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Evaluation of antimicrobial activity, total phenolic compounds , antioxidant activity and nutritional value of fresh spinach (*Spinacia oleracea*)extracts.

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ABSTRACT

Antimicrobial activity, total phenolic compounds(TPC) and total antioxidant activities (TAA) of three different spinach extracts (Spinacia Oleracea) were determined.The antibacterial activities of spinach different extracts (with aqueous ethanol(50%) and ethanol(100%) as polar and petroleum ether as non polar solvents) via diameter of inhibition zones (mm) against the Gram positive test strains Bacillus cereus, Listeria monocytogens and Staphylococcus aureus and Gram negative test strains Salmonella typhi, Escherichia coli O157:H7 , were investigated. Also, antifungal activities of the different extracts were carried out against Aspergillus niger and Saccharomyces cerevisiae. Results revealed that the aqueous ethanol(0.277g/5ml crude extract) as polar extract (100%) showed the highest antibacterial activity against Staphylococcus aureus. Meanwhile, the polar and non polar spinach extracts appeared to have no antibacterial activity against the test strains of Bacillus cereus and Listeria monocytogens. Also, Results revealed that only the non polar petroleum ether(0.100g/5ml) spinach extract have the antibacterial activities against both of the test strains Salmonella typhimurium and Escherichia coli O157:H7. The antifungal activities of spinach extracts revealed that the aqueous ethanol(0.277g/5ml) and ethanol(0.3021g/5ml) as polar extracts (100%) showed antifungal activities against Saccharomyces cerevisiae and Aspergillus niger. Levels of TPC ranged from 6.9 to 112.5 mg of Gallic acid equivalent per gram extract .The highest content in TPC was found in ethanol extract of S.oleracea .Antioxidant activity ranged from 31.1% to 63.7% . The highest value of TAA was found in aqueous ethanol extract (50%) . The proximate analysis showed that S.oleracea examined contained a high level of moisture with low fat content and crude fiber. Results indicate that spinach can be used as potential source of natural antioxidants and antimicrobials agent .

Keywords: Antimicrobial activity , spinach, phenolic , antioxidants ,proximate composition

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INTRODUCTION

A renewed interest has occurred in the last decade to search for photochemical of native and naturalized plants for pharmaceutical and nutritional purposes with the recognition that plant-derived products have great potential as sources for pharmaceuticals and food additives. Spinacia oleracea in an annual herb belongs to the family Chenopodiaceae and it is widely distributed, cultivated in India [1] or Basella rubra L. as "Indian spinach"[2] known as “Spinach”. It is native to South-West Asia and cultivated throughout the world as vegetable. It is a rich source of vitamins A, C, E, B6, B2 and minerals such as magnesium, manganese, iron, calcium, potassium, and low levels of proteins and carbohydrates[3]. Spinach is also packed with a number of antioxidants components like polyphenols, flavonoids and carotinoids which are shown to possess anti-inflammatory effects, anti-mutagenic potential, antineoplastic effects, as well as chemo-preventive activities[4, 5].Spinach as Basella rubra contained photochemicals as tannins and alkaloids which have been found to possess antimicrobial activity against some organisms [6,7].

The increasingly high numbers of bacteria that are developing resistance to classical antibiotics drive much of the current interest on natural antimicrobial molecules in hope that they may provide useful leads into anti-infective drug candidates. Food borne pathogenic bacteria as Salmonella, Escherichia coli, Listeria monocytogens, Bacillus cereus, and Staphylococcus aureus causing food borne disease continues to be a common and serious threat to public health [8] and found in diarrhea cases and different foods in Egypt [9,10,11]. The potential of developing a new antimicrobial from plants are rewarding towards different uses for the benefit of mankind [3].

