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IDENTIFICATION OF BIOACTIVE INGREDIENTS IN *SONCHUS OLERACEUS* BY HPLC AND GC/MS

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Abstract

This study aims to evaluate the successive extraction of the active ingredients and their anticancer activity of arial parts (stem, leaves and flowers) of *S. oleaceous*. Therefore, *Sonchus oleraceus* aerial parts were subjected to a successive extraction by using solvent of different polarities; each fraction was evaluated for its anticancer. Each active extract was farther biogided fractionated to determine and identify the active compound/s present in each sub-fraction. All the tested species were extracted using four solvents: hexane, ethyl acetate, methanol and water. Phenols, flavonoids and tannins were quantified for all extracts. The chemical analysis proved it to will be a good source of protein, fat and carbohydrates. The results showed that the percentages of moisture content (92.72%), ash content (15.14%), crude protein (25.94%), crude lipid (4.05%) and carbohydrate (54.87%) respectively of the *S. oleraceus L*. the highest content of total phenolic (TP), total flavonoid (TF) and total tannin (TT) was obtained by methanol fraction. The active ingredients were evaluated as well employing gas chromatography coupled to mass spectrometry (GC-MS) and high-performance liquid chromatography (HPLC). Gas chromatography mass spectrometry (GC-MS) data showed the identification of ten volatile compounds in hexane fraction of the *Sonchus oleraceus*, among them two were found to be the major components 9 Octadecenamide (CAS) 40.92% and 1 Hexacosanol 21.01%. The HPLC analysis of phenolic compounds confirmed that the water fraction of *Sonchus oleraceus L* detected high amounts of pyrogallol and chlorogenic respectively. The result showed that pronounced cytotoxic activity of cancer cells at 100 µg/ml in aqueous fraction in breast cancer (94.8%), while in Methanol extract in Colon Carcinoma (88%) On the other hand, ethyl acetate fraction of the *Sonchus Oleraceus* showed the highest percentage of colon cancer cells (82.6%) and breast cancer (80%), while the ethyl acetate extract in hepatocellular carcinoma cell line showed moderate effect (46.3%). Thus, our results highlight of *Sonchus oleraceus L* for its possible clinical use to oppose malignancy development against mostly breast and colon cell line as a bio agent in pharmaceutical industries.

Key words: *Sonchus oleraceus*; HPLC; GC/MS; anti-proliferative.

Introduction

Herbs are still used by 80% of world population, because of their easy availability and very negligible side effects. From ancient period medicinal plants are used for pharmacological studies. The *Asteraceae* (commonly known as sunflower) is a large and widespread family which contain many genera. *Sonchus oleraceus* is a plant belongs to family *asteraceae* (Hussain *et al*., 2010). The *Sonchus oleraceus* plant, popularly known as milkweed, milkweed-smooth is a cosmopolitan species, found in many agricultural regions like weed crops. The *S. oleraceus* is native to Europe, North Africa and West Asia. It has spread to North and South America, India, China, southern Australia (Cameron, 2000). The genus *Sonchus* comprises about 60 species and three of them have become common weeds around the world. *Sonchus* contains variety of phytochemical compounds such as sesquiterpene lactones of the eudismanolide and guaianolide structures (ElkHayat, 2009) also contain flavonoids, flavanols, proanthocyanins, total phenol, saponins, phytate and alkaloids (Jimoh *et al*., 2011). High concentration of fatty acids, vitamin C, carotenoids, oxalic acid and high mineral contents which gave this plant high

value in as nutritional supplements (Guil-Guerrero *et al*., 1998). Currently, studies have indicated that the extract have many bio-activities, including antioxidant, antibacterial motion, headaches, general pain, rheumatism and even as a general tonic, anxiolytic, antinociceptive, anti-ageing, antitumor and anti-inflammatory properties (Elgazali, 2003; Yin *et al*., 2007 and Vilela *et al*., 2009) as well as has hepatoprotective activity, antitumor effect, cardiovascular therapy.

So, the aim of this study will be focused on fractionation of *Sonchus oleraceus* and investigate *in vitro* study their biological effects of each fraction as antitumor agent.

