Journal of Fundamental and Applied Sciences

ISSN 1112-9867

Available online at http://www.jfas.info

Research Article

SOLVENT EXTRACTION EFFECTS ON PHYTOCHEMICAL PROFILES, ANTIOXIDANT ACTIVITIES AND SUPEROXIDE ANION RADICAL INTERACTIONS OF WHOLE FRUIT, PULP AND SKIN OF *CUCURBITA PEPO* L.

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Received: 24 June 2020 / Accepted: 30 August 2020 / Published online: 01 September 2020

ABSTRACT

In this study, hexane and ethanol were used separately as solvent in the extraction of different part of fresh fruit of *Cucurbita pepo* L. For each solvent, total phenolic content, total flavonoid content, total flavanol content and antioxidant activity in skin, pulp and whole fruit extract were investigated using in vitro assays. The results showed that skin part is the rich one en compounds and ethanol is the best solvent for the extraction of total phenolics (231.16 mg GAE/g), total flavonoids (8,4 mg RE/g) and total flavanols (4,16 mg QE/g). For evaluation of antioxidant activity, using DPPH• radical scavenging and total antioxidant activity, skin extract obtained by ethanol showed the highest value respectively (IC50= 0,42 mg/mL) and (19,72 mg/g). The superoxide scavenging assay of ethanol extract of whole fruit and skin showed the highest value respectively (IC_{50}= 0,015 mg/ml) and (0.016 mg/ml).

Keywords: *Cucurbita pepo* L., solvent effect, total phenolics, total flavonoids, total flavonols, antioxidant activity, DPPH, CAT, cyclic voltammetry, superoxide anion radical.

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doi: http://dx.doi.org/10.4314/jfas.v12i3.14

1. INTRODUCTION

Fruits and vegetables are considered in dietary guidance because of their high concentrations of dietary fiber, vitamins, minerals, especially electrolytes; and more recently phytochemicals, especially antioxidants [1]. In particular, there is an association between an increased level of fruits and vegetables in the diet and a reduced risk of some life-threatening diseases such as cardiovascular disease and cancer. There is also growing acceptance that many phenolic secondary metabolites present in plant derived foods exert beneficial effects in the prevention of these degenerative diseases because of their activities [2]. Natural phenolic and flavonoid compounds are plant secondary metabolites that hold an aromatic ring bearing at least one hydroxyl group. Phenolic compounds are good electron donors because their hydroxyl groups can directly contribute to antioxidant action [3]. These compounds are generated naturally during steeping and sprouting. These are further synthesized during normal growth and development as well as in response to mild stress conditions like sub- or supra-optimal radiation, salinity, water potential, minerals, and heavy metals [4]. There is high interest on determination of antioxidant capacity of natural products. *Cucurbita pepo L. (Cucurbitaceae*) a small summer marrow or green squash, has a similar shape to a ridged cucumber. It is usually marketed in fresh, being eaten raw in salads and always with skin, or served cooked in soups or other recipes [4]. Advantageous characteristics of *Cucurbita pepo L.* include its nutrient content, short growing period, ease of storage and transportation, and medicinal value [5]. Active component was found in the seed and pulp of Cucurbita pepo L. that have antidepressant, antiulcerant, hypoglycemic, hypolipidemic and cytotoxic effect [6]. Several studies revealed the importance of this vegetable by studying the composition, activity, chemical profile, total phenolics [7-12]. Different solvent systems have been used for the extraction of polyphenols from plant material. The yield and antioxidant activity of natural extracts is dependent on the solvent used for extraction. Several procedures have been proposed: extraction using fats and oils, organic solvents, aqueous alkaline solutions and supercritical carbon dioxide. Aqueous mixtures of ethanol, methanol and acetone, are commonly used [13].

the present work was aimed to evaluate, for the first time the effects of extraction solvents on

total phenol content, total flavonoid content, and antioxidant activity of whole fruit, pulp and skin of *Cucurbita pepo* L. Two different antioxidant assays were chosen for the measurement of antioxidant function in samples and compared : DPPH• radical scavenging and total antioxidant activity [14,15]. For the second time cyclic voltammetry method was used to evaluate the interaction between extracts of whole fruit, pulp, skin and superoxide anion radical ($O_2^{\bullet-}$)[16-18]. It was selected because of its presence in human body it has longer half life and is capable of generating other harmful radicals such as hydroxyl radical [19]. Only few researchers have investigated this electrochemical method [20-23].