Antioxidants are substances that prevent or delay oxidative damage of lipids, proteins and nucleic acids caused by reactive oxygen species as well as free radicals. The most well known antioxidant constituents of fruits and vegetables, which may play the role of prevention and protection, are vitamins C and E, carotenoids, minerals (selenium and zinc), some peptides and phenolic compounds [12,13]. The antioxidants obtained from plants are of greater benefit in comparison to synthetic ones [14]. Synthetic antioxidant like butylated hydroxyl toluene (BHT), butylated hydroxyanisole (BHA), are known to ameliorate oxidative damage. They are widely used in the food industry due to their abilities to prevent food deterioration and to extend the shelf life of foods [15] but they have been restricted due to their carcinogenic and harmful effect on the lungs and liver [16]. Several data have revealed their high antioxidant capacities and their health promoting effects. Indeed, phenolic compounds have been reported to inhibit the development of cancerous tumours and to have anti-bacterial, anti-viral, anti-inflammatory, antispasmodic and anti diarrhoeic properties ([17,18]. S.oleracea is known to be rich in flavonoids, phenolic acids and pigments such as lutein and chlorophyll which are also antioxidants [19**]**. It is a very good source of dietary fiber, protein and Omega -3-fatty acids, zinc and vitamin $B_{1,}$whilethis mixture of conventional nutrient gives spinach a unique status in the antioxidant and anti-inflammatory department [20].

The present study was carried out to evaluate the antimicrobial activity, total phenolic compounds , antioxidant activity and nutritional value of polar and non-polar extracts of Spinacia oleracea, as spinach retailed in Cairo market, on the most common food borne illness bacteria and some moulds.

MATERIAL AND METHODS

Materials

Fresh samples of spinach (Spinacia Oleracea) were purchased from local supermarket , The vegetables were randomly sampled from the shelf.

Chemicals

All chemical and solvents were purchased from Sigma Chemicals Company (USA).

Preparation of different extracts

The homogenized sample of the aerial parts of spinach was weight and washed by running tap water, then cutting into small pieces. Sample of the prepared aerial parts of spinach was placed in a continuous

extraction apparatus (soxhlet) and subjected to extraction using petroleum ether (40-60 ^{o}C).Another two samples of aerial parts of spinach were subjected to extraction in soxhlet apparatus using ethanol or aqueous ethanol (50%).The solvent of each extract was completely removed by evaporation under reduced pressure at a temperature not exceeding 40^{0}C. All extracts were kept in deep freeze till used [21].

Methods

Determination of antimicrobial activity

The antimicrobial activity of vegetable's extract was determined by the agar well diffusion method [22]. The five pathogenic indicator bacteria strains were obtained from the stock cultures of the Dairy Microbiological Lab. , National Research Centre , Cairo, Egypt . Escherichia coli 0157 : H7 ATCC 6933 , Bacillus cereus ATCC 33018 , Staphylococcus aureus ATCC20231, Salmonella typhimurium ATCC 14028 , Listeria monocytogenes ATCC 7644 ,Saccharomyces cerevisiae and Aspergillus niger . Each strain was activated in Tryptone soy broth by fermention at 37C^{0} for 24 h. One ml culture of the activated indicator strain (104 Cells/ml) was inoculated into 20 ml of Mueller- Hinton agar (Becton Dickinson , USA) and poured in petri dishes .After solidification of the agar , wells of 5 mm in diameter were cut from the agar with a sterile borer and 50 µL of extract delivered in each well.

Control negative were sterile phosphate buffer. All tests were carried out in triplicates . The plates were incubated at 37 C^{0} for 24 h.

The antimicrobial activity was expressed as the diameter of the zone of inhibition (ZOI); whereby a diameter > 1mm around the well was considered as a positive result and the greater the diameter of the ZOI, the higher is the antimicrobial activity. The % inhibition was calculated according to National Committee for the Clinical Laboratory (NCCLS).

The zone diameter of wells cut in nutrient agar medium was 5.0 mm and the diameter of inhibition zone (DIZ) of negative a control for each bacterium was also 5.0 mm. If the DIZ value is 5.0 mm, that means the sample has no inhibitory activity against that bacterium.

