



Full Length Research Paper

Effect of *Apium graveolens* extract on Rat Liver Mitochondria, Cytosolic and Microsomal Fractions *In-Vitro*.

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Abstract

Different organic solvents of *Apium graveolens* extract were determined for their antioxidant activity by applying: DPPH (1,1 diphenyl-2-picryl hydrazyl) assay at different concentrations "25, 50, 100, and 200 µg/ml"; Fe²⁺/Ascorbate induce lipid peroxidation in isolated rat liver mitochondria, microsome, and cytosolic fractions by two concentrations "0.5 and 1.0 mg"; hydroxyl radical scavenging (OH[•]) activity at "50, 250, and 500 µg/ml" concentrations; total phenolic contents, and total flavonoids were examined in each extract against two standard antioxidants "Silymarin & Rutin". Also, GC mass was determined. The results revealed that the antioxidant activity of ethyl acetate extract proved to be of higher activity than other extracts as compared to high dose, followed by the chloroform and the methanolic extracts in DPPH scavenging assay as compared to Rutin as standard antioxidant. It was observed that the high concentration of Rutin showed highly percentage of inhibition for DPPH assay, hydroxyl radical, and Fe²⁺/Ascorbate in microsomal > cytosolic > mitochondrial fractions. The chloroform extracts revealed highly antioxidant activity at the concentration of 1.0mg which exerted higher reducing power on lipid peroxidation of rat liver mitochondria, microsome, and cytosolic fractions as compared to the high dose, the inhibition percentage of Fe²⁺/Ascorbate for lipid peroxidation revealed variable activity according to the concentration "dose dependent" and the organic solvent of each extract "type as solvent". Hydroxyl radical (OH[•]) scavenging activity, total phenolic and total flavonoids contents of methanolic extract appeared to be of high values, the ethyl acetate and chloroform extracts were less. The total phenolic contents of different extracts were dependent on each solvent. In addition, GC/MS analysis of the plant extract was identified to look for the active constituents which have an antioxidant activity. It was obviously that the major components of *Apium* extract appeared to be limonene by 50.5% and α - phellandrene by 21.4%. It was concluded that the antioxidant activity of *Apium* extract could be attributed to their higher phenolic contents and the total flavonoids. The inhibitory effect was dose-dependent and type of extract. Also, the activity was dependent on the cell fraction.

Keywords: *Apium graveolens*, DPPH, Hydroxyl radical; Fe²⁺/Ascorbate, Total phenolic and flavonoids contents, volatile oil and GC / GC-MS.

Introduction

Antioxidants are compounds that protect biological system against the potentially effects of processes of reactions that generate reactive oxygen species, several antioxidant dietary compound classes have been suggested to have health benefits [21]. Evidences show that consumption of these products lead to decrease in various pro-inflammatory and/or oxidative stress biomarkers [42].

Apium graveolens exerted an antioxidant effect [34]; hepatoprotective activity [38], antitumor and anti-inflammatory effects [35], and hypolipidemic effect [28] some of these are widely used in food. Three new triterpenoids and two new flavonoids, apigenin and chrysoeriol were isolated from the whole plant of fresh celery (*Apium graveolens*), together with 10 known flavonoids. The inhibitory effects of the compounds isolated on nitric oxide production in lipopolysaccharide-activated macrophages were evaluated [46].

Silymarin is a flavonolignan complex, silibinin is the most active and abundant constituent present in *Silymarin* [37]. In both animal and cell culture stress have shown that *Silymarin*, a naturally occurring polyphenolic flavonoids antioxidant, prevents anticancer effect against skin cancer by scavenging free radicals and reactive oxygen species (ROS) and strength antioxidant system [37].

Rutin is a well known a plant containing flavonoids, which exhibit multiple pharmacological activities including: Antibacterial, antiviral, and anti-inflammatory and it's a source of quercetin flavonoids [34]. The antioxidant effect of *Rutin* as a polyphenolic flavonoid compound was investigated by [21].

