced the levels of nitrite (a Nitric oxide metabolite) in culture medium to mean basal levels. Results revealed dose dependant decrease in the levels of NO in culture medium of hepatocytes with maximal effect at concentration of 10µg/mL, the maximum tested concentration.

Table 4: LDH leakage percentage, IC_{50}, initial hepatoprotection dose, Glutathione Reductase and Nitric oxide in culture medium of rat hepatocytes

Sample	LDH leakage after 2h (µg/mL)	Hepatoprotection (µg/mL)	Therapeutic index %	Glutathione Reductase/10^6 cells (100µg/mL)	Nitric Oxide (µmol nitrite/10^6 cells) (100µg/mL)
Total alcoholic extract	<250	<12.5	20	4692.6	20.56±2.2
Ethyl acetate fraction	1000	<12.5	80	5163.25	18.67±1.3
Comp. 1	25	1.25	80	5062.60	23.83±3.1
Comp. 2	12.5	1.25	80	5721.72	14.47±2.4
Comp. 3	<100	1.25	80	5917.33	10.08±1.7
Comp. 4	12.5	10	1.25	5147.00	14.67±1.1
Comp. 5	<100	1.25	80	5186.52	7.25±1.8
Comp. 6	100	>10	<10	4915.93	6.58±2.6
quercetin	1000	12.5	80	5428.33	11.11±1.7
Silymarin	500	25	20	5139.63	7.50±3.3

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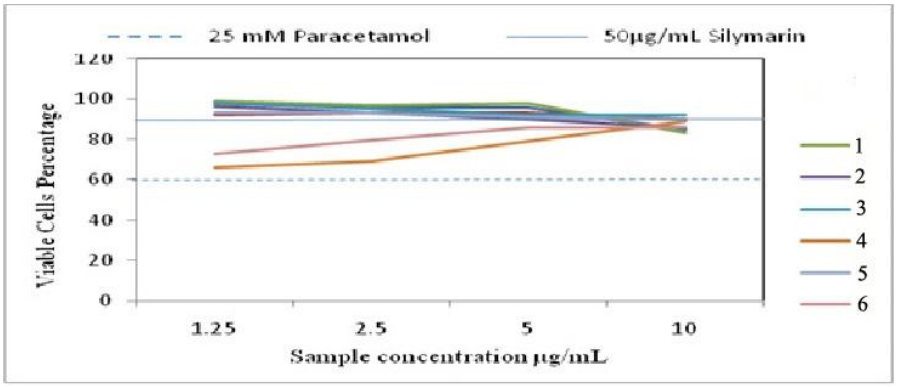

Figure 1: Viable cell absorbance showing the hepatoprotective effect of the tested compounds against the hepatotoxic effect of paracetamol (25mM) using MTT assay

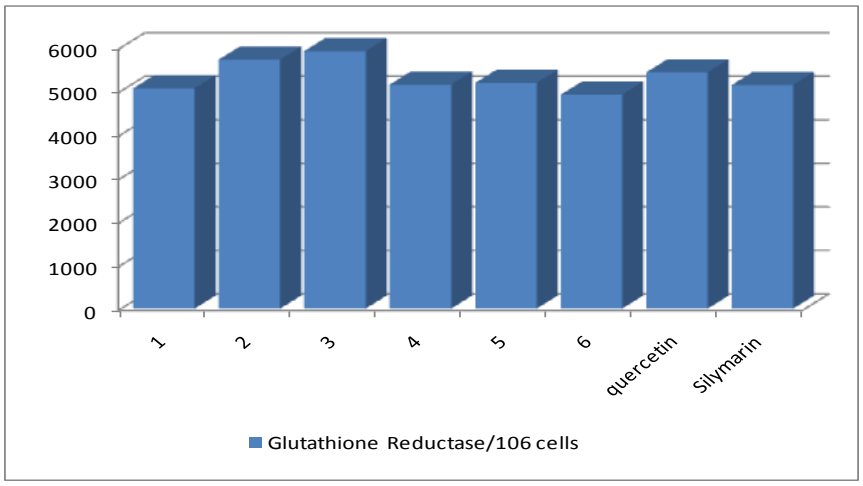

Figure 2: Glutathione (GSH) concentrations of primary rat hepatocytes in each group. Data are mean±SD for 3 hepatocyte preparations.