Antibiotic assay as control positive

Muler Hinton Agar and Nutrient agar were used for agar well diffusion assay. Amoxicillien (10 mg. for gram +ve), Gentamycien (10 mg. for gram –ve) and Fluconazole (10 mg. for moulds) were used as positive control [23].

Determination of total phenolic compounds in extracts

Total phenolics were determined colorimetric in the extracts using Folin-Ciocalteu reagent [24]. Absorbance was measured at 765 nm using UVPC spectrophotometer. The total phenolic content was expressed as gallic acid equivalent (GAE) in milligrams per gram extract.

Evaluation of Antioxidant Activity

Antioxidant activities of different extracts were carried out using thiocyanate method [25]. Extracts (4 mg) were added to a solution mixture of 4.1 ml linoleic acid (2.52% in absolute ethanol), absolute ethanol (4 ml) and 0.05 M sodium phosphate buffer (pH 7, 8 ml). Distilled water 3.9 ml was added to the mixture. The solution was incubated at 40°C and the degree of oxidation was measured according to the method of [26] where 9.7 ml of ethanol (7.5%), 0.1 ml of an aqueous solution of ammonium thiocyanate (30%), 0.1 ml of sample solution and 0.1 ml of ferrous chloride solution (20 mM in 3.5% HCl) being added sequentially. The mixture was stirred for 3 min. The absorption values of mixtures were measured on the seventh day of incubation at 500nm. A control was performed with linoleic acid but without the extract. BHT (4 mg) was used as positive control. The maximum peroxidation level observed at 7 days of the control was used as a test point. The percent inhibition of linoleic acid peroxidation, 100 – [(Absorbance of sample at the seventh day/Absorbance of control at the seventh day) X 100] was calculated to express antioxidant activity.

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Evaluation of nutritional values

The Proximate composition of the composite S.oleracea were determined according to the methods of [27] , the total carbohydrate content were calculated by difference , the energy value(Calarific value) was determined as kcal per 100g of composite plant by multiplying protein and carbohydrates contents by 4.0 and fat content by 9.0 [28].

