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Phytochemical and antioxidant studies of some green leafy vegetables consumed in Yemen as salad

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Abstract
In present study four green leafy vegetables which widely consumed in diet as salad in Yemen have been evaluated for antioxidative constituents and free radical scavenging activities. Phytochemical screening of 70% methanol leaf extracts of *Eruca sativa, Lactuca sativa* L, *Raphanus sativus* and *Allium porrum* was showed present carbohydrates, steroids, triterpenoids, polyphenols, flavonoids and alkaloids. All these extracts exhibited antioxidant activity in rapid screening by dot-blot assay and TLC analysis with DPPH. Free radical scavenging activities were ranged from 32.23±4.18% for *Allium porrum* to 91.33±4.01% for *Lactuca sativa*. Results of this study may confirm the nutrients, pharmacological properties and explain partially the uses of selected green leafy vegetables in prevention of many illnesses.

Keywords: Yemen, vegetables, constituents, antioxidant

Introduction
Plants are the good sources for foods and drugs. Phytochemicals are bioactive, non-nutrient plant compounds in fruits, vegetables, grains and other plant foods that have been linked to reducing the risk of major degenerative diseases. These non-nutrients or phytochemicals carry out their healing activities by combining with vitamins or with other nutrients [1, 2]. The usefulness of plant materials medicinally is due to the presence of phytochemical such as alkaloid, tannins, flavonoid and phenolic compounds [3]. They may offer a variety of health benefits such as antioxidant, antibacterial, anti-inflammatory or anticancer activity [4]. Recent researches into free radicals has confirmed that foods rich in antioxidants play an essential role in the prevention of cardiovascular diseases and cancers [5, 6, 7], and neurodegenerative diseases, including Parkinson's and Alzheimer's diseases [8]. Also Synthetic antioxidants have been widely used in food, however, some side effects were registered [9]. They are wonderful green-leafy vegetables often recognized as of the functional foods for its wholesome nutritional, antioxidants and anti-cancer composition. In this study, four green leafy vegetables including, *Allium porrum, Lactuca sativa, Raphanus sativus* and *Eruca sativa* used as salad by Yemeni people were selected to phytochemical analysis and evaluate antioxidant activity. Although fresh juices of Leek, lettuce, Horseradish leaves showed antioxidant activity toward lipid peroxidation in liver homogenate [10], present study was aimed to evaluate antioxidative constituents and free radical scavenging activities by using the stable radical DPPH.

Materials and methods
Collection and identification of plant material
The fresh leaves of studied vegetables were collected from the local market in Aden, Yemen on August 2016, dried in the shaded area and then manually grinded and stored at room temperature for further analysis.

Preparation of the extracts
Preparation of extracts was achieved by standard method [11, 12]. Thirty grams of each dried powdered leaves were successively extracted in a Soxhlet's apparatus with petroleum ether (boiling point 60-80 ºC), 70% methanol (80-90 °C) and water separately. The solvents in the extracts were removed by distillation and the concentrated extracts so obtained were further dried at a temperature not exceeding 40 ºC in water bath and then stored at 4°C in refrigerator till further use. The yield values were calculated.

Qualitative phytochemical analysis

Phytochemical screening was conducted to detect various classes of chemical constituents in the extracts of studied vegetables, using the procedures described previously for qualitative phytochemical screening [13, 14].

Antioxidant studies

Dot-blot assay for antioxidant

Rapid screening of antioxidant activity was performed by dot-blot and DPPH staining. Each diluted sample of the studied green leafy extracts was carefully loaded onto a 20 cm × 20 cm TLC layer (silica gel G 60 F254, thickness 0.2mm, Allugram- Germany) and allowed to dry (5 min). Drops of each sample were loaded, in order of decreasing concentration (0.50, 0.25, 0.125 and 0.062mg/ mL), along the row. The staining of the silica plate was based on the procedure. The sheet bearing the dry spots was placed upside down for 10 s in a 0.05% 2, 2-diphenyl-1-picrylhydrazyl (DPPH) methanol solution. Then the excess of solution was removed with a tissue paper and the layer was dried with a hair-dryer blowing cold air. Stained silica layer revealed a purple background with yellow spots at the location where radical-scavenger capacity presented. The intensity of the yellow color depends upon the amount and nature of radical scavenger present in the sample [15].