Several natural antioxidant flavonoids exerted antioxidant properties and were reported to prevent the development of free radical induced diseases. Therefore, antioxidants are in use therapeutically for treatment of more than one hundred diseases [24]. In addition, plant extracts with antioxidant activities are traditionally used to strengthen the natural immune defenses [16].

The aim of this work was to determine the antioxidant activities of *Apium graveolens* extract by different organic solvents in different cell particles "mitochondrial, microsomal, and cytosolic fractions" of rat liver against two antioxidant standards "*Rutin and Silymarin*". Identification of the active ingredients of the extract by GC mass was investigated.

Materials and Methods

Materials

All chemicals, solvents and reagents were used in analytical and pure grade. DPPH was purchased from Fluke chemical company (Switzerland). *Rutin and Silymarin* as antioxidant standard antioxidants were purchased from Sigma chemical Co., St. Lewis, USA.

Plant preparation

Plant sample was authenticated via plant herbarium of Orman Garden, Ministry of Agriculture. Fresh aerial parts of *Apium graveolens* was purchased from local market and washed several times in distilled water, it was cut into small pieces, cleaned, and dried on lyophilizer. The dried leaves of the plant were grinded and extracted by soaking in methanol 96%, then the solvent was filtered and then evaporated under reduced pressure and lyophilized (-50°C) [24], also, other organic solvent such as: chloroform, and ethyl acetate were used at the same way. The residual materials was kept and stored at -20°C until used for further study [11, 2].

Determination of total volatile oils by hydro distillation

The essential oil was prepared from the fresh Aerial parts of *Apium graveolens* cultivated in Egypt (50g) by hydro distillation using the Egyptian Pharmacopeia (E.P.) apparatus. The distilled were extracted with distilled ether after saturated with sodium chloride. The ether extract were dehydrated over anhydrous sodium sulfate. Solvents were removed under reduced pressure at 20°C. The volatile constituents was packed in dark container and kept in refrigerator till analysis. Percentage yields determined according to [13].

Organoleptic Characters of oil

As: Odor (Spicy warm), Color (Yellowish to white liquid) and Taste (Sweet taste) this method according [13].

Physical characters of oil

Specific gravity (0.870), refractive indexes (1.4800 at 20°C), optical rotation (+48°) and solubility in alcohol (1ml of oil in 8ml 90% ethanol) according to [13], the percent of oil 4% (V/W).

Investigation of volatile oil

A) Gas Chromatography (GC). The GC analyses were carried out on an Agilent Technologies 6890 gas chromatograph, fitted with a HP1 30m x 0.25mm x 0.25µm film thickness capillary column and FID. The column temperature was programmed from 60 to 250 °C at initial rate of 10 °C/min. The injector and detector temperatures were programmed at 250 and 275 °C, respectively. Nitrogen was used as the carrier gas at a flow rate 1 ml/min. this method was determined according to [14, 30].

B) Gas Chromatography-Mass Spectrometry (GC-MS). The GCMS analyses were performed on the Agilent 5989B, VL MS Detector system operating in EI mode (equipped with a HP1MS 30m x 0.25mm x 0.25µm film thickness capillary column), using He (1 ml/min) as the carrier gas. The initial temperature of the column was 60 °C. The column was heated gradually to 280 °C with a 3 °C/min rate. Identification of the compound were compared with reference spectra in NIST library and Wiley library spectra and confirmed via retention indices of compound from literature from GC spectrum [1.29.39].

Cell fractionation Techniques of mitochondria, microsomal, and cytosolic fractions from rat liver

Ten male albino rats weighing between 200-250g were used to separate liver mitochondria (*in-vitro*). The rats were obtained from the animal house of the National Organization for Drug Control and Research (NODCAR), Egypt. The animals were kept under standard laboratory conditions of light/dark cycle (12/12h) and room temperature. Preparation was performed by using the method of [44], Rats were killed by decapitation after fasting for 24h and the liver tissues were quickly removed and cut into small pieces in 0.25 M sucrose buffer (pH 7.4) at 4°C and then homogenized with nine volume of sucrose buffer using Teflon homogenizer (CAT R₁₈).