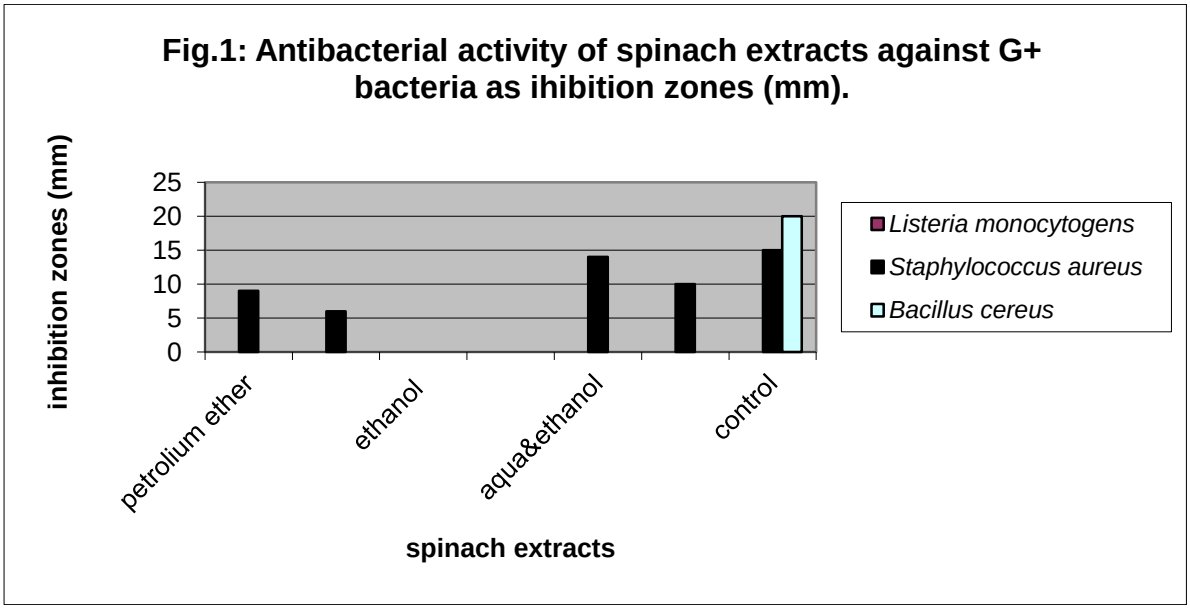
RESULATS AND DISCUSSION

The antibacterial activities of spinach extracts (with polar and non polar solvents) as the diameter of inhibition zones (mm) against the Gram positive test strains Bacillus cereus, Listeria monocytogens and Staphylococcus aureus were shown in Table (1).

Table 1: Antimicrobial activity of spinach extracts as inhibition zones (mm)

	Extract 1		**Extract 2**		**Extract 3**	
	100%	50%	100%	50%	100%	50%
Gram positive bacteria						
Listeria monocytogens	0	0	0	0	0	0
Bacillus cereus,	0	0	0	0	0	0
Staphylococcus aureus	9	6	0	0	14	10
Gram negative bacteria						
Salmonella typhi	8	6	0	0	0	0
Escherichia coli O157:H7	8	6	0	0	0	0
Moulds			0			
Saccharomyces cerevisiae.	0	0	6	0	7	0
Aspergillus niger	0	0	11	0	0	0

Extract 1: petroleum ether (0.1005g/5ml), Extract 2: ethanol (0.3021g/5ml), Extract 3: aqueous ethanol 50% (0.277g/5ml)= 100%.
50% extract = extract with distilled water (1:1)

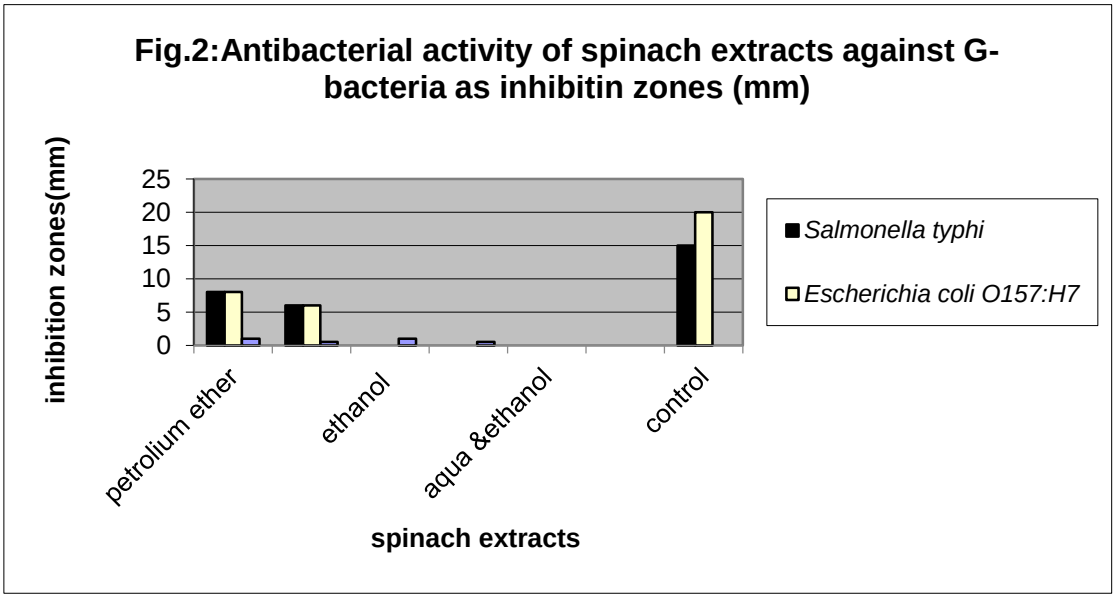


Results revealed that the aqueous ethanol as polar extract (50%) showed the highest antibacterial activity against Staphylococcus aureus. The non polar extract (100%petroleumether)showed lower activity, while ethanol (100% and 50%) extract showed no effect. Meanwhile, the polar and non polar spinach extracts appeared to have no antibacterial activity against the test strains of Bacillus cereus and Listeria monocytogens. Similar antibacterial activity of aqueous and ethanol extracts of spinach was shown against Staphylococcus