2. MATERIALS AND METHODS

2.1 Collection of plants

For this present investigation the plant Cucurbita *pepo Linn*. (Family: *Cucurbitaceae*) was collected on February 2018 from Hassani Abdelkarim, El Oued region.

2.2. Preparation of samples

The freshly harvested fruits of *Cucurbita pepo L.* were rinsed with tap water followed by distilled water to remove the dirt on the surface and were manually separated into three different parts whole fruits, skin and pulp using a sharp knife. Pulp was the portion remaining after removal of the skin and included seeds. All samples were cut into small pieces.

2.3. Reagents and standards

All chemicals used were of analysis. Ethanol, hexane, folin-Ciocalteu reagent, sodium carbonate, gallic acid, rutin, aluminum chloride, quercetin, sodium acetate, 1,1-diphenyl-2-picrylhydrazyl (DPPH), ammonium molybdate, ascorbic acid, dimethylformamide (DMF), tetrabutylammonium tetrafluoroborate (TBFB). All reagents including solvents were purchased from the scientific company (Sigma; Aldrich) and from other common sources.

2.4. Extraction procedure

For extract preparation, 60 g of fresh samples; whole fruit, pulp and skin were macerated separately three times in 600 mL of ethanol solvent (100%), 600 mL of Hexan solvent (100%), at room temperature for 24 hrs. After filtering with Whatman No. 1 filter, each extract was

concentrated to dryness at 55 ◦C on the rotary evaporated attached to a vacuum pump, each extract obtained was kept in the desiccators until the time for analysis. The yield of dried extracts was calculated according to the following equation 1:

$$Yield(\%) = \frac{(W_1 \times 100)}{W_2} \qquad (1)$$

Where W_1 was the weight of extract after evaporation of solvent and W_2 was the fresh weight of the sample.

2.5. Total phenolic content

Total Phenolic Content of the extract was determined using the Folin-Ciocalteu method with some modifications [24]. 1 ml of prepared extract solution (0,8 mg/ml) was combined and mixed with 500 µl of the Folin-Ciocalteu reagent that previously diluted to ten-fold with distilled water in the test tube. and allowed to stand at room temperature for 5 min in the dark. This reaction was neutralized by adding 2 ml of sodium bicarbonate. The samples were incubated for 30 min. The absorbance of the resulting blue colour solution was measured at wavelength 760 nm [25] on an UV–Vis recording spectrophotometer. Total phenolic content was quantified by a calibration curve from measuring the absorbance of a known concentration of gallic acid (GA) standard (0,03 - 0,3 mg/ml). The content of phenolic in extracts was expressed in terms of gallic acid equivalent (GAE) per gram dry weight extract (mg GAE eq. /g of extract).

2.6. Total flavonoid content

The flavonoid content of samples was measured using a aluminium chloride method with some modification [26]. 1,5 of prepared extract solution (2 mg/ml) was mixed with 1,5 ml of 2% aluminum chloride solution ($AlCl_3$), was incubated for 1 hour in the dark at room temperature, the absorbance of the resulting yellow colour solution was measured spectrophotometrically at 420 nm [27]. Total flavonoid content was determined using a standard plot of rutin (0,02 - 0,1 mg/ml), then expressed as (mg RE eq./g of extract).

2.7. Total flavonol content

Total flavonols in the plant extracts were estimated using the described method of Kumaran and Kumaran with some modifications [28]. 1 ml of prepared extract solution (3 mg/ml) was

mixed with 1 ml of 2% $AlCl_3$ ethanol and 1,5 ml (500 mg/ml) sodium acetate solutions. The mixture was incubated for 2,5 hours in the dark at room temperature, the absorbance of the resulting green colour solution was measured spectrophotometrically at 440 nm [29]. Total flavonols content was calculated from the standard calibration curve constructed with various concentrations of quercetin calculated as (mg QE eq./g of extract).