Materials and Methods

1. Plant Material

The arial parts (stem, leaves and flowers) of *Sonchus oleraceus* were collected from the farm, faculty of Agriculture, Cairo University in November 2016. The harps were identified by Prof. Dr. Mohamed El-Khateeb, Ornamental Department –faculty of Agriculture, Cairo University. The leaves were used for extraction of active constituents which was followed by identification of each compound present in each fraction.

2. Solvents

n-Hexane, ethyl acetate, methanol, Dimethyl sulfoxide (DMSO). All solvents were of analytical grade (from Sigma-Aldrich), purified and distilled.

3. Preparation of Plant Material

The basic steps include drying of plant materials and grinding the plant sample to obtain a homogenous sample.

4. Chemical studies

The moisture, ash, crude protein, total lipid and total carbohydrate were determined according to (A.O.A.C., 2005).

5. Extraction of bioactive compounds in *Sonchus oleraceus* extracts

The freshly arial parts (stem, leaves and flowers) of *Sonchus oleraceus* were dried in air and protected from direct sunlight and mechanically grounded to powder. The powder of leaves was macerated with organic solvents, namely n-hexane solvent (non-polar), followed by ethyl acetate (semi-polar), methanol (polar) and ended with aqueous (polar). The leaves were macerated with n-hexane, ethyl acetate, methanol and aqueous solvents, filtered and the filtrate obtained was concentrated in a rotary evaporator vacuum to obtain concentrated extracts. The maceration processes lasted for 48 hours, with three times solvent additions. Finally, n-hexane, ethyl acetate, methanol and aqueous extracts concentrated on the *Sonchus oleraceus* leaves were prepared, respectively according to (Simorangkir *et al.*, 2018).

6. Secondary metabolites analysis of *Sonchus oleraceus*

(a) Determination of total phenolic compound content: The total phenolic (TP) was determined by Folin Ciocalteu reagent assay at 750 nm by spectrophotometer (Unicum UV 300), using Gallic acid as a standard. Total phenolics were expressed as mg Gallic acid equivalents (GAE)/g dry weight. Samples were analyzed in triplicates according to Singleton and Rossi, (1965).

(b) Determination of total tannin content: Total tannin (TT) of *Sonchus oleraceus* fractions was measured by Folin Ciocalteu's reagent at 775 nm by spectrophotometer (Unicum UV 300), using tannic acid as a standard. Total tannins were expressed as mg tannic acid equivalent (TAE)/g dry weight as described by (Tempel, 1982; Schanderi, 1970).

(c) Determination of total flavonoid content: Total flavonoid (TF) of *Sonchus oleraceus* extracts was determined by the aluminum chloride method at 510 nm by spectrophotometer (Unicum UV 300), using quercetin as a standard. Total flavonoids were expressed as mg quercetin equivalents (QE)/ g dry weight according to Hostettmann and Hostettmann, (1982)

(d) Identification and quantification of phenolic and flavonoid compounds of *Sonchus oleraceus* extracts *by HPLC:* the dried sample were dissolved in 2 ml methanol. HPLC spectral grade by vortex mixing for 30 min. 31 The HPLC system is Agilent 1100 series coupled with a DAD detector following the method of (Mattila *et al*., 2000). Sample injections of 5 µl were made from auto-sampler. The chromatographic separations were performed on a C18 column (4.6×250 mm, particle size 5 µm). A constant flow rate of 1 ml/min was used with mobile phases: (A) 0.5% acetic acid in distilled water at pH 2.65 and solvent (B) 0.5% acetic acid in 99.5% acetonitrile. The elution gradient was linear starting with A and ending with B over 50 min, using a DAD detector set at wavelength 280 nm. The results expressed as mg phenolic/100 g dry weight (Fernandez de Simon *et al*., 1990)

(e) Identification of non-polar compound (methylated hexane fraction) of *Sonchus oleraceus* by GC/MS analysis: The bioactive fractions were subjected to GC/MS analysis to identify the non-polar compounds present in the non-polar fraction. Identification of the constituents was carried out by comparison of their retention times and fragmentation patterns of mass with those of published

data (Adams 1995, 2007) and/or with those of the Wiley 9 and NIST 08 mass spectra libraries.

7. Biological studies

• *In vitro* antitumor activity of *Sonchus oleraceus* by MTT Cell Viability assay: To find out the long-term cytotoxicity of the plant extracts on various cancer cell lines using the colorimetric MTT assay.