aureus zone of inhibition, whereas the minimum activity is reported against the Bacillus subtilis**,** respectively[23].Also, [29]found moderate significant of ethanolic fresh spinach extract again Staphylococcus aureus.

The potential antibacterial activity of the spinach different spinach extracts against the Gram positive bacteria in comparison with the antibiotic Amoxicillin (10 mg) was shown in Figure (1).

Results reveal that aqueous ethanol and petroleum ether extracts potentiate about 93% and 60% of Amoxicillin (10 mg) against Staphylococcus aureus. However, the higher the potential was gained by [23]for spinach ethanol extract against Staphylococcus aureus for the same antibiotic. The antibacterial activities of spinach extracts (with polar and non polar solvents) as the diameter of inhibition zones (mm) against the Gram negative test strains Salmonella typhimurium and Escherichia coli O157:H7 were shown in Table (1). Results reveal that only the non polar petroleum ether spinach extract have similar antibacterial activities against both of the test strains Salmonella typhimurium and Escherichia coli O157:H7, while aqueous and ethanol extracts showed no antibacterial activities against the two test strains. Similar results were obtained by [3], who found that spinach water and ethanol extracts showed very low antibacterial activity against Escherichia coli, while the non polar extract petroleum ether showed higher activity than the obtained one. Also**,** [3]reported the higher the antibacterial activity of petroleum ether spinach extract against Escherichia coli. However, the current results for the polar extracts contradict results found by[23]**,** who reported he antibacterial activity of spinach aqueous and ethanol extracts against Salmonella colerassius and Escherichia coli. The potential antibacterial activity of the different spinach extracts against the Gram negative bacteria in comparison with the antibiotic Gentamycien (10 mg) was shown in Figure (2).



Results reveal that aqueous ethanol and petroleum ether extracts potentiate about 40% and 53% of Gentamycien (10 mg) against Escherichia coli and Salmonella typhimurium, respectively. However, the higher the potential was gained by[23]for spinach aqueous and methanol extracts against Salmonella colerassius and Escherichia coli for the same antibiotic.

The antifungal activities of spinach extracts (with polar and non-polar solvents) as the diameter of inhibition zones (mm) against the mould test strains Saccharomyces cerevisiae and Aspergillus niger were shown in Table (1). Results revealed that the aqueousethanol and ethanol as polar extracts (100%) showed antifungal activity against Saccharomyces cerevisiae and Aspergillus niger. Higher antifungal activities were shown by [23]using aqueous and methanol spinach extracts against Aspergillus niger, Penicillium crysogenum and Candida albicans . Similar antimicrobial activity had been reported by[30].The potential antifungal activity of the different spinach extracts against Saccharomyces cerevisiae and Aspergillus niger in comparison with the antibiotic Fluconazole (10 mg) was shown in Figure (3).