2.8. Total antioxidant activity

2.8.1. DPPH Radical scavenging activity

The free radical scavenging capacity of extracts were determined using 2,2-diphenyl-1-picrylhydrazyl (DPPH), as described earlier with some modifications [14,15,30]. 1,5 ml of different concentration (1, 2 and 3 mg/ml) of sample solutions was mixed with 1.5 ml of DPPH solution (2 mg of DPPH were dissolved in 50 ml of methanol). the tubes were incubated in the dark. After 30 minutes, the absorbance was read at 517 nm [14,15,31]. The percentage of scavenged DPPH was calculated using the following equation 2:

$$\text{DPPH scavenging activity, \%} = 100 \text{ x } \frac{Ac - As}{Ac} \qquad (2)$$

where *A*c is the absorbance of the control and *A*s is the absorbance of the sample. The calculated IC_{50} values denote the concentration of the sample required to decrease the absorbance at 517 nm by 50 %.

2.8.2. Total antioxidant capacity

The total antioxidant capacity (TAC) of extracts and was determined by the phosphomolybdenum assay using the method described by earlier with some modifications [32]. Briefly, 0.1 ml of a 2.5 mg/ml extract solution in methanol was mixed with 1 mL phosphomolybdenum reagent (28 mM sodium phosphate and 4 mM ammonium molybdate in 0.6 M sulphuric acid) in capped test tubes. Incubation was then carried out for 60 min in a water bath at 95 °C. After cooling to room temperature, the absorbance of the solutions was measured using a UV-visible spectrophotometer at 695 nm. Results were given in mg ascorbic acid equivalent (AAE)/g by using calibration curve prepared from solutions with ascorbic acid.

2.9. Elechtrochemical study

2.9.1. Interaction with superoxide anion radical

Cyclic voltammetric measurements were carried out using PGZ301 potentiostat / galvanostat (radiometer analytical SAS) and Data acquisitions were accomplished with a computer using VoltaMaster4 software version 7.08 (radiometer analytical SAS). Graphs and calculus were carried out using OriginPro9. In an electrochemical cell of 25 ml, conventional three electrode system was employed. Glassy Carbon electrode was used as a working electrode having area 0.013 cm^2. Electrode surface was polished before each measurement. Platinum wire was used as a counter electrode and Hg/Hg_2Cl_2 reference electrode saturated with KCl was used as a reference electrode. Tetrabutylammonium tetrafluoroborate (Bu_4NBF_4) was used as supporting electrolyte and its concentration was kept 0.1 M. Superoxide anion radical was generated in DMF, the scan rate was kept at 100 mV/s, and potential window was from −1.6 to 0.0 V, the extract solutions were added incrementally to the in situ generated radical. The ability of the test sample to quench $O_2^{\cdot-}$ (% inhibition of $O_2^{\cdot-}$) was calculated using the following equation 3 [16-18]:

$$\%O_2^{\cdot-} \text{ radical scavenging activity}, \% = 100 \text{ x } \frac{i_o - i_s}{i_o} \qquad (3)$$

Where i_0 and i_s are the anodic peak current densities of the superoxide anion radical in the absence and in the presence of extract.

3. RESULTS AND DISCUSSION

3.1. Extraction yield

The yield of crude extracts from the whole fruit, pulp and skin of *Cucurbita pepo L.*, obtained by maceration method using polar solvent (ethanol) and non-polar solvent (hexane), were calculated using Equation 1 and the results were shown in Table 1. The result revealed that the highest extract yield was obtained by the ethanol solvent in comparaison with hexane solvent. For ethanol exract, the result showed that whole fruit extract (2.68%) > pulp extract (2.37%) > skin extract (1.9%). and the lowest yield was obtained in Hexane, with the value skin extract (0.18%) > pulp extract (0.15%) > whole fruit extract (0.12%). This difference

may be attributable to the higher solubility of extractable bioactive components in ethanol than in hexane. The variation in the yields of extracts could be attributed to the difference in solvent polarities used which also plays a key role in increasing the solubility of phytochemical compounds.