Cell viability was investigated using MTT 3-(4, 5-Dimethyl-2-thiazolyl)-2, 5-diphenyl-2H-tetrazolium bromide. (MTT) is a water-soluble tetrazolium salt yielding a yellowish solution when prepared in media or salt solutions lacking phenol red Dissolved MTT is converted to an insoluble purple formazan by cleavage of the tetrazolium ring by mitochondrial dehydrogenase enzymes in metabolically active cells Dead or non-viable cells do not cause this change. This water-insoluble formazan can be solubilized using isopropanol or other solvents and the dissolved material is measured spectrophotometrically yielding absorbance as a function of the concentration of converted dye (Mosmann, 1983 and Wilson, 2000). MTT is converted by the succinate dehydrogenase enzyme in the cell, into an insoluble colored formazan product. It is solubilized in DMSO and the cell viability depends on its absorbance value. The absorbance of untreated cells was taken as the 100 % viable cells.

Statistical Analysis

Data were statistically analyzed using Costat statistical package data according to **(**Snedecor and Cochran, 1989)

Results and Discussion

Proximate analysis

The results of chemical composition of *Sonchus oleraceus* as g/100g dry weight are shown in table 1.

The moisture content of whole plant of *Sonchus oleraceus* was found to be 92.72% higher than that obtained by (El-Tantawi, 2002), 90.62% in the leaves of *S. oleraceu,* 89.9 in S. *oleraceus* and 85.37% *in* S. *asper* (Jimoh *et al*., 2011). This result indicated low shelf life of the fresh plant hence long storage would lead to spoilage due to its susceptibility to microbial attack. This supports the practice of storage in dry form by users (Onwuka, 2005). The Ash content 15.14% was obtained as a result of *Sonchus oleraceus*. Ash in food is *a* reflection of the amount of mineral elements present in the samples. The ash content of *S. oleraceus* displayed lower content than that found by (El-Tantawy, 2002) which presented 23.97%. Protein is an essential component of diet needed for survival for animals and human being to supply adequate amounts of essential amino acids. The highest crude protein content was obtained 25.94% in *S. oleraceus* which was higher than that found by (Jimoh *et al*., 2011). In the leaves of *S. asper* and *S. oleraceus* which represented 13.25% and 17.50% respectively. The crude lipid content obtained for *S. oleraceus* was 4.05%. Lipid provides very good sources of energy and aids in transport of fat soluble vitamins, insulates and protects internal tissues and contributes to important cell processes (Pamela *et al*., 2005). The crude lipid was found in *Sonchus oleraceus* 4.05% which was higher than obtained (El-Tantawy, 2002) 3.79% and (Hussain *et al*., 2009) 1.48%, based on dry weight and lower than that found by Jimoh *et al*., (2011) for *S. asper* and *S. oleraceus* which represented 7.75% and 7%, respectively based on dry weight. Furthermore, the carbohydrate content showed the highest content was (54.87%) which was higher than that obtained by Hussain *et al*., (2009) and (Jimoh *et al*., 2011), which represented 36.56%, 41.92% and 46% in leaves of *Sonchus oleraceus, S. asper and S. oleraceu* respectively.

Photochemical constituents of *Sonchus oleraceus*

(a) Phenolic, flavonoid and tannin content of *Sonchus oleraceus* T: The results of the total phenolic, total flavonoids and total tannins and the extraction yield in *Sonchus oleraceus* fractions: Hexane, ethyl acetate, methanol and aqueous fractions are tabulated in table 2. Among the various solvent used methanol fraction showed the highest values of total phenolic 21.08mgGE/g DW, flavonoid 16.91 mg Q E /g DW and Tannin 321.66% mg TE/gDW compared to hexane which showed the lowest values total phenolic 0.92 mg G/gDW, flavonoids 1.72 mg QE/gDW and Tannins 1.68 mg TE/gDW.