Liver from rats were homogenized rapidly in ice-cold 0.25M sucrose and centrifuged at 700, 5000, and 57000xg/ 10, 10, and 60 min successively to obtain mitochondrial, microsomal, and cytosolic fractions. The microsomes and cytosolic fractions were washed with ice-cold 0.15M KCL and used for the fractionation techniques [25, 33]. The protein content was determined using [27].

Lipid peroxidation assay

Lipid peroxide formation was determined as thiobarbituric acid reactive substances (TBARS) according to the method of Fe⁺⁺/Ascorbate induce lipid peroxidation [31].

Free radical scavenging assay

The free radical scavenging effect of the three organic solvents of the extract was assessed by the discoloration of a methanolic solution of DPPH according to [5. 41].

Determination of Hydroxyl radical scavenging assay

It was determined according to the deoxyribose method of [36].

Determination of total phenolic contents

The concentration of total phenolic compounds were determined spectrophotometrically using the Folin-Ciocalteu reagent which is a mixture of phosphomolybdate and phosphotungstate used for the colorimetric assay of phenolic antioxidants and polyphenol antioxidants, according to [12. 18].

Determination of total flavonoids

Determination of the total flavonoid contents were done colorimetrically by using aluminum chloride solution. Standard curve was done using different concentrations of apigenin in methanol (six serial 2 fold dilution to give 100-3.2 µg/ml). 100µl of each extract (previously prepared) were added to a 96 micro well plate and then 100µl of 2% aluminum chloride solution in methanol. After 10min, their absorbance was measured using HP spectrophotometer at 415nm using methanol as blank and the concentration of total flavonoids was calculated [10. 20. 22].

Results

DPPH radical scavenging activity

Table (1) illustrated a significant increase in the concentration of DPPH radical due to the scavenging ability, the scavenging effect of *Rutin* and *Apium* extracts on the DPPH radical increased in the order 20% at low concentration (25µg/ml) to 93% at high concentration (200µg/ml) for Ethyl acetate extract as compared to *Rutin* 80% at low concentration to 90% at high concentration. The highest percent of inhibition was observed for Ethyl acetate extract were the lowest percent of inhibition was observed for chloroform extract as compared to *Rutin*.

Table 1. The Antioxidant activity of *Apium graveolens* extract in different organic solvents as: chloroform; methanolic and ethyl acetate on the free radical scavenging (DPPH) in comparable against *Rutin*.

Parameters	Yields	Free radical scavenging activity ^a "DPPH" decoloration % of I			
		25µg/ml	50µg/ml	100µg/ml	200µg/ml
<i>Rutin hydrate</i>	-----	80%	84%	86%	90%
<i>Chloroform extract</i>	0.997g	48%	70%	79%	90%
<i>Methanolic extract</i>	4.284g	20%	65%	85%	87%
<i>Ethyl acetate extract</i>	0.723g	20%	65%	90%	93%

N. B.: ^a (%): It was expressed as the absorbance of sample versus the absorbance of control, the highest % of I for high antioxidant power as compared against *Rutin*.

Fe⁺⁺/Ascorbate for lipid peroxidation

The results of *Rutin* on non-enzymatic peroxidation are shown in Table (2), the inhibition of lipid peroxidation of *Rutin* and *Silymarin* increased with increasing the concentration (0.5 and 1.0mg) as all other extracts of *Apium*. The percent of inhibition of lipid peroxidation by 0.5 mg & 1.0 mg/ml of *Rutin* and *Silymarin* was found to be 37%, 40% & 57%, 49%, respectively. The Cytosolic fraction was higher inhibition at the high concentration of (1.0mg/ml) than microsomal and mitochondrial fractions on chloroform extract as compared to *Rutin* and *Silymarin*

Table 2. The Antioxidant activity of *Apium graveolens* extract in different organic solvents as: chloroform; methanolic and ethyl acetate on Fe⁺⁺/Ascorbate in rat liver mitochondria, microsomes and cytosolic fractions in comparable against *Rutin* and *Silymarin*.