TLC analysis for phytochemical and antioxidant constituents

Drops of each sample were loaded on TLC plate (20 cm X 20 cm). The plates were developed in (solvent system) BAW (3:1:1) to separate different constituents and various spraying reagents were used. One plate sprayed with 0.05% DPPH reagent to give antioxidant constituents in the deferent extracts (fractions). The antioxidant constituents were analyzed by DPPH technique [16, 17]. For this 0.05% of DPPH solution in methanol was sprayed on the surface of developed TLC plate and incubated for 10 min in dark place at room temperature. The active antioxidant constituents of the extracts were detected in sunlight as yellow spots produced via reduction of DPPH by resolved bands against purple back ground on the TLC plate. Second developed plate, after air-dried, the spots were visualized by spraying with ferric chloride (2% in methanol) and the third developed plate was sprayed with Aluminum chloride (5% in methanol) to identify the respective compounds. Chromatograms were evaluated under UV light and in sunlight before and after derivatization with reagents. The colour of the spots was noted and Rf values were calculated [18, 19, 20].

DPPH radical scavenging activity assay

The free radical – scavenging activity of each extract was determined as described by Chan *et al*. with slight modification [21]. Different dilutions of the extract (20, 50, 70 and 100 µg/ml) were prepared. DPPH solution was also prepared by dissolving 6.0 mg of DPPH in 100 mL methanol. Then, 1 mL of extract from each dilution was added into the test tube containing 2 mL of DPPH solution. Control was prepared by adding 1 mL of methanol to 2 mL of DPPH solution. The mixture was shaken vigorously and was left to stand in the dark for 30 min. The absorbance of the resulting solution was measured spectrophotometric ally at 517 nm. The scavenging activity of extract on DPPH radical was calculated using the following equation:

$$\textbf{Inhibition \%} = [(A_0 - A_1)/A_0] \times 100;$$

A_0 i

s the absorbance of control and $A_1$is absorbance of test. Antioxidant activity of ethanolic leaf extract expressed as IC50 values and compared with standard. The 50% inhibition (IC50) of antioxidant activity was calculated as the concentrations of samples that inhibited 50% of scavenging activity of DPPH radical's activity under these conditions [22]. The data were presented as mean values ± standard deviation (n = 3).

Statistical analysis

Analysis of variance of data was evaluated by Student's t test P-values less than 0.05 was considered to be statistically significant.

Results and Discussion

Qualitative phytochemical analysis

Fruit and vegetables are rich sources of phytochemicals with many reported human health promoting benefits beyond basic nutrition. Epidemiological studies have shown that there may be significant positive associations between intake of fruits and vegetables or cereals and reduced rate of heart disease mortality, common cancers and other degenerative diseases as well as ageing [23]. Extractive value of 70% methanol extracts of selected green-leafy vegetables was determined and the result showed in the Table 1. Phytochemical Screening was showed that all studied extracts are rich source of Steroids, Triterpenoids, Polyphenols, Flavonoids, Alkaloids and Carbohydrates (Table 2, Figures 4 to 12).

Table 1: Percent extractives and colors of 70% methanol extracts of the studied green-leafy vegetables

S. No.	leaf vegetable	Weight (GM)	Percentage of Yield (%)
1	*Allium porrum* L	30	39.50
2	*Lactuca sativa* L	30	27.71
3	*Raphanus sativus* L	30	32.83
4	*Eruca sativa* Miller	30	42.36

Table 2: Results of phytochemical screening of 70% Methanol extracts of selected green-leafy vegetables

Phytochemical Screening		*Eruca sativa* Miller	*Lactuca sativa* L	*Raphanus sativus* L	*Allium porrum* L
Alkaloids	Wagner's test	-	-	-	-
	Mayer's test	-	-	-	-
	Dragendorff's reagent	++	+++	+++	++
Amino acid/ Protein	Ninhydrin test	-	-	-	-
Carbohydrates	Molisch's test	+	+	+	+
	Fehling's test	+++	+	++	+++
Saponins	Foam test	-	-	-	-