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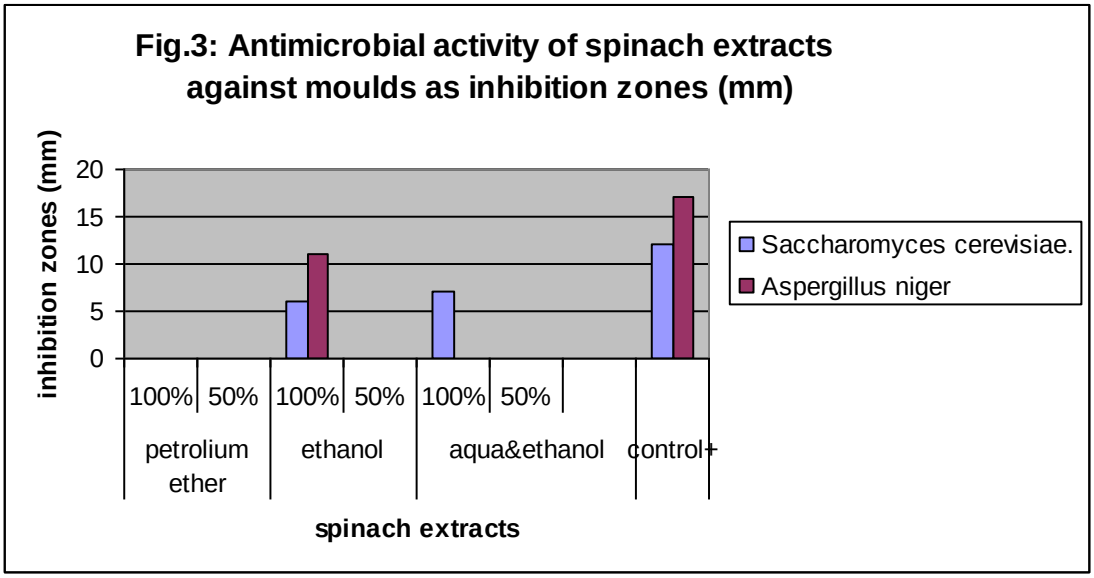

Table (2) Total phenolic contents (TPC) of fresh S. Oleracea

Extract	Total phenolic compounds (mg GAE /g extract)
Petroleum ether extract	$67.9 \pm 4.399^{*}$
Ethanol extract	112.5 ± 4.240
Aqueous ethanol extract (50 %)	87.5 ± 2.053

