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DETERMINATION OF ANTIOXIDANT PROPERTIES OF EGGPLANT FRUIT CALYX IN DIFFERENT TYPES OF SOLVENTS

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ABSTRACT

This study was concerned with eggplant(Solanum melongena) calyx and enabling the preparation of three plant extracts from it, using multiple extraction methods, in different solvents (ethanol 70%, distilled water, methanol), then the phenolic content and the antioxidant activity of each one of these extracts was calculated, as another two extracts of eggplant calyx were prepared by Soaking method for 48 hours in two different solvent (70% ethanol, distilled water). The results of the antioxidant activity of all five extracts, were compared with an aqueous extract prepared from three months, and with previous studies results, in addition to the use of the TLC technique for the first three extracts. The ethanolic extract 70% of the eggplant calyx showed a higher result due to the phenolic content and the antioxidant activity followed by the aqueous extract then the methanolic extract of the eggplant calyx. Ethanolic extract (70%) and aqueous extract with heat-based extraction methods showed higher antioxidant activity than methanolic extract and other soaking extracts. Aqueous extract preserved in the refrigerator at -20 ° showed a decrease in the antioxidant activity. The R2 correlation coefficient of the total polyphenol content of the three extracts (aqueous, ethanol (70%), methanol) was compared with its antioxidant capacity by DPPH method, and the results were as follows (correlation coefficient -0.86 at O.OO3 significance), indicating That the antioxidant activity is mainly due to its polyphenol content.

KEYWORDS: phenols, antioxidants, thin layer chromatography, Gallic acid.

INTRODUCTION

Living organisms produce ROS (Reactive oxygen species) as a natural result of metabolism where these species at low and moderate concentrations play a role in the cell's functioning,[1] As it is considered necessary for gene expression, cellular growth and protection from infections, it also sometimes plays a stimulating role for the chemical production of compounds in the cell,[2] but at high concentrations, it causes modifications to cellular compounds such as fats, proteins, and genetic material,[1] When oxygen is metabolized in the body, it produces unstable molecules called free radicals. Free radicals are defined as unstable and highly interacting molecules because they contain a single electron in their atomic orbit, and they are also able to exist independently.[3]

The vandalism caused by the large number of free radicals for a long time occurs irreversibly, And leads to many diseases occurrence, including heart diseases, nervous diseases, liver diseases and some types of cancer.[4]

Oxidation processes can be accelerated due to external influences, including cigarette smoke, environmental pollution, radiation, some medications, pesticides, industrial solvent and ozone.[5]

All aerobic organisms have an antioxidant system to work against the harmful effects of free radicals, and when antioxidant protraction system fails, the organisms must be supplied with external antioxidants.[6] The phenols and flavonoids, which are found in many fruits, vegetables, and teas, are the main compounds responsible for the antioxidant activity.[7]

Antioxidants act as free radical suppressing compounds that inhibit hyperoxidation and all other interactions with these free radicals, and are therefore able to protect human body from oxidative damage.[