	Haemolysis test	-	-	-	-
Sterols/ Triterpenes	Salkowski test	++	++	++	++
	Liebermann-Burchard test	++	++	++	++
Polyphenols	Ferric chloride test	++	++	++	++
Tannins	Ferric chloride test	++	++	++	++
	Gelatin test	-	-	-	-
Flavonoids	Shinoda test	++	++	++	++
	Ammonia (10%) Test	++	++	++	++
	Lead acetate test	++	++	++	++
	Aluminium solution test	++	++	++	++

+++ = Most intense, ++ = moderately intense, + = Least intense, - = absent.

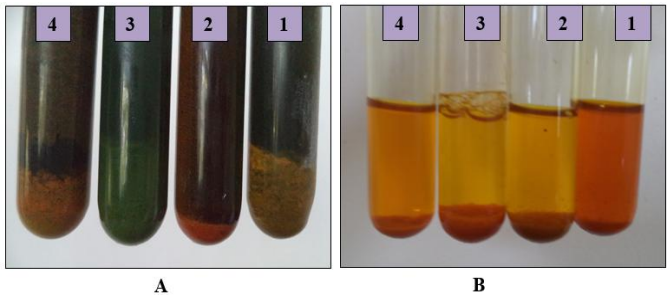

Fig 1: Test tubes with Fehling's test (A), Dragendorff's reagent (B) and 70% methanol extracts of the leaves of *Eruca sativa* (1), *Lactuca sativa* (2), *Raphanus sativus* (3) and *Allium porrum* (4).

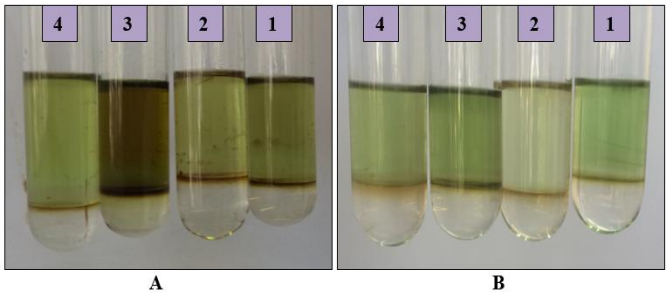

Fig 2: Test tubes with Salkowski Reactions (A), Liebermann-Burchard Reactions (B) and 70% methanol extracts of the leaves of *Eruca sativa* (1), *Lactuca sativa* (2), *Raphanus sativus* (3) and *Allium porrum* (4).

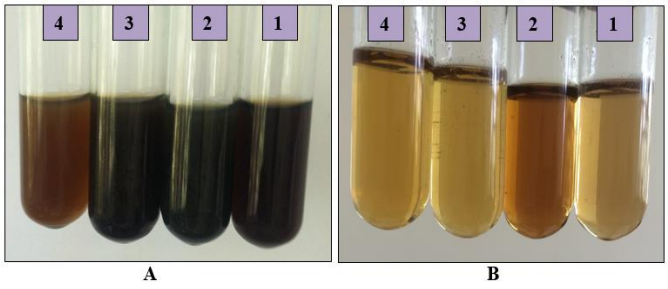

Fig 3: Test tubes with Ferric chloride (A), Shenoda Reactions (B) and 70% methanol extracts of the leaves of *Eruca sativa* (1), *Lactuca sativa* (2), *Raphanus sativus* (3) and *Allium porrum* (4).

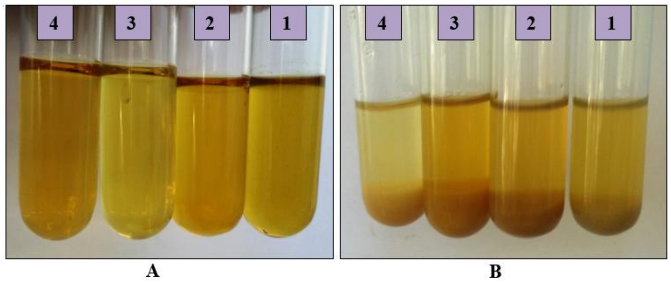

Fig 4: Test tubes with 1% Aluminum chloride (A), Lead acetate (2%) and 70% methanol extracts of the leaves of *Eruca sativa* (1), *Lactuca sativa* (2), *Raphanus sativus* (3) and *Allium porrum* (4).