***Each value is the average of three replicates ± SD.SD = Standard deviation**

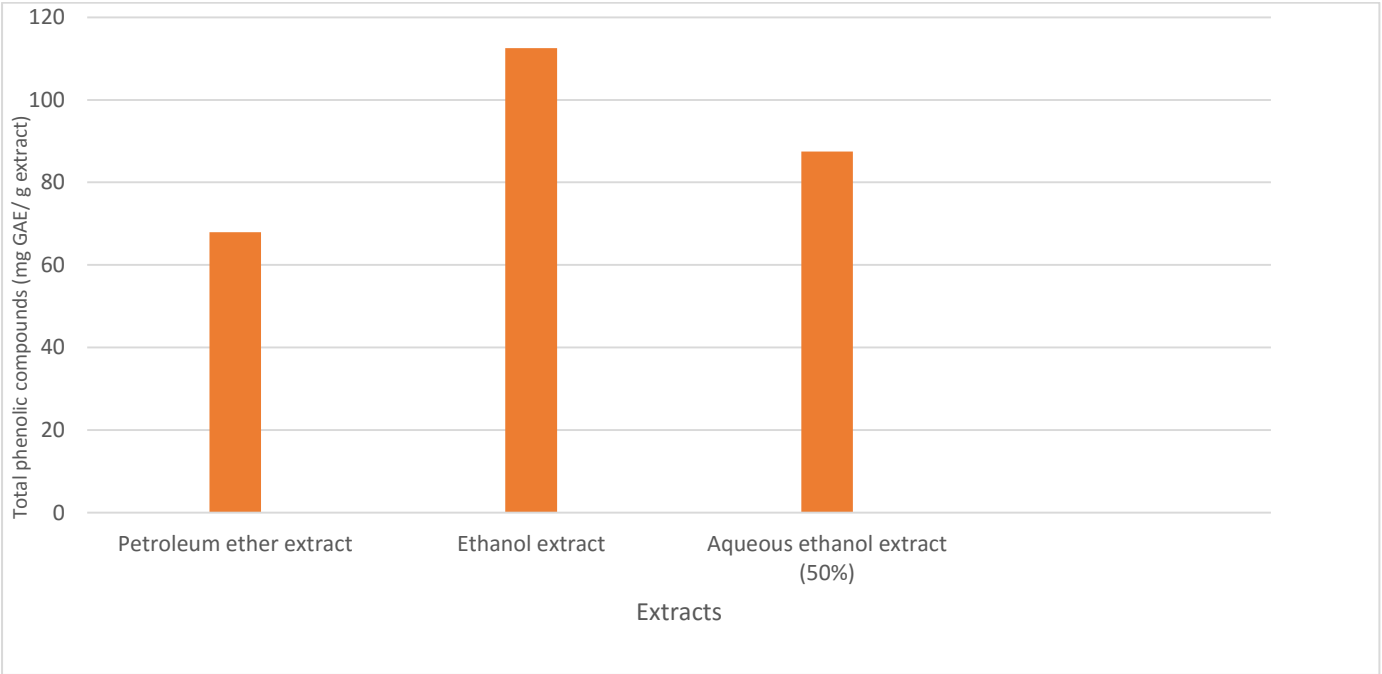

Fig. (4) Variation of TPC of different extracts of S. Oleracea

Results reveal that ethanol and aqueous ethanol extracts potentiate about 58% and 64% of Fluconazole (10 mg) against Saccharomyces cerevisiae and, Aspergillus niger, respectively. Similar potentials were gained by [23] for spinach aqueous extracts against Aspergillus niger, Penicillium crysogenum and Candida albicans,60%, 82% and 73%, respectively, but they gained the higher the potential of methanol extract, for the same antibiotic.The antimicrobial activities shown by leafextracts of Spinacia oleracea in

different extracts may be due to one or more of the Phytochemical constituents of the spinach as steroids, saponins, phenols, flavonids, alkaloids, tannins, carbohydrates, amino acids, glycosides, carbonyl and anthroquinone using the polar water and ethanol solvents, while the none polar petroleum ether extracted only the terpenoids, phenols and glycosides[3].Hence, the present work emphasize the states of searching new antimicrobials is very important in recent times considering the escalating levels of antibiotic resistance among pathogenic microorganisms[31].The total phenolic compounds in the different plant extracts ranged from 67.9 to 112.5 mg GAE/ g. as shown in Table (2) and figure (4).

The total phenolic compounds of ethanol extract showed highest value (112.2 mg GAE/g) followed by aqueous ethanol extract (50%) and it was (87.5 mg GEA / g). Petroleum ether extract contains considerably smaller value of phenolic compounds (67.9 mg GEA /g). In this respect [32,33,34]declared that the total phenolic compounds in plant extract of S.oleracea depends on the type of extract , i.e. the polarity of solvent used in extraction . High solubility of phenols in polar solvents provides high concentration of these compounds in the extracts obtained using polar solvents for the extraction. Antioxidant activity of the three different plant extracts from S.oleracea are shown in Table (3) and figure (5).

Table (3) Total antioxidant activity (TAA) of fresh Spinacia Oleracea

Extracts	Total Antioxidant Activity %
Petroleum ether extract	$31.1 \pm 0.451^{*}$
Ethanol extract	62.6 ± 0.225
Aqueous ethanol extract (50 %)	63.7 ± 0.390
BHT (standard)	65.6 ± 0.518