Table 1. Extract yield of whole fruit, pulp and skin of *Cucurbita pepo* L.

Part of	Extract yield % (w/w)	
Plant \ Solvent	Hexane	Ethanol
Whole fruit	0.12	2.68
Pulp	0.15	2.37
Skin	0.18	1.9

3.2. Total phenolic content

The total phenol contents of different extracts were quantified. The regression equation of standard curve of gallic acid was y = 3.54 X – 0.039 with R^2 = 0.996. The differences in phenolic contents between the analyzed extract is due to solubility of the phenolic compounds in different solvent polarity. As known, ethanol is polar protic solvent, while hexane non-polar aprotic solvent**.** The result in Table 2 showed variations in the levels of total phenolic contents of all extracts. As shown, the total phenolic content varied from 113.34 to 231.16 mg GAE/g and the highest amount was obtained with ethanol for skin followed by whole fruits, the lowest value was obtained for hexane extract in pulp. Phenolic compounds have redox properties, which allow them to act as antioxidants [33].

Table 2. Absorbance and total phenolic content of whole fruit, pulp and skin of *Cucurbita pepo* L.

Solvent	Part of plant	Total phenolic content (mgGA/g)	Absorbance
Hexane	Whole fruit	127.47	0.40
	Pulp	113.34	0.36
	Skin	148.9	0.45
Ethanol	Whole fruit	134.53	0.42
	Pulp	120.4	0.38
	Skin	231.16	0.53

3.3. Total flavonoid content

The total flavonoid contents of different extracts were quantified. The regression equation of standard curve of rutin was y = 21.3x – 0.04 with R^2 = 0.997. The ethanol extract contains relatively more flavonoid compounds than hexane extracts. However, in ethanol extract the skin (8.4 mgQE/g) apparently contain more flavonoid compounds than the whole fruit (7.04 mgQE/g) and the pulp (6.34 mgQE/g). The same in hexane extract, the skin contain (7.51 mgQE/g) more than the whole fruit (6.34 mgQE/g) and the pulp (5.64 mgQE/g). Results obtained as shown in Table 3. The importance of flavonoids in foods and herbal extracts appears in their protective effects against a variety of diseases related to ROS through their capacity to transfer free radical electrons, chelate metal catalysts, activate antioxidant enzymes, reduce α-tocopherol radicals and inhibit oxidation [34].

Table 3. Absorbance and total flavonoid content of whole fruit, pulp and skin of *Cucurbita pepo* L.

Solvent	Part of plant	Total flavonoid content (mgRE/g)	Absorbance
Hexane	Whole fruit	6.34	0.23
	Pulp	5.64	0.20
	Skin	7.51	0.28
Ethanol	Whole fruit	7.04	0.26
	Pulp	6.34	0.23
	Skin	8.4	0.32

3.4. Total flavonol content

The total flavonol contents of different extracts were quantified. The regression equation of standard curve of quercetin was Y= 24.74 X- 0.009 with R^2 = 0.993. Results obtained as shown in Table 4. The ethanol extract contains relatively more flavonol compounds than hexane extracts and skin part in Ethanol (8.4 mgRE/g) and Hexane (7.51 mgRE/g) contain relatively more flavonols than the whole fruit and pulp.

Table 4. Absorbance and total flavonol content of whole fruit, pulp and skin of *Cucurbita pepo* L.