The results of S. *oleraceus* are in the same trend of those reported by Lin *et al.,* (2011) they found that the Polyphenols content in acid methanol extracts of leaves of *S. oleaceous* was found to be 29.80 mg gallic acid /g DW. While Yin *et al*., (2007) evaluated total phenolic content of different extracts from *S. oleraceus* such as water, ethanol, 70% ethanol, methanol and 70% methanol and recorded the highest content for phenol 74.8 mg, 73.6 mg, 71.2 mg, 54.1 mg,

Table 1: Proximate analysis of dehydrated *Sonchus oleraceus* (%)

Plant	Moisture (On fresh weight)	Ash	Crude Protein	Crude Fat	Total carbohydrate
Sonchus oleraceus	92.72±0.85	15.14±4.17	25.94±0.19	4.05±0.07	54.87±4.23
All values demonstrate as mean ± SD					

Table 2: Total Phenolic, flavonoid, tannin content and extraction yield *S. oleraceus* fractions.

Extracts	Total phenolic (TPC) mg GAE/gDW	Total flavonoid (TFC) mg QE /gDW	Total tannin (TTC) mg TE /g Dw	Yield %
Hexane	0.92^{a} ±0.05	1.72^{a} ±0.04	1.68^{a} ±0.55	1.68^{a} ±0.55
Ethyl acetate	5.05^{b} ±0.07	3.49^{b} ±0.03	4.79^{b} ±1.07	4.79^{b} ±1.07
Methanol	21.08^{d} ±0.04	16.91^{d} ±0.9	21.66^{d} ±0.47	21.66^{d} ±0.47
Aqueous	8.74^{c}±0.09	7.73^{c} ±0.11	11.15^{c} ±0.30	11.15^{c} ±0.30
LSD at 0.05	0.15	0.94	1.34	1.34
All values demonstrate as mean ± S.D				

40.5 mg and 39.5 mg/g, respectively.

• The total flavonoid was 16.91mgQE/gDW which was higher than that obtained with Khan *et al.,* (2012) for flavonoid compounds of *Sonchus asper* methanol extract *11.4 mg*. while, Morales *et al.,* (2014) found that total flavonoid of *S. oleraceas* was found to be 51.33 mg GAE/g extract. Also, are accordance with Gaafar *et al*., 2020) in Jojoba methanol extract. The yield percentages of *S. oleraceus* fractions are tabulated in table 2. The methanol and aqueous extracts displayed the higher values being 21.66% and 11.15% respectively because of they have the polarity index of around 5.1 and 9.0. They are the best solvent system for enhanced the solubility of both the methoxylated and hydroxylated compounds and hence improved the overall extraction yield of entire group of compounds.This could be due to the higher polarity. While ethyl acetate yielded 1.90% due to having moderate polarity which holds semi-polar compounds such as terpenes, phenolics, flavonoids, glycosides compounds. However, a different composition of phenolic compounds was detected in that study which could be due to different extraction method. And the solvent polarity which plays a key role in the extraction of the plant processes and increasing phenolic solubility. On the other hand, Morales *et al*., (2014) have reported that a lower portion of total phenols consists of flavonoids, which could be due to different growing conditions and cultivation practices (wild versus cultivated species), as well as to different geographical origin (Schaffer, Schmitt-Schillig, Müller and Eckert, 2005).

(b) Identification and quantification of phenolic compounds of different fractions of *Sonchus oleraceus:* Natural products have always been a preferred choice of all as it plays a great role in discovering new medicines. Extraction and separation of chemical constituents of plants depend on selective solvents through standard procedure. Fractionation is the best method to separate each group of constituents alone when the plant contains several groups of constituents (Ahmed *et al.,* 2015). HPLC is technique for quantification of phenolic compounds in plant extract is affected by the chemical nature of the analytic, as well as assay method, selection of standards and presence of interfering substances. The HPLC is frequently used for the quantification of polyphenols from *S. oleraceus* extracts due to its accuracy, reliability and repeatability. The HPLC chromatogram of three solvent fractions of *Sonchus oleraceus* showed the presence of different compounds of phenolics in variable amounts as presented in (Table 3). The highest phenolic content was observed for aqueous fraction followed by methanol and ethyl acetate fractions. The water fraction of *Sonchus oleraceus* shows remarkable content of pyrogallol (411.60 µg/g DW) followed by chlorogenic (142.11 µg/g DW) and benzoic (68.71 µg/g DW). Small amounts of coumarin (0.84 µg/g DW), caffeic (1.26 µg/g DW) were observed (Table 3).