***Each value is the average of three replicates ± SD**
SD = Standard deviation

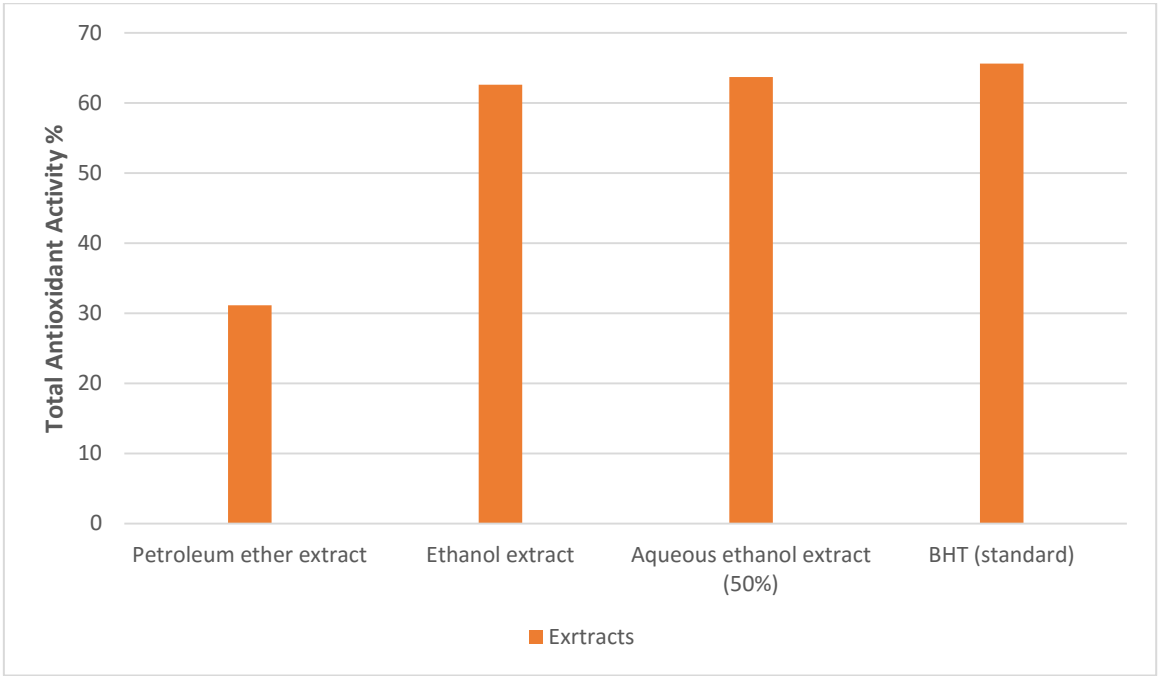

Fig. (5) Variation of TAA of different extract of fresh S.Oleracea

The synthetic antioxidant butylated hydroxyl toluene (BHT) as standard showed the highest antioxidant activity (65, 6%). The examination of antioxidant activities of S.oleracea extracts under study showed different values ranged from 31.1% to 63.7% . Aqueous ethanol extract (50%) showed the highest antioxidant activity (63.7%) , compared to petroleum ether extract (31.1%). A moderate antioxidant activity was found for ethanol extract (62.6%) . The lowest value of petroleum extract of S.oleracea due to low activity of different plant extract depends on the polarity of solvent used in the extract preparation [32,35].The extracts that perform the highest antioxidant activity as shown in Table (3) and Figure (5) have the highest concentration of phenolic compounds. Phenols are very important constituents because of their scavenging

ability on free radicals due to their hydroxyl groups. Therefore, the phenolic content of plants may contribute directly to their antioxidant action [32,36].

Table (4) presents the macronutrient contents of the plant included in the study . It could be observed that the fresh S.oleracea is characterized by high moisture content (88.05 %). Meanwhile the carbohydrate value was (4.17%) , protein content was (3.33%) , the ash value was (2.4%) , the fibre content was (1.8%). On the other hand the fat content was (0.48%) . The calculated energy value was 34.33 (kcal). Such given data in Table (4) are in general agreement with those previously reported by [37].

Table (4) Proximate analysis and energy value of fresh S.Oleracea (g /100g).

Fresh spinacea	Moisture %	Protein %	Ether extract %	Total Carbohyd. %	Crude Fibre %	Ash %	Energy value (kcal)
Per100 g	88.05	3.33	0.48	4.17	1.13	1.83	34.32

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