Antioxidant studies

A great number of TLC techniques have been developed and successfully applied for qualitative and quantitative analysis of antioxidants [24], and the stable free radical 2, 2-diphenyl-1-picrylhydrazyl (DPPH) was often used as a derivatization reagent for this purpose [25]. Stained silica layer revealed a purple background with yellow spots at the location where radical-scavenger capacity presented. The intensity of the yellow color depends upon the amount and nature of radical scavenger present in the sample [15].

Dot-blot assay for antioxidant

The antioxidant potential activity of 70% methanol extracts of studied plant materials was determined by eye-detected semi-quantitatively by a rapid DPPH staining-TLC technique. Each diluted samples were applied as a dot on a TLC layer that was then stained with DPPH solution. Rutin was used as a positive control. Stained silica layer revealed a purple background with yellow spots at the location where radical-scavenger capacity presented (Figure 5). Methanol extracts of *Lactuca sativa* L at concentration of 0.50 mg /lm, 0.25 mg/ml, 0.125 mg/ml and *Raphanus sativus* L. at concentration of 0.50 mg /lm, 0.25 mg/ml showed higher scavenging activity, while *Eruca sativa Miller* and *Allium porrum L* showed weak scavenging activity when compared with control (rutin) at similar concentrations.

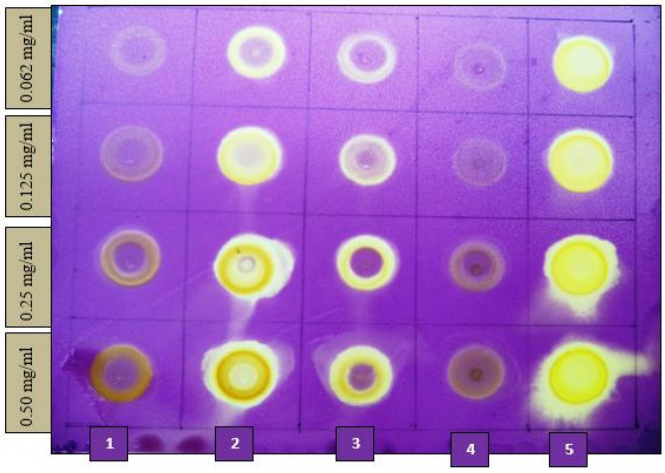

Fig 5: Dot blot assay of 70% methanol extracts of *Eruca sativa Miller* (1), *Lactuca sativa L* (2), *Raphanus sativus L* (3) and *Allium porrum L* (4) and Rutin (5) on a silica sheet stained with a DPPH solution. All dots at different concentration were applied

TLC analysis for phytochemical and antioxidant constituents

Thin Layer Chromatography (silica gel G 60 F254 TLC plates of layer thickness 0.2mm, Allugram- Germany) of prepared extracts was performed. Solvent systems such as Butanol – Acetic acid glacial–Water (4:1:5), Butanol –Acetic acid glacial–Water (3:1:1), Butanol –Acetic acid glacial –Water