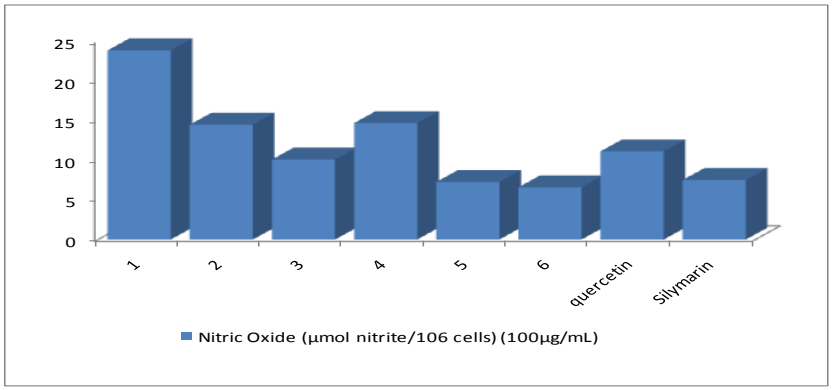

Figure 3: Bar chart showing the difference in nitrite activity using nitric oxide kit assay of some tested samples

CONCLUSION

Compound 3 (caffeic acid) and compound 5 **(**quercetin-7- O- glucoside) showed high antioxidant activity as well as hepatoprotective activity and very low toxicity on liver cells.

This result coincides with Yang *et al.* 2013 for caffeic acid. Quercetin-7- O- glucoside is a flavonol glycoside compound where this group was reported before by Singab *et al.* 2005 to poses hepatoprotective and antioxidant activities.

DISCUSSION

Fractionation of Onopordum alexandrinum seeds constituents was performed by applying column chromatography. Moreover, further purification was achieved using successive chromatographic techniques, viz: preparative thin layer chromatography (PTLC), paper chromatography (PPC), as well as sephadex LH-20 column chromatography.

Identification of the isolated compounds was carried out through chromatographic means and spectroscopic analyses (viz: UV, MS and NMR).

In this respect, the investigation of the ethyl acetate fraction from Onopordum alexandrinum seeds resulted in the isolation of β-sitosterol compound and arctiin; a lignan glucoside compound. Moreover, the study of flavonoidal constituents of the alcoholic extract from Onopordum alexandrinum seeds resulted in the isolation of caffeic acid and three flavonoid compounds, which were identified as 7- methoxy luteolin, Quercetin 7- Oglucoside and kampferol 7- O- glucoside. These compounds were isolated and identified for the first time from the genusOnopordum.

Hepatoprotection activity of the different extracts of Onopordum alexandrinum seeds (ethyl acetate, methanol and the isolated compounds) was studied using hepatocytes monolayer culture from rat. The monolayer cells were exposed to certain hepatotoxic doses of paracetamol, which was capable of damaging half the isolated cells and was used as a control group (20Mm), compared with 50 µg/ ml silymarin as a control. The results showed that ethyl acetate fraction and the alcoholic extract of seeds had hepatoprotective activity on hepatocytes against paracetamol cytotoxicity at concentration 12.5 µg/mL, where the LC50 (concentration that killed half of the cells) were >1000 µg/mL.

Compound 3 (caffeic acid) and compound 5 (quercetin-7- O- glucoside) showed high antioxidant activity as well as hepatoprotective activity and very low toxicity on liver cells.

This result coincides with Yang et al. 2013 for caffeic acid. Quercetin-7- O- glucoside is a flavonol glycoside compound where this group was reported before by Singab et al. 2005 to possess hepatoprotective and antioxidant activities. Consequently, these fractions were assumed to be promising with regard to their antioxidant, hepatoprotective and hepatotoxic activities.

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