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Effects of Extraction Techniques on Total Flavonoids, Phenols and Antioxidant Activity of Different Plants Extract

Indu Bharti¹, Aradhita Ray²

² Professor & Head, Department of Food Technology, Guru Jambheshwar University of Science and Technology

Hisar, Haryana, India

Abstract: Among the known fruits and vegetables, dark coloured fruits and vegetables have been reported to be good sources of phenolics, including flavonoids, anthocyanins and carotenoids and are recognized as more healthy to human body. The plants (such as *Mentha*, *Zingiber officinale*, *Allium cepa* and *Prunus*) were extracted by two methods i.e. ultrasonicator and solvent extractor. These plants were determined for moisture content and the extracts were analyzed for total phenolic content (TPC), total flavonoids (TFC) and antioxidant activity (DPPH radical scavenging assay). The results showed that, there was significant difference ($P < 0.05$) in TPC, TFC and antioxidant activity for *Mentha*, *Zingiber officinale*, *Allium cepa* and *Prunus*. A significant and positive correlation found between TPC and DPPH activity using ultrasonicator ($r^2 = 0.75$) and solvent extractor ($r^2 = 0.80$). TFC also showed a positive correlation with antioxidant activity as rutin standard ($r^2 = 0.86$) and ($r^2 = 0.88$) for ultrasonicator and solvent extractor for various plants extracts. This indicated that phenolic compounds including flavonoids were the main contributors of antioxidant activity in plants extracts.

Keywords: flavonoids, phenolics, antioxidant activity, ultrasonicator, solvent extractor,

I. INTRODUCTION

Plants provide abundant natural antioxidants, which are vitally important for human health. Flavonoids are one of them, commonly found in plants is biologically active substances having antiseptic, antimicrobial and pharmacological activities such as radical scavenging and metal chelating properties. Flavonoids come from Latin word *flavus* which means yellow; however some flavonoids are red, purple, blue or white. Best sources of flavonoids include berries, red wine, onion, grains, barks, roots, stems, flowers, and tea and soy products. (Bylka *et al.* 2004). Flavonoids are polyphenolic compounds that have benzo-y-pyrone structure which is ubiquitous in fruits and vegetables. (Rajendran *et al.* 2004).

Flavonoid compounds are common dietary bioactive compounds found in fruits, vegetables and cereals. (Yao *et al.* 2004) The flavonoids are attracting more and more attention because it reduces the risk of coronary heart disease and also possess the antioxidant properties, anti-inflammatory agents, antispasmodic, anti-allergic and anti peroxidation effects. Some studies have shown the positive correlation of increased dietary intake of flavonoid antioxidants with reduced coronary heart disease and cancer mortality. (Prasad *et al.* 2012) Flavonoids can interfere with not only the propagation

reactions of the free radicals, but also with the formation of the radicals, either by chelating the transition metal or by inhibiting the enzymes involved in the initiation reaction. (Saskia *et al.* 1996).

Traditionally extraction of flavonoids was done by the marinated extraction and reflux method. But now there are varied methods of extraction like solvent extraction, ultrasonic extraction, high pressure extraction, microwave assisted extraction etc.

In present study extraction was done by solvent extractor and ultrasonic extractor. In solvent extractor the sample was placed in the thimble, which was immersed in the extracting solvent in a beaker and then it was set in a solvent extractor. The time & temperature was set according to the solvent required. Thereafter ultrasonic extraction developed in order to decrease less consumption of solvent and time, extraction done by the changes in cell structure by sonic waves so that the flavonoids extracted easily at the room temperature. (Wang and Weller 2006).

Studying the mechanism of polyphenols antioxidant activity, it can be noticed that these compounds showed low redox potentials, which allow them to act as reducing agents. The

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aim of the research was to investigate total phenols, flavonoids and antioxidant activity of alcoholic extracts of various plant extracts such as *Mentha*, *Zingiber officinale*, *Allium cepa* and peels of *Prunus*. The literature illustrated that these plants contain some useful polyphenolic compounds with good antioxidant activity (e.g. flavonoids and other polyphenols). The total phenols (TP) and flavonoids content of plant extracts was spectrophotometrically estimated by using Gallic acid, rutin and, quercetin as standards respectively. The relationship between antioxidant activity and total phenols was studied.