(8:1:1), Ethyl acetate – Formic acid – Acetic acid glacial – Water (100:11:11:26) and others were tested to obtain best results. The best results were obtained with Ethyl acetate – Formic acid – Acetic acid glacial – Water (100:11:11:26), the *Eruca sativa* Miller extract revealed 3 spots, the *Lactuca sativa* L extract revealed 6 spots, the *Raphanus sativus L* revealed 6 spots and the *Allium porrum L* revealed 4 spots. Developed plates were visualized by spraying with various spraying reagents to find different compounds or classes present in the studied extracts. Plats developed in Ethyl acetate – Formic acid – Acetic acid glacial – Water (100:11:11:26); and sprayed by Ferric chloride showed that in methanol extract of *Lactuca sativa* L, *Raphanus sativus* L *and Allium porrum* L present 6, 6, and 4 spots respectively, which are represent phenolic compounds. With Aluminum chloride reagent, in methanol extract of *Lactuca sativa* L and *Raphanus sativus* L present 6, 6 spots respectively, which are represent flavonoids. With 0.05% DPPH, in methanol extract of *Lactuca sativa* L, *Raphanus sativus* L *and Allium porrum* L present 5, 3, and 1 spots respectively, which are represent antioxidant constituents. Rutin was used as reference compound. Phenolic compounds such as phenolic acid, flavonoid and tannin are commonly found in plants, and they have been reported to have multiple biological effects, including antioxidant activity [26, 27, 28]. Photos of the plates were taken in UV chambers before derivatization with reagents and in sunlight after derivatization, then Rf values were calculated (Table 3) and (Figures 6 to 8).

DPPH radical scavenging activity assay

The antioxidant activity of 70% methanol extract of selected green-leafy vegetables was determined. Antioxidants activity ranged from 32.23 ±4.18% for *Allium porrum* to 91.33 ±4.01% for *Lactuca sativa*. The decreasing order of antioxidant activity is *Lactuca sativa* > *Raphanus sativus* > *Eruca sativa*> *Allium porrum*. The percentage (%) scavenging of DPPH free radical was found to be concentration dependent, i.e. concentration of the extract between 20-100 µg/ml greatly increasing the inhibitory activity (Table 4) and (Figure 9).

Table 3: Observations of thin layer chromatographic studies of 70% Methanol extracts of selected green-leafy vegetables in Ethyl acetate – Formic acid – Acetic acid glacial – Water (100:11:11:26)

`Extract of / Compound	Rf	Colour of the spot before derivatization		Colour of the spot in daily light after derivatization with			Assigned substance
		At 254 nm	At 365 nm	ferric chloride reagent	Aluminum chloride reagent	0.05% DPPH reagent	
Eruca sativa	0.44	Light dark	Blue	-	-	-	
	0.55	Deep blue	Sky blue	-	-	-	
	0.66	Light dark	Blue	-	-	-	
Lactuca sativa	0.31	Light dark	Light blue	Light brown	Deep yellow	Light yellow	Flavonoid
	0.46	Light dark	Blue	Light brown	Deep yellow	Light yellow	Rutin
	0.55	Deep blue	greenish blue	Deep brown	Deep yellow	_	Phenol -
	0.66	Bark green	greenish blue	Brown	Deep yellow	Deep yellow	Flavonoid
	0.75	Light dark	Light blue	Brown	Deep yellow	Light yellow	Flavonoid
	0.88	Light dark	greenish blue	Brown	Deep yellow	Deep yellow	Flavonoid
Raphanus sativus	0.31	Deep dark	Light blue	Deep brown	Yellow	Light yellow	Flavonoid
	0.46	Deep dark	Blue	Deep brown	Deep yellow	Light yellow	Rutin
	0.55	Deep blue	greenish blue	Deep brown	Deep yellow	Light yellow	Flavonoid
	0.63	Bark green	Sky blue	Brown	Deep yellow	_	Phenol
	0.71	Bark green	Blue	Light brown	Deep yellow	_	Phenol
	0.76	Dark	Blue	Light brown	Deep yellow	_	Phenol
Allium porrum	0.31	_	Blue	Light brown	-	Light yellow	Flavonoid
	0.55	Deep blue	greenish blue	Light brown	-	-	
	0.66	Dark	Blue	Light brown	-	-	
	0.78	Dark	Blue	Light brown	-	-	
Rutin	0.46	Deep dark	Deep blue	Deep brown	Deep yellow	Deep yellow	Rutin