Parameters	Fe ⁺⁺ / Ascorbate in rat liver (% of I)					
	Mitochondrial fraction		Microsomal fraction		Cytosolic fraction	
	0.5mg/ml	1.0mg/ml	0.5mg/ml	1.0mg/ml	0.5mg/ml	1.0mg/ml
<i>Rutin hydrate</i>	37.0	57.0	78.3	80.6	52.3	77.8
<i>Silymarin</i>	40.0	49.0	86.1	90.1	50.2	69.1
<i>Chloroform</i>	42.0	69.0	65.2	70.5	88.6	92.3
<i>Methanolic</i>	29.0	40.0	32.4	52.7	44.2	55.7
<i>Ethyl acetate</i>	32.0	38.0	45.0	59.0	54.2	61.1

Fe⁺⁺/Ascorbate: the highest % of I for high antioxidant activity as compared to *Silymarin* and *Rutin*

The comparative study between the percentage inhibitions of all plant extracts on DPPH assay and the % inhibition in rat liver mitochondria microsomal, and cytosolic fractions

As revealed in table (3) that the high percent of inhibition on DPPH reaction for the ethyl acetate extract by 93%, chloroform extract by 90%, then the methanolic extract by 87%. On the other hand, the percent of inhibition on the activity of the extracts was altered in rat liver mitochondria Fe^{++} /Ascorbate reaction, the microsomal and cytosolic fractions. It was obviously that the chloroform extract exerted the lowest percentage by 69%, methanolic by 40% and ethylacetate by 38% for the mitochondrial fraction, while in the microsomal fraction were (71%, 53%, 59%) but the highest effect were at the cytosolic fraction by 92%, 56%, 61% respectively.

Hydroxyl radical scavenging activity

Table (4) shows the percentage of ($OH\cdot$) scavenging effect by *Rutin* and *Silymarin* in comparison with the effect of different extract of *Apium* at the concentrations of 50, 250, and 500 μ g/ml. The percentage of $\cdot OH$ scavenging effect of *Rutin* and *Silymarin* at the high concentration of 500 μ g/ml was found to be 89% and 93%, respectively, while the ratio at this concentration for the highest activity in methanolic extract was found to be 75%, ethylacetate extract was found to be 68%, and then chloroform extract was found to be 52%.

Total phenolic and total flavonoids contents

Table (4) show the total amount of phenolics (gallic acid equivalents from 5 μ g/ml to 100 μ g/ml gallic acid) was higher in methanolic extract by 89.5 μ g then in ethyl acetate by 11.2 μ g. The total flavonoids appeared to be high activity in methanolic then ethyl acetate and chloroform extracts. The highest content of flavonoids in methanolic extract appeared to be (105 μ g), and then ethyl acetate (30 μ g) and chloroform extract (8.5 μ g).

Table 3. Comparison of the Antioxidant activity of *Apium graveolens* extract in different organic solvents as: chloroform; methanolic and ethyl acetate between DPPH and Fe^{++} /Ascorbate in rat liver mitochondria, microsomes and cytosolic fractions in comparable against *Rutin* and *Silymarin*.

Parameters	Comparison between %I of DPPH and Fe^{++} /Ascorbate in rat liver mitochondria, microsomes, and cytosolic fractions			
	%I by DPPH at 200 μ g/ml	Mitochondrial fraction 1.0mg/ml	Microsomal fraction 1.0mg/ml	Cytosolic fraction 1.0mg/ml
<i>Rutin hydrate</i>	90	57	80.6	77.8
<i>Silymarin</i>	-----	49	90.1	69.1
<i>Chloroform extract</i>	90	69	70.5	92.3
<i>Methanolic extract</i>	87	40	52.7	55.7
<i>Ethyl acetate extract</i>	93	38	59.0	61.1

Table 4. Antioxidant activity of *Apium graveolens* extract in different organic solvents as: Chloroform; methanolic, and ethyl acetate on hydroxyl radical ($OH\cdot$), total phenolic and total flavonoids contents in comparable against *Silymarin* and *Rutin*.