Previous studies confirmed our results, they studied the composition of selected phenolic compounds in *S.*

Table 3: HPLC analysis for Phenolic compounds of *Sonchus oleraceus* in ethyl acetate, methanol and aqueous fractions.

Phenolic compounds µg/g DW	Ethyl acetate	Methanol	Aqueous
Pyrogallol	9.415	101.87	411.60
Gallic	0.806	3.64	2.61
3- OH tyrosol	4.575	30.15	30.90
Protocatchuic	1.787	4.15	8.16
Catechol	0.838	3.86	-
4-Amino-benzoic	1.723	5.43	2.99
Catechein	5.394	36.55	
Chlorogenic	12.912	97.73	142.11
p- OH- benzoic	4.651	-	-
benzoic	11.338	69.99	68.71
Caffeic	0.913	3.46	1.26
Vanillic	4.494	17.42	6.29
Caffeine	1.999	29.71	15.30
Ferulic	2.825	18.76	6.64
Iso- Ferulic	0.926	8.99	5.23
α- coumaric	1.324	25.65	9.76
Coumarin	5.305	53.32	0.84
3,4,5-Methoxy-cinnamic	2.790	39.42	10.50

Table 4: Main compounds identified in hexane fraction of *S. oleraceus* by GC/MS.

S.N.	R_t[a]	Compound name	Area%[b]	Molecular formula
1	13.59	Methyl 14methylpentadecanoate	6.64	$C_{17}H_{34}O_2$
2	17.38	9,12Octadecadienoic acid, methyl ester	1.20	$C_{19}H_{34}O_2$
3	17.53	6,9,12,15Docosatetraenoic acid, methyl ester (CAS)	4.96	$C_{23}H_{38}O_2$
4	19.37	1Heptadecanamine	3.20	$C_{17}H_{37}N$
5	23.11	9Octadecenamide (CAS)	40.92	$C_{18}H_{35}NO$
6	27.12	cis11Eicosenamide	1.00	$C_{20}H_{39}NO$
7	30.84	13Docosenamide, (Z)	11.79	$C_{22}H_{43}NO$
8	32.78	1Hexacosanol	21.01	$C_{26}H5_4O$
9	13.59	Methyl 14methylpentadecanoate	6.64	$C_{17}H_{34}O_2$
10	17.38	9,12 Octadecadienoic acid, methyl ester	1.20	$C_{19}H_{34}O_2$

[a]Rt: retention time (min). [b]e percentage composition was computed from the gas chromatography peak areas

oleraceus, the result showed that presence of quercetin in the ethyl acetate fraction and apigenin in n-butanol fraction of Leaves of *S. oleraceus* (Alrekabi *et al.*, 2018). In another study (Gaafar *et al.,* 2016) Identified moringa defatted extract by HPLC found that presence of quercetin. Similar results were found by Saxena *et al.,* (2020) who tested the phytochemical on floral extract of *Sonchus oleraceus* for identifying its chemical constituents. The result showed that the Phytochemical constituent was Quercetin and apigenin. Also, Chen *et al*., (2019) determined phenolic compounds from *Sonchus oleraceus* by HPLC. After analysis the result showed that presence of seven major pheno-lic compounds namely, Chlorogenic Acid (CGA, 10.85mg/g), caffeic acid (3.95 mg/g), rutin (4.52 mg/g), astragalin (2.28 mg/g), isoquercetin (2.66 mg/g), quercetin (4.02 mg/g) and apigenin (2.52 mg/g).

(c) Dentification of bioactive non-polar compounds present in non-polar fractions (n-hexane fraction) of *Sonchus oleraceus* by GC/MS Analysis