Solvent	Part of plant	Total flavonol content (mgQE/g)	Absorbance
Hexane	Whole fruit	3.22	0.23
	Pulp	2.81	0.20
	Skin	3.9	0.28
Ethanol	Whole fruit	3.62	0.26
	Pulp	3.35	0.24
	Skin	4.16	0.30

3.5. Antioxidant activity

3.5.1. DPPH Radical-scavenging activity

Scavenging activity of DPPH is based on one-electron reduction which represents the free radical reducing activity of antioxidants [14,15,35]. Lower IC_{50} values thus indicate a higher DPPH free radical scavenging activity. All the samples of *Cucurbita pepo* L. in ethanol and hexane solvent showed significant DPPH radical scavenging activity (IC_{50} from 0.42 to 2.4 mg/ml), Table 5. The DPPH radical scavenging activity differs considerably according to solvent and part of the plant, the ethanol extracts of whole fruit, pulp and skin of *Cucurbita pepo L* showed higher activity than the hexane extracts. The ethanol extract of the skin showed higher DPPH radical scavenging activity than that of the constituent parts (the whole fruit and pulp) indicating that the antioxidant property in one part of the fruit was e0nhanced by the presence of phenolic or other compounds in another.

Table 5. DPPH radical scavenging activity of whole fruit, pulp and skin of *Cucurbita pepo* L.

Part of	IC_{50} (mg/ml)	
Plant \ Solvent	Hexane	Ethanol
Whole fruit	2.4	1.5
Pulp	1.7	1.2
Skin	0.89	0.42

IC50 = concentration that inhibits 50% of the initial concentration of DPPH radical.

3.5.2. Total antioxidant activity by phosphomolybdenum

Table 6 presents the total antioxidant capacity obtained through the phosphomolybdenum assay for each extract. In ethanol extract, skin (19.72 mg/g) was the best antioxidant as demonstrated by the highest value of TAC compared to the whole fruit (17.20 mg/g) and pulp (15.31 mg/g). Similarly, In hexane extract, skin (13.11 mg/g) was the best antioxidant as demonstrated by the highest value of TAC compared to the whole fruit (11.84 mg/g) and pulp (10.27 mg/g).

Table 6. Total antioxidant activity of whole fruit, pulp and skin of *Cucurbita pepo* L.

Solvent	Part of plant	Total antioxidant activity (mg/g)	Absorbance
Hexane	Whole fruit	11.84	0.52
	Pulp	10.27	0.56
	Skin	13.11	0.47
Ethanol	Whole fruit	17.20	0.69
	Pulp	15.31	0.77
	Skin	19.72	0.63

3.6. Voltammetric studies of extract interaction $O_2^{\cdot-}$

The superoxide anion radical was generated by one electron reduction of the atmospheric molecular oxygen (O_2) dissolved in DMF at room temperature and the resultant cyclic voltammogram response is presented in Figure 1. The cyclic voltammogram of superoxide anion radical showed one electron reversible process having well developed and clear

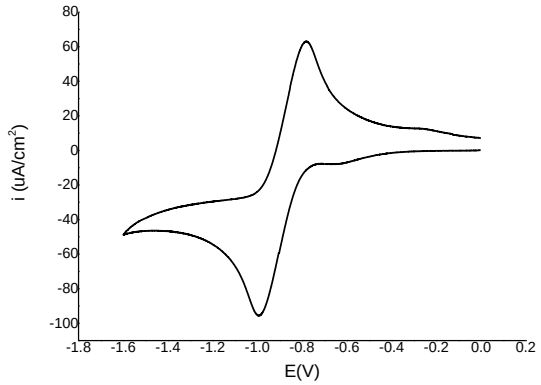
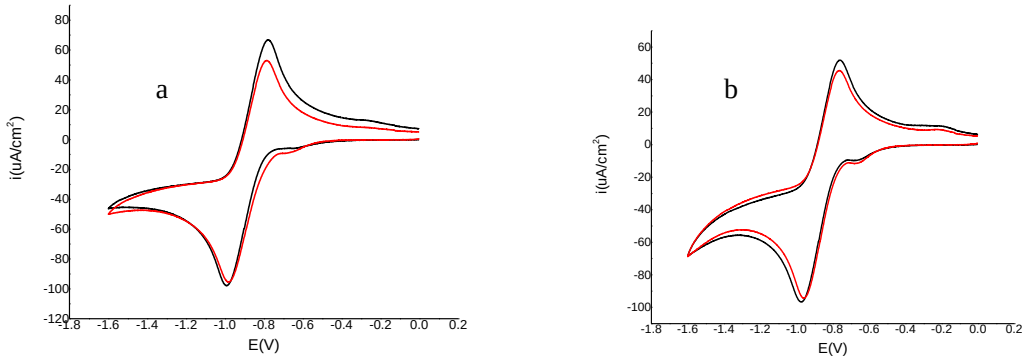