parameters	^a Hydroxyl radical "I %" scavenging activities			^b Total phenolic contents μ g%	^b Total flavonoids μ g%
	50(μ g/ml)	250(μ g/ml)	500(μ g/ml)		
<i>Rutin hydrate</i>	83.0%	84.0%	89.0%	-----	-----
<i>Silymarin</i>	70.0%	85.0%	93.0%	-----	-----
<i>Chloroform extract</i>	11.0%	32.0%	52.0%	1.1 μ g \pm 0.1	8.5 μ g \pm 0.08
<i>Methanolic extract</i>	20.0%	38.0%	75.0%	89.5 μ g \pm 0.1	105 μ g \pm 1.05
<i>Ethylacetate extract</i>	12.0%	15.0%	68.0%	11.2 μ g \pm 0.1	30 μ g \pm 0.3
<i>Powder</i>	-----			101.8	143.5

N. B.: ^a (%): It was expressed as the absorbance of sample versus the absorbance of control, the Control (Without antioxidant) = 100% toxicity

^b: Data are expressed as mean \pm S.E., the results are given as mean of five measurements of percentage.

GC and GC/MS analysis for oil of *Apium graveolens*.

As revealed from GC in Table (5) and figure (1) that thirteen compounds are present in the extract; six of them have a relative percentage more than 2.0%, five compounds were identified as fewer components than 1.0% by their mass spectrum. The major components were Limonene (50.45 %) and α - phellandrene (21.38%).

Table 5. Chemical composition for volatile of *Apium graveolens* using GC and GC/MS.

No.	Retention time	RI	%	Compound name
1	0.56	934	2.12	α - pinene
2	1.03	973	0.99	β - pinene
3	1.23	999	21.38	α - phellandrene
4	1.65	1007	0.77	1,4 cineol
5	1.72	1022	50.45	Limonene
6	1.87	1025	4.55	1,8 cineol
7	2.03	1050	0.55	γ - terpinen
8	2.15	1086	2.58	Linalool
9	2.60	1105	0.56	α - fenchene
10	3.46	1137	0.79	Menthone
11	3.73	1153	3.29	Borneol
12	4.09	1165	8.78	Terpene 4-ol
13	4.22	1270	3.19	Bornyl acetate

RI: RI: Retention index/kovats index.

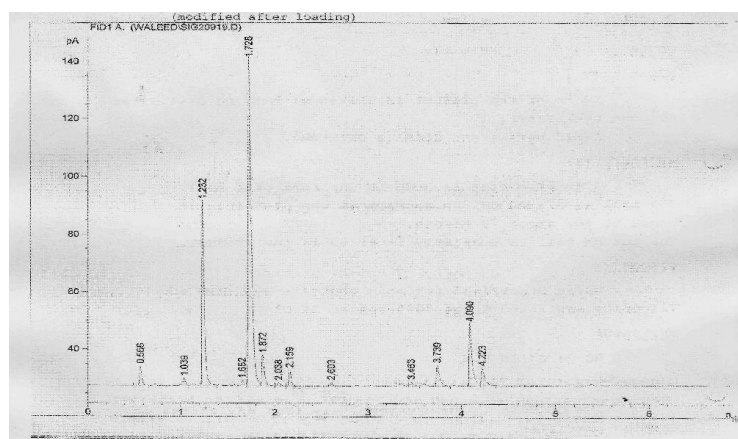


Figure 1. GC Chromatogram for volatile oil of *Apium graveolens*

Discussion

DPPH radical scavenging activity

The effect of antioxidant activity on DPPH radical scavenging is thought to be due to their hydrogen donating ability. DPPH is a stable free radical and accepts an electron or hydrogen radical to become a stable diamagnetic molecule. The reduction capability of DPPH radicals was determined by the decrease in its absorbance induced by antioxidants, it is visually noticeable as a discoloration from purple to yellow.