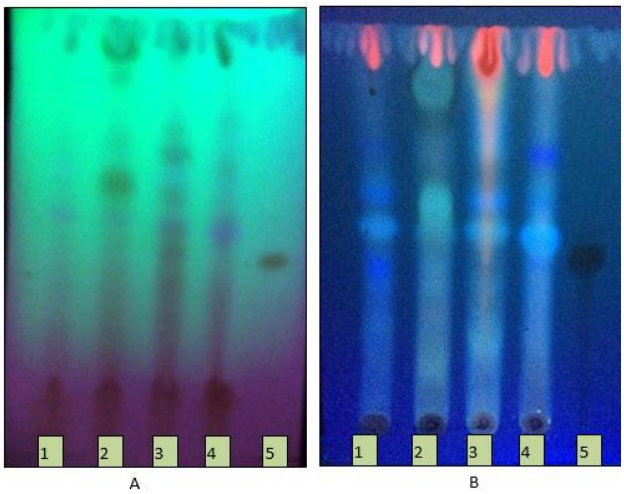

Fig 6: TLC plate of 70% methanol leaf extracts of *Eruca sativa Miller* (1), *Lactuca sativa* (2), *Raphanus sativus* (3), *Allium porrum* (4) and rutin (5) in EFAW (100-11-11-26): A- under UV 254 nm, B- under UV 365.

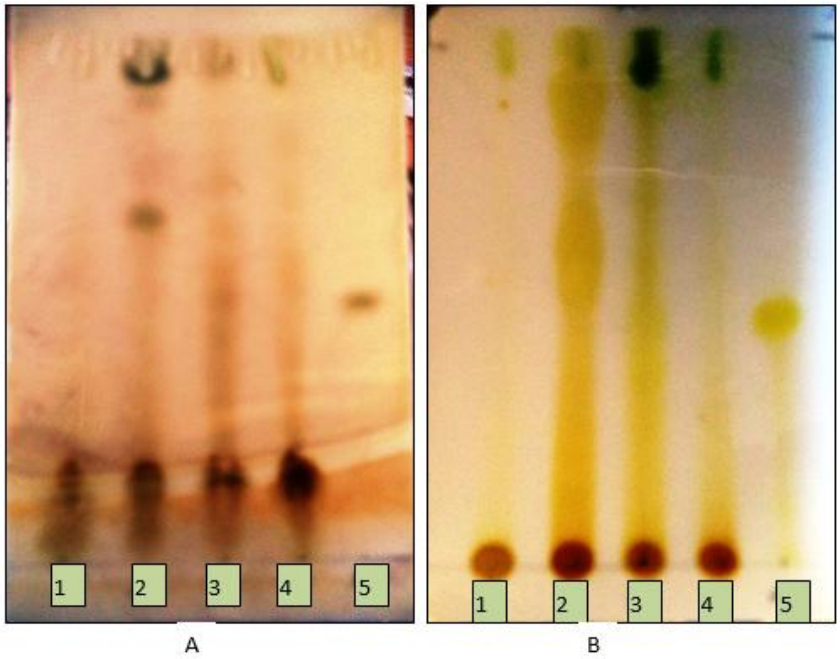

Fig 7: TLC plate of phenolic constituents of 70% methanol leaf extracts of *Eruca sativa Miller* (1), *Lactuca sativa* (2), *Raphanus sativus* (3), *Allium porrum* (4) and rutin (5) in EFAW (100-11-11-26): A-after derivatization with FeCl3-A and Al Cl3-B reagents.