Fig.1. Cyclic voltammograms of oxygen in saturated DMF/0.1 TBFB on GC as working electrode

oxidation and reduction peaks. Next, the voltammetric performance of the ethanol extracts of the whole fruit, pulp and skin of *Cucurbita pepo* L. were investigated one by one in the DMF. All extracts were found electroactive and the obtained response is shown in Figure 2. The extracts were added incrementally (0.1 ml to 0.8 ml) to investigate their effect on superoxide anion radical. The corresponding voltammograms of samples are presented in Figure 3. In all the three cases the addition of the extract causes a proportional decrease in anodic current while the effect on the cathodic current appears to be negligible. By adding 0.1 ml of samples solution in DMF, the significant decrease in anodic peak confirm the



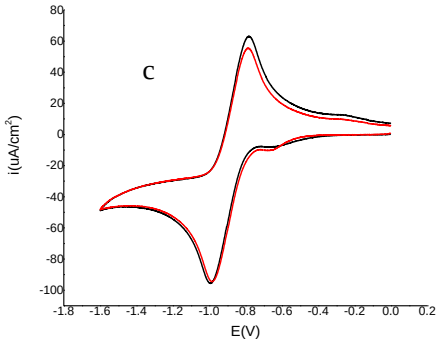


Fig.2. Cyclic voltammograms of oxygen in saturated DMF/0.1 TBFB in the absence (black line) and presence (red line) of 0.1 ml of (a) whole fruit (b) pulp and (c) skin on GC as working electrode. Scan rate 100 mV. s^{-1}

Interaction of extract with superoxide anion radical, is attributed to the presence of some active components (antioxidants) in the extracts which react with superoxide radical and decrease its concentration at/around the electrode surface, Table 7. No change in the reduction wave also imparts that there is no interaction between the extract and the molecular oxygen.

Table 7. Shifts in peak potential and decrease in anodic peak current of $O_2^{\cdot-}$ bound forms of whole fruit, pulp and skin of *Cucurbita pepo* L.

Compound	$ip_a(\mu A)$	$Ep_a(V)$	$Ep_c(V)$	$E_f^0(V)$	$\Delta E_f^0(mV)$	$\Delta ip_a\%$	$Kox/Kred$
$O_2^{\cdot-}$	93.35	- 0.7744	- 0.9870	-0.8807	–	–	–
$O_2^{\cdot-}$–Whole fruit	78.50	- 0.7825	- 0.9788	-0.8807	0.000	15.908	0.998
$O_2^{\cdot-}$	81.28	- 0.7609	- 0.9707	-0.866	–	–	–
$O_2^{\cdot-}$–pulp	75.94	-0.7595	-0.9558	-0.858	-0.008	6.570	0.728
$O_2^{\cdot-}$	87.69	- 0.7798	- 0.9924	-0.886	–	–	–
$O_2^{\cdot-}$–skin	79.28	- 0.7866	- 0.9815	-0.884	-0.002	9.591	0.923

The molecular weight of the whole fruit, pulp and skin extracts could not be calculated because it contains various compounds.