Rutin exhibited a high scavenging effecting toward DPPH radicals, while chloroform extract exhibited similar effect on the activity as compared to *Rutin* [8], these results indicated that *Rutin* has a noticeable effect on scavenging free radicals. Free radical scavenging activity was also increased with an increasing of the concentration [36]. These data clearly indicated that *ethylacetate* is a powerful free radical inhibitor or scavenger as compared to *Rutin* at the high dose (200 μ g/ml). Also, the resulting inhibition at high dose were 90% of *Rutin* and *Apium*, these results were in agreement with [46].

Fe^{++} /Ascorbate for lipid peroxidation

The reducing capacity of a compound may serve as a significant indicator of its potential antioxidant activity. There is a direct correlation between antioxidant activities and reducing power of components [45]. Measurements of reductive ability of $Fe^{+++} \rightarrow Fe^{++}$ transformation at the presence of *Rutin* was investigated by the method of [6]. Higher absorbance of the reaction mixture indicates greater reducing power. The reducing power of *Rutin* displayed a concentration-dependent manner. Lipid peroxides are likely involved in numerous pathological events, including inflammation, metabolic disorders and cellular aging [3]. As indicated from Table (2) that the antioxidant activity of the extracts on the sub-cellular fractions by percentage inhibition appeared to be variable for the three organic solvents of *Apium*. The activity was higher at the high dose than the low dose, also the cytosolic fraction exerted a highly values of inhibition then the microsomal and mitochondrial fractions. As well as, the chloroform extract revealed a highly activity than ethyl acetate and methanolic extract, this may be due to the active ingredient and the flavonoids and the total phenolic compounds as approved by [26. 40]. These results indicated that the plant extract was found to be dose-dependent; these findings are in agreement with the results of [43].

The comparative study between the percentage inhibitions of all plant extracts on DPPH assay and the % inhibition in rat liver mitochondria microsomal, and cytosolic fractions showed a variable effect, this may be due to the penetration of the active

ingredients of plant extracts through mitochondrial and other fractions membrane, also due to the active constituents of each extract [26].

Hydroxyl radical scavenging activity

The hydroxyl radical (OH[·]) in the cells can easily cross cell membranes at specific sites, react with most biomolecules and further more cause tissue damage and cell death. Thus, removing (OH[·]) is very important for the protection of living systems [43]. As indicated from these results that the inhibitory effect of the extract was dose dependent, these results were similar to the results reported by [9].

Total phenolic and total flavonoids contents

The total amount of phenolics (gallic acid equivalents from 5µg/ml to 100 µg/ml gallic acid) was high in methanolic extract (89.5 µg) then in ethyl acetate (11.2 µg), the estimated values were in good agreement with the data reported by [4, 46]. It was approved that the estimated values of the total phenolic contents in different extracts was dependant on the type of the plant extract. These results were in agreement with [7]. *Rutin* contains quercetin flavonoids [32], as well as, *Silymarin* containing a flavonolignan complex; silibinin is the most active constituent present in *Silymarin* [37]. These data clearly indicate that *Rutin* is a powerful free radical inhibitor or scavenger, this result are in agreement with the results of [43]. There is a correlation between antioxidant activity and phenolic content of extracts obtained from various natural sources [26, 40]. It was reported that the solvent may also be important in the antioxidant activity of the extract, phenolic and flavonoid contents of an *endophytic xylaria* sp. were higher in methanol extracts than in hexane extracts [26].

The highest content of flavonoids in methanolic extract appeared to be (105µg) then ethyl acetate (30µg) and chloroform extract (8.5 µg). These results revealed that the total flavonoid contents were changed according to each extract and plant species. It was found that phenolics substances such as flavonoids displayed strong antioxidant capacity (AC) [16, 17].

Conclusion

In the present study, it was found that different organic solvents extracts of *Apium graveolens* has different variability in the antioxidant activity. These effects were dose and organic solvent dependent. Also, it was indicated that the antioxidant activities were attributed to the phenolic contents and other factor such as the functional groups as well as the position of hydroxyl groups. Therefore, it is suggested that further work should be performed on the mechanism of the antioxidant capacity (AOC) of *Rutin*, *Silymarin* and the active ingredients of the antioxidants extract from the tested plant.

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