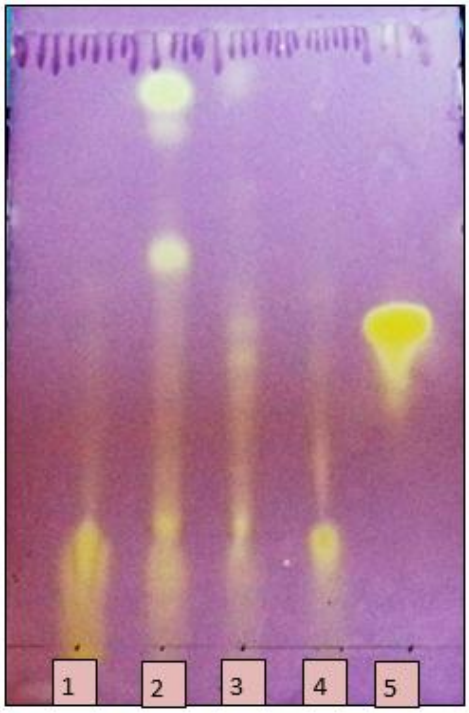

Fig 8: TLC plate of antioxidant constituents in 70% methanol leaf extracts of *Eruca sativa Miller* (1), *Lactuca sativa* (2), *Raphanus sativus* (3), *Allium porrum* (4) and rutin (5) in EFAW(100-11-11-26: in daily light after derivatization with 0.05% DPPH in methanol.

DPPH radical scavenging activity assay

The antioxidant activity of 70% methanol extract of selected green-leafy vegetables was determined. Antioxidants activity ranged from 32.23 ±4.18% for Allium *porrum* to 91.33 ±4.01% for Lactuca sativa. The decreasing order of antioxidant activity is *Lactuca sativa* > *Raphanus sativus* > *Eruca sativa*> *Allium porrum*. The percentage (%) scavenging of DPPH free radical was found to be concentration dependent, i.e. concentration of the extract between 20-100 µg/ml greatly increasing the inhibitory activity (Table 4) and (Figure 9).

Table 4: The DPPH free radical scavenging activity of the methanolic extract of selected leaves and Rutin

Cconcentration µg/ml	Radical scavenging effect (%)				
	Rutin	**Eruca sativa**	**Lactuca sativa**	**Raphanus sativus**	**Allium porrum**
20	80.6	40.44 ±2.11	74.6 ±2.11	70.6 ±3.04	32.23 ±4.18
50	87.3	54.32 ±2.18	85.1 ±3.22	75.1 ±3.22	45.12 ±2.18
70	91.8	60.11 ±2.91	89.8 ±3.18	78.8 ±4.15	48.86 ±3.21
100	95.33	64.12 ±1.18	91.33 ±4.01	81.33 ±3.44	53.21 ±4.14

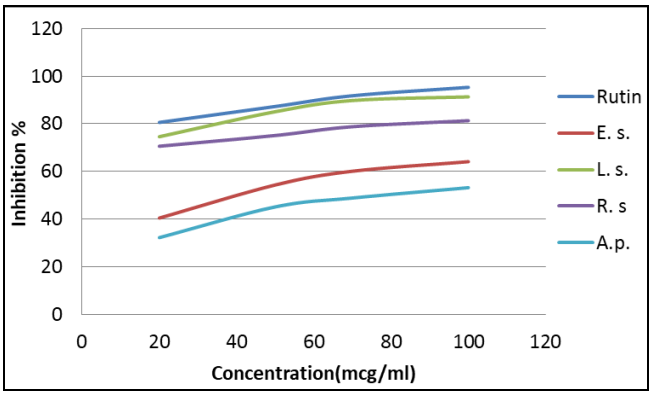

Fig 9: The DPPH free radical scavenging activity of methanolic extract of selected leaves and Rutin

Conclusion

Study of medical and food plants, is actually and necessary due to the widespread uses by local peoples. Four green leafy vegetables; *Eruca sativa*. *Lactuca sativa. Raphanus sativus* and *Allium porrum* used as Salad, were selected for phytochemical and antioxidant analysis. In all studied extracts were detected the present of phytoconstituents such as Steroids, Triterpenoids, Polyphenols, Flavonoids, Alkaloids and Carbohydrates, which play important roles in human health and diseases. Phenolic compounds and antioxidant constituents were revealed in studied samples and antioxidants activity was evaluated by Dot-blot assay, TLC and spectrophotometric method. Study of phytochemicals, antioxidative constituents and antioxidant evaluation by Dot-blot assay, TLC of selected four vegetables grown in Yemen were carried out and reported for first time. It may confirm the nutrients, pharmacological properties and explain partially the uses of these green leaves in prevention of many illnesses. More studies should be carried out on the fractions in order to isolate, purify and characterize the active chemical compounds. Daily use of studied green leaves is necessary to the maintenance of health and protection from coronary heart disease, cancer and others.

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