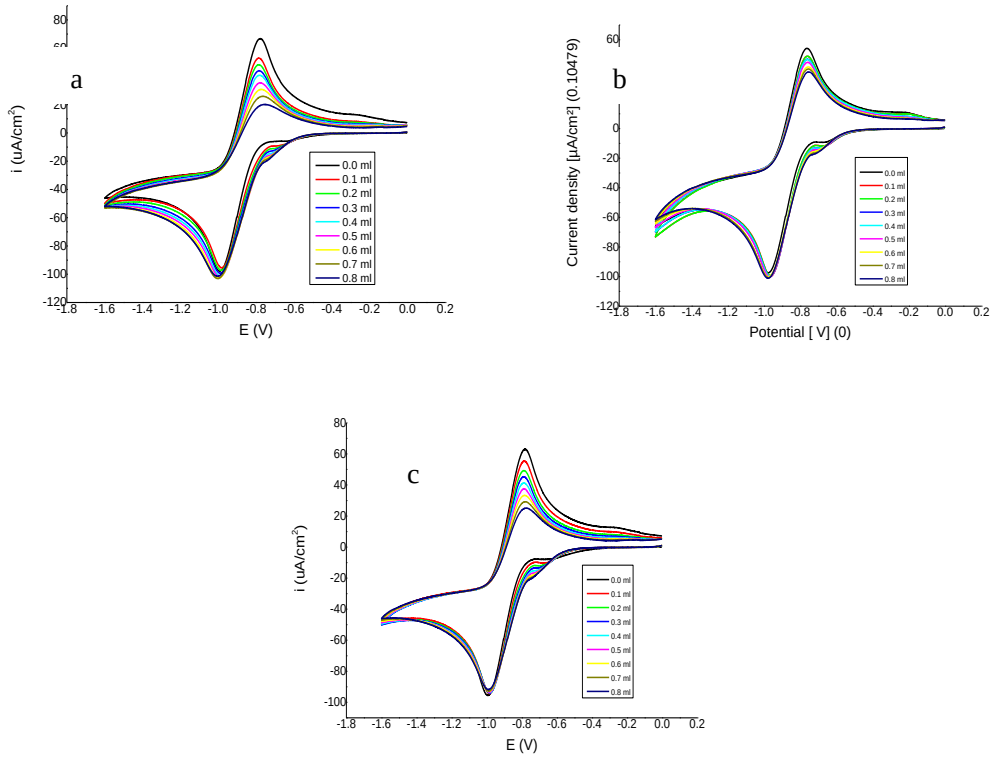


Fig.3. Cyclic voltammograms of oxygen-saturated DMF/0.1 TBFP on a GC electrode in the absence and presence of 0.1 ml, 0.2 ml, 0.3 ml, 0.4 ml, 0.5 ml, 0.6 ml,0.7 ml and 0.8 ml of extract

The augmentation of concentration of the added plant extracts causes the augmentation in radical scavenging activity $O_2^{\cdot-}$%. Which was calculated by Equation 3. The systematic decrease in anodic current, upon further addition of the extract, ensures the consumption of the $O_2^{\cdot-}$. We can present graphs of $O_2^{\cdot-}$% changes in terms of concentrations Figure 4. and we can calculate IC_{50} values. IC_{50} values were determined by interpolating the 50% inhibition point on a straight line fitted through concentrations.