Phytochemical analysis of plants, exploring the possibilities of complex using of raw materials, creation a new drug, it had recently got a significant relevance. It is due to the high efficiency of biologically active substances (BAS) of plant materials and their low toxicity. The separation and analysis of hexane extract components of *S. oleraceus* L plant was identified using the GC-MS method and they are given in table 4. Analysis of S. *oleraceus* L by the GC-MS method was found out the 36 compounds. For the *S. oleraceus* each of the bioactive compounds of hexane fraction has been identified it was found out the highest quantity of 9 Octadecenamide (CAS) 40.92%, 1 Hexacosanol 21.01%, 13 Docosenamide, (Z) 11.79%. Identified compounds have a wide range of biological properties. Especially higher hydrocarbons namely pentadecane, hexadecane, heneykozan, trykozan, Tetracosane, Fitola is diterpene acyclic alcohol and it is a precursor of vitamin E, K1 and it is preventive measure against the epoxide -induced breast cancer (Yu *et al*., 2005). Previous studies confirmed our results Ibrahim *et al.,* (2015) identified chemical constituents of the diethyl ether extract of *Sonchus asper* and *Sonchus oleraceus* by using GC-MS. Twelve compounds were identified from *Sonchus asper,* the main constituents were found to be Phytol 33.89% and trans-anethole 20.22% in *Sonchus asper.* While, eighteen compounds were identified from *Sonchus oleraceus*, among them Ethyl linoleate 43.05%, (E)-9-Octadecenoic acid ethyl ester 24.02% were found to represent the major constituents.

Table 5: Anti-proliferative activity of the *S. oleraceus* extract.

Fractions	Remarks % at 100 µg/ml		
	Human Colon Carcinoma HCT-116	**Human hepatocellular carcinoma** HepG2	**Human Caucasian breast adenocarcinoma** MCF-7
***n*- Hexane**	0	4.8	48.5
Ethyl acetate	82.6	46.3	80
Methanol	88	-	19.3
Aqueous	-	-	94.8

Biological studies

• *In vitro* cytotoxicity effects of the *S. oleraceus:* Cancer is a global health problem with high morbidity and mortality and poses both economic and psychological challenges (Moyad and Carroll, 2004). To find out the long-term cytotoxicity of the plant extracts on various cancer cell lines using the colorimetric MTT assay. It is known that different cell lines might exhibit different sensitivities towards an antiproliferative compound, so the use of more than one cell line is therefore considered necessary in the detection of antiproliferative compounds. The effects of hexane,

ethyl acetate methanol and water fractions of *Sonchus oleraceus* arial parts on human cancer cell lines: colon carcinoma (HCT116), Caucasian breast adenocarcinoma (MCF7) and hepatocellular carcinoma cell line (HePG 2) are present in table 5. The results showed that pronounced cytotoxic activity of cancer cells in water fraction in breast cancer (94.8%), while in Methanol fraction in Colon Carcinoma (88%) On the other hand, ethyl acetate fraction of the *Sonchus Oleraceus* showed the highest percentage of colon cancer cells (82.6%) and breast cancer (80%), while the ethyl acetate fraction in hepatocellular carcinoma cell line showed moderate effect (46.3%). While methanol fraction and water fraction did not show anticancer effects against HepG2 and colon cancer.

Hence, it can be revealed that water fraction of *S. oleraceus* plant has promising anti-cancer bio- efficacy and must have to contain some chemical moiety which might be responsible for observed beneficial effect. This anticancer activity due to presence of major chemical constituents in the extract such as 9 Octadecenamide and 13 Docosenamide, (Z) which reported for their anticancer activity (Phutdhawong *et al*., 2004 and Komiya, 1999). The extract of this plant has been recognized to contains compounds capable of therapeutic properties including anti-inflammatory, antitumor, antihypertensive, antihyperglycemic, immunomodulatory as well as antidiarrhea and diuretic. The structures of the bioactive fatty acid's amide are shown in table 4. According to the recent studies polyphenol compounds have anticancer properties. Ahmida *et al*., (2018) and (Bin Sayeed and Ameen, 2015) showed that consumption of polyphenols such as rutin (a kind of flavonoids) and b-sitosterol (a kind of sterols) are the major components of *S. oleraceus*. Rutin modulates multiple biological functions with anticancer antiviral, anti-bacterial and anti-inflammatory activities due to its appreciable free radical-scavenging and antioxidant capacities.

Conclusion

The present study confirmed that the high availability and valuable bioactive compounds in *S. oleraceus* L with wide range of medicinal and biological effects as anticancer activities. Thus, ethyl acetate fraction of *S. oleraceus* L may be profitable in styling new protocols for cancer diseases. Further *in vivo* studies are desired to investigate their active components mechanism of action.

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