II. MATERIAL AND METHODS

CHEMICALS

Ethanol, petroleum ether, gallic acid, folin ciocalteu reagent, sodium bicarbonate, methanol, aluminium nitrate, sodium nitrate, sodium hydroxide, aluminium chloride, potassium acetate, rutin, quercetin, catechin, L-ascorbic acid, DPPH (1, 1, diphenyl- 2 picrylhydrazyl) .All chemicals and solvents used in this study were of analytical grade.

COLLECTION OF SAMPLES

The samples were procured from the Local Market, Hisar (Haryana).In order to investigate the flavonoid content from different plant sources like *Mentha*, *Zingiber officinale*, *Allium cepa* and *Prunes* samples collected according to availability. The fresh samples were washed with water.

PREPARATION OF PLANT EXTRACTS

Samples were lyophilized (Lypholizer model no HRIST-Alpha 2/4 LD plus Germany) for 24 hours, and then crushed into powder by a grinder. The powder of the samples were kept in zipped pouch and stored in deep freezer. Ultrasonicator and solvent extractor type of extraction techniques were used.

1. Ultrasonicator

One gram powder was accurately weighed and placed in sealed vessel by adding 70 ml of 96% ethanol solvent, and then the vessel was placed in an ultrasonic cleaning bath (model no Power Sonic 410) for extraction for 60 min at (40°C). After extraction the extract was stored in glass vials at refrigerated temperature for further analysis.

2. Solvent extractor

Powder of the sample (1 gm) was accurately weighed and placed in thimble. The 96% ethanol solvent poured, followed

by extraction for 1.5 hrs by Solvent Extractor (VELP Scientifica SER148 Solvent Extractor) and then extract were stored.

DETERMINATION OF TOTAL PHENOLS CONTENT

Total phenols were determined by the (AOAC 1985) method. Extract prepared using above said procedure. 0.2ml of different samples was taken in a test tube, 0.5ml FC reagent (1:1 diluted with distilled water) and 1ml of saturated sodium carbonate solution added. Made up the final volume with water. Thus the mixture was allowed to stand for 15 min. Thus the absorbance was read at 746 nm using UV-vis Spectrophotometer (model no-Thermo Scientific Genesys 10S UV-VIS Spectrophotometer). The standard was prepared by 0- 250 mg/ml solutions of Gallic acid water. Total phenol values were expressed in terms of Gallic acid equivalent (mg/g dry mass).

DETERMINATION OF TOTAL FLAVONOID CONTENT

As Rutin Standard:

Total Flavonoids was determined by the method (Zangh *et al* 2008) with some modifications.2 ml of the extract was taken in 10 ml volumetric flask then 0.6 ml NaNO₂ (5%) solution was added to it .It was then shaken to mix properly and allowed it to stand for 6 min. After that 0.5ml Al(NO₃)₃(10%) solution was added to the volumetric flask, it was shaken for some time and was left to stand for 6 min, finally 3.0 ml of the NaOH (4.3%) solution was added to the flask followed by addition of distilled water up to mark, shaken and left to stand for 15 min before determination. Standard solution of rutin was prepared (0.16 mg/ml) with 70% ethanol solution in a 100 ml volumetric flask.Flavonoid content was determined using a standard curve of rutin at (1-5 mg/ml).Then absorbance was determined at 500 nm against blank by UV –visible spectrophotometer.Similarly the sample was taken using the above said procedure. Results were expressed as mg/ml rutin equivalents.

As Quercetin Standard:

Total flavonoids content (TFC) of the extract was measured by the aluminium chloride colorimetric assay described by (Prasad *et al*). 2ml of the sample was mixed with 0.2ml of 5% sodium nitrite. After 5 min, 0.2 ml of 10% aluminium chloride was added to the mixture and mixed. After 6 min, 2ml of 1M sodium hydroxide was added to the mixture. The end volume of the reaction mixture was made up to 5 ml with aqueous ethanol and mixed thoroughly. Absorbance of the reaction mixture was measured at 510 nm against a blank. The

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flavonoid content was determined using a standard curve of Quercetin at 0-50µg/ml and the results were expressed as µg/ml quercetin equivalents (QE).

DPPH RADICAL SCAVENGING ACTIVITY

The DPPH activity was determined by the method of (Brand-Williams *et al* 1999). A 0.200 gm sample in a 100 ml volumetric flask was taken & made up the volume with methanol or water as suitable. Then 5 ml sample was diluted to 50 ml with same solvent. Then from this solution 50, 100,200,300 & 400 micro litre sample was taken for analysis. A set of clean and dry test tubes prepared and then add sample from 50-400 microlitre in each test tube containing methanol (Total volume of methanol + Sample should be 1 ml) and 2 ml of 0.1mM DPPH solution Mix thoroughly and kept in dark for 1 hr. Similarly control was prepared by mixing 2 ml of DPPH and 1 ml methanol. The absorbance was measured at 517 nm in a UV-VIS Spectrophotometer using methanol as blank. Methanolic solution of standard ascorbic acid (0.5mg/ml) was prepared and added in range of 10-100 µg/ml in test tubes containing methanol and DPPH reagent solution as a positive control.

Percentage of DPPH scavenging activity was calculated as

$$\% \text{ Reduction} = \frac{\text{Abs control} - \text{Abs sample}}{\text{Abs control}} \times 100$$

ultrasonicator, while *Allium cepa* had the lowest yield of extraction among all samples (23.6%) in solvent extractor and 5.24% in ultrasonicator.

SELECTION OF EXTRACTION METHOD

Two different extraction methods were selected ultrasonicator and solvent extractor were investigated and flavonoids of these samples were determined by UV-Vis spectrophotometer. The results were shown in Table 1; we can find that solvent extractor had the highest extraction efficiency in comparison with ultrasonicator.

TOTAL PHENOLIC CONTENT (TPC)

Constructed a plot between concentration vs. % reduction in absorbance of DPPH by adding the ascorbic acid and calculated the IC₅₀ (Concentration of Ascorbic acid required for 50% reduction in absorbance)

STATISTICAL ANALYSIS

All extractions and determinations were conducted in triplicate. Data are expressed as means's and two way ANOVAs. The means were compared using the one-way and multivariate analysis of variance followed by Duncan's multiple range tests. The differences between individual means were deemed to be significant at p<0.05. All analyses were performed using the "Statistica, version 5.1" software.

III. RESULTS AND DISCUSSION

MOISTURE CONTENT AND EXTRACTION YIELD

Results showed that, mean moisture content of *Mentha*, *Zingiber officinale*, *Allium cepa* and peels of *Prunes* in this study was 87.89%, 91.26%, 85.20%, and 76.63% respectively. Analysis of extraction showed that solvent extractor had the highest yield when compared with the ultrasonicator. The yield of *Mentha* extracted was 27.8% in solvent extractor and 8.06% in

The total phenols of different plant extracts were determined using Folin- Ciocalteu reagent that reacts non-specifically with phenolic compounds. Plant phenolics constitute one of the major groups of compounds acting as primary antioxidants or free radical terminators (Cai *et al*2004). The TPC value varies among different plant samples as shown in Table 1, it was higher in *Allium cepa* i.e 11.68, 23.14 mgGAE/100g and lower in *prunes*, 3.01, 6.13 mgGAE/100g in ultrasonicator and solvent extractor. There was significant difference between the value of total phenols in *Allium cepa*> *Zingiber officinale*>*Menthe*>*Prunes*. The results clearly reveal that the TPC obtained using a Solvent extractor is higher as compared to ultrasonicator. TPC in plants grown over the world differ significantly.

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TABLE I.
MEAN ± SD OF TOTAL PHENOLIC CONTENT OF PLANT EXTRACTS

Sample	Total Phenolic Content(mgGAE/100g extract)	
	US	SE
<i>Mentha</i>	6.20±0.21 ^b	15.41±.025 ^b
<i>Zingiber officinale</i>	9.00±0.02 ^c	16.42±.025 ^c
<i>Allium cepa</i>	11.68±0.02 ^d	23.14±.015 ^d
<i>Prunes</i>	3.01±0.03 ^a	6.13±0.03 ^a
Mean	7.47±3.36	15.27±6.32

Values are mean (n=3) ± standard deviation. Values with the same superscript letter within each column are not significant different (p>0.05).

TOTAL FLAVONOIDS CONTENT (TFC)

Flavonoids are a group of polyphenolic compounds, naturally present in most of the fruits and vegetables. Use of these natural antioxidants in food and other biological materials is presumed safe and has nutritional and therapeutic value (Tamuly et al 2013). It was concluded from the results that the TFC (as rutin standard) in ultrasonic extract, *Prunes* (3.0900 mg/g) were found significantly higher than other plants. In the same way, the value of TFC in solvent extractor was significantly higher for *Allium Cepa*(.03267mg/g). It was also observed that there was significant difference in TFC between the different plants (Table 2).Further the higher value of TFC was observed in solvent extractor, i.e. 6.3150mg/g for *Allium Cepa* and lower value i.e. 1.8963mg/g for *Prunes* respectively.

In ultrasonic extracts the TFC (quercetin as standard) value found higher i.e. 5.3610µg/g in *Menthe*, Lower value of 1.6880µg/g and 1.5550µg/g in *Zingiber Officinale* & *Allium Cepa* .it also observed that there was no significant difference between the *Zingiber Officinale* and *Allium Cepa* (table 2). Whereas the solvent extractor shows the higher value of TFC in *prunes* i.e. 6.3150 µg/g and lowest value in *Allium Cepa* i.e.1.8963 µg/g (quercetin as standard).

TABLE II.
COMPARATIVE MEAN ± SD OF TOTAL FLAVONOIDS OF PLANT EXTRACTS USING DIFFERENT STANDARD

Sample	Total flavonoids(rutin)(mg/g)		Total flavonoids (quercetin)(µg/g)	
	US	SE	US	SE
<i>Mentha</i>	1.87±0.01 ^b	4.53±0.10 ^b	5.36±.104 ^c	5.66±0.08 ^c
<i>Zingiber officinale</i>	2.85±0.01 ^c	4.99±0.06 ^c	1.68±.104 ^a	2.95±0.04 ^b
<i>Allium cepa</i>	.326±0.01 ^a	6.31±0.01 ^d	1.55±.104 ^a	1.89±0.01 ^a
<i>Prunes</i>	3.09±.014 ^d	1.89±0.02 ^a	2.51±0.06 ^b	6.31±.024 ^d
Mean	2.03±1.13	4.43±1.67	2.77±1.60	4.20±1.91

Values are mean (n=3) ± standard deviation. Values with the same superscript letter within each column are not significant different (p>0.05).

DPPH ACTIVITY

The DPPH is a stable free radical which dissolves in methanol and its colour shows a characteristic absorption at 517 nm.The antioxidant activity of different plant samples was significantly different for both the extraction processes. Structure-activity relationships of flavonoids have been determined in many antioxidant activity assays and they vary with the protocols.(Wolfe and Liu 2008) It was interpreted that the antioxidant activity or DPPH was higher in *Allium Cepa* for both ultrasonicator and solvent extractor 87.62%, 82.44% respectively. The value observed lower in *Prunes* extract i.e. 51.60% for ultrasonicator and 65.97% for solvent extraction. Similar results were shown by Annegowda *et al.*2012.

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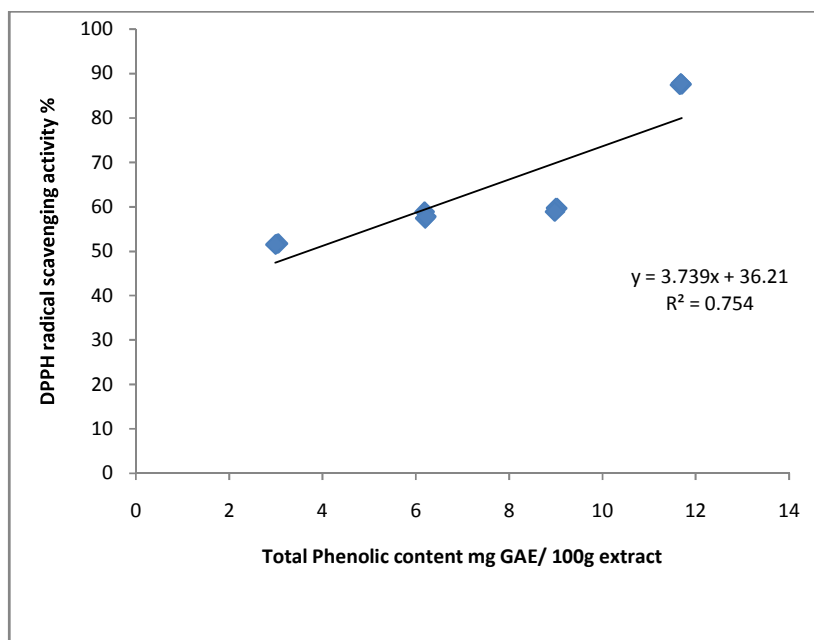
TABLE III
DPPH INHIBITION/ANTIOXIDANT POWER ASSAY OF PLANT EXTRACT

SAMPLE	DPPH INHIBITION%	
	US	SE
<i>Mentha</i>	58.03±0.77 ^b	68.36±0.55 ^{ab}
<i>Zingiber officinale</i>	59.43±0.46 ^c	72.13±1.12 ^b
<i>Allium cepa</i>	87.62±0.17 ^d	82.44±0.13 ^c
<i>Prunes</i>	51.60±0.11 ^a	65.97±0.11 ^a
Mean	64.17±14.47	72.23±6.59

Values are mean (n=3) ± standard deviation. Values with the same superscript letter within each column are not significant different (p>0.05).

CORRELATION BETWEEN TOTAL PHENOLS (TPC) AND ANTIOXIDANT ACTIVITY

Correlation plots (fig1&2) were performed to determine the relationship between TPC and antioxidant activity. It came out from the results that there was a good correlation found different plant samples (*Mentha*, *Allium Cepa*, *Zingiber Officinale* and *Prunes*) using ultrasonicator ($r^2=0.75$) and solvent extractor ($r^2=0.80$). This result suggest that the 80% of antioxidant capacity of plant extracts results from the contribution of phenolic compounds in solvent extractor as compared to ultrasonicator in which the contribution is 75%. Our results compared favourably with previous studies on different plant extracts (Popa *et al* 2012), and showed equivalent or higher antioxidant activity.



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Fig. 1 The correlation between total phenolic content and antioxidant activity (DPPH free radical-scavenging activity) of plants extracts by ultrasonic extraction method

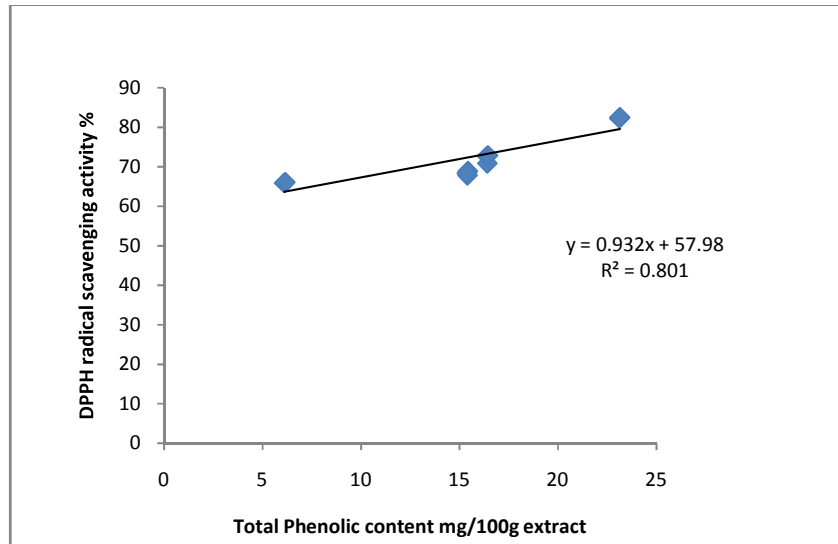
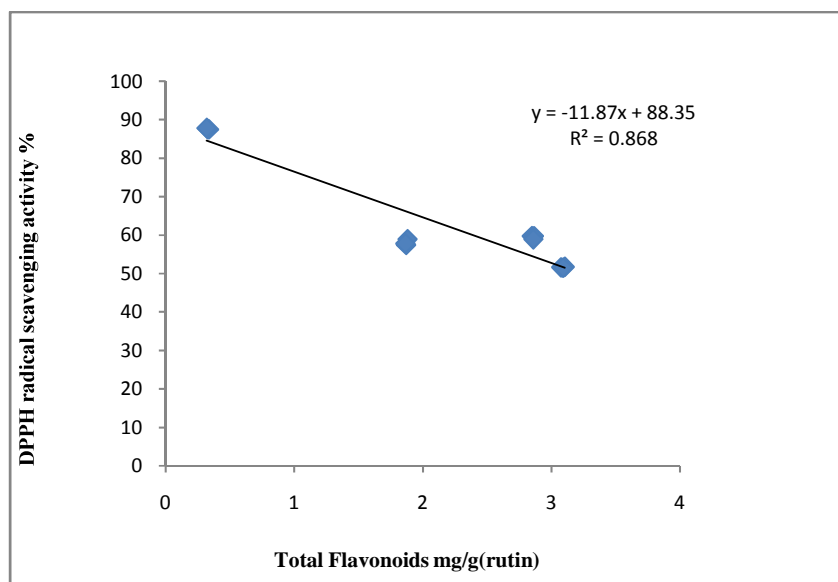


Fig. 2 The correlation between total phenolic content and antioxidant activity (DPPH free radical-scavenging activity) of plants extracts by Solvent extractor method

CORRELATION BETWEEN TOTAL FLAVONOIDS (TFC) AND ANTIOXIDANT ACTIVITY

It was revealed that the TFC showed a positive correlation with antioxidant activity as rutin standard ($r^2=0.86$) and ($r^2=0.88$) for ultrasonicator and solvent extractor in fig 3, 4. This result was similar to findings for total flavonoids and antioxidant activity (Kim et al., 2003). However, a weak positive correlation was obtained between TFC and antioxidant activity when quercetin used as a standard in solvent extractor and ultrasonicator. The variation in correlation among antioxidant activity of total phenols and total flavonoids in the selected plant species may be due to a different reaction mechanism (C. Tamuly et al 2013). The results mentioned above are in agreement with the fact that the total flavonoids content are major contributors to the antioxidant activity of different plant extracts, which is in accordance with the literature. (Zhang et al 2011&Kim et al 2003).



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Fig. 3 The correlation between Total Flavonoid (rutin) and antioxidant activity (DPPH free radical-scavenging activity) of plants extracts by ultrasonicator methods

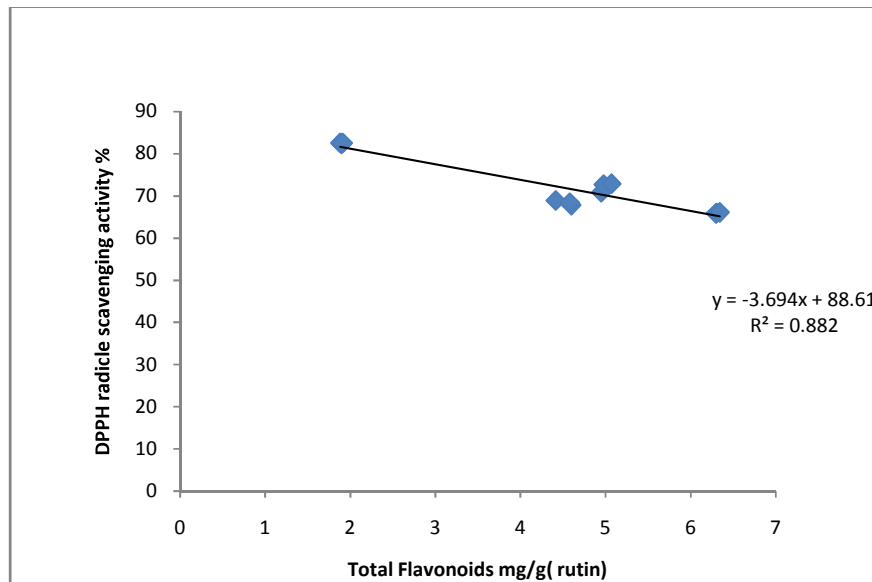


Fig. 4 The correlation between Total Flavonoid (rutin) and antioxidant activity (DPPH free radical-scavenging activity) of plants extracts by soxhlet extraction method

IV. CONCLUSION

In this study, TPC, TFC and antioxidant activities of 4 different selected plants were evaluated. Between the extractions processes studied, the solvent extractor presented the highest yield for all the plant extracts. Novel extraction techniques has removed the technical barriers, improved the design and scaled up the new extraction systems for their

industrial application. A good correlation between TPC and TFC with antioxidant activity of the analyzed plant extracts was established. The obtained data were in good agreement with literature data regarding similar plant extracts. High correlation was observed in extracts of *Menthe*, *Allium Cepa*, *Zingiber Officinale* and *Prunes* using solvent extractor as compared to the ultrasonicator.

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