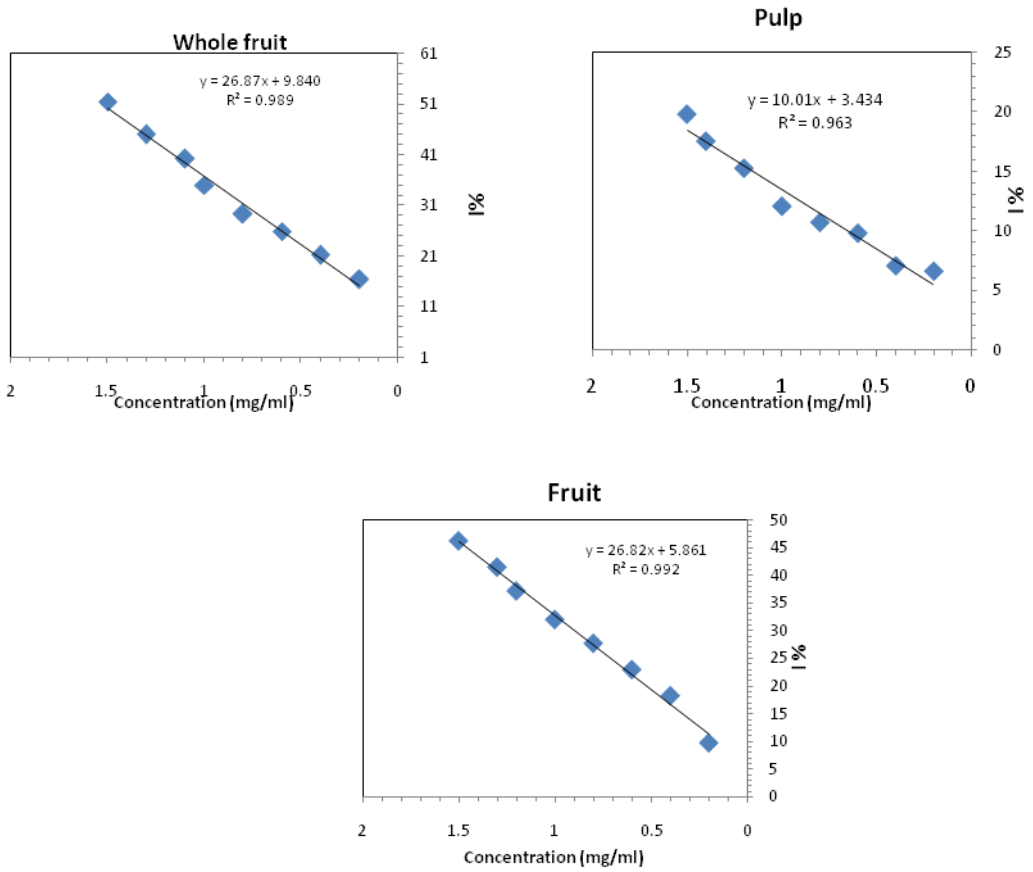


Fig.4. Graphs of $O_2^{\cdot-}$% changes in terms of concentrations of whole fruit, pulp and skin of *Cucurbita pepo* L.

The extract of whole fruit and skin of *Cucurbita pepo* L. showed powerful anti radical activity (0.015 mg/ml) and (0.016 mg/ml) than pulp part (0.047 mg/ml), Table 8.

Table 8. The value of IC_{50} of the whole fruit, pulp and skin of *Cucurbita pepo* L.

Extract samples	Equation	R^2	IC_{50} (mg/ml)
Whole fruit	y=26.87x+9.840	0.989	0.015
Pulp	y=10.01x+3.434	0.963	0.047
Skin	y=26.82x+5.861	0.992	0.016

4. CONCLUSION

In our experimental, data showed that all the crude extracts from whole fruit, pulp and skin of *Cucurbita pepo* L. has a significant amount of total phenols, total flavonoids and total

flavonols. Among the prepared extracts, ethanol contains the maximum of phenol contents, flavonoid contents and flavonol contents. The highest values were obtained from skin extracts. In addition, the antioxidant activity of the six prepared extracts showed also the good inhibition. skin part was the best antioxidant as demonstrated by the highest value of DPPH assay and TAC compared to the other parts. From electrochemical method, by using cyclic voltammetric technique superoxide was generated at glassy carbon electrode by reduction of molecular oxygen in DMF solvent. Therefore, this method is cost effective and the result was very significant, It was shown clearly that the inhibitory activities of the whole fruit and skin part were stronger than the pulp part. This study confirms that *Cucurbita pepo* L. is a source of natural phenolic antioxidants.

5. ACKNOWLEDGEMENTS

Authors are grateful to Mr. Ali Tliba from Laboratoire de Valorisation et Technologie des Ressources Sahariennes (VTRS) staff for his assistance in electrochemical analysis.

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How to cite this article:
Boubékri C, Koraichi B. solvent extraction effects on phytochemical profiles, antioxidant activities and superoxide anion radical interactions of whole fruit, pulp and skin of *cucurbita pepo* l. J. Fundam. Appl. Sci., 2020, *12(3), 1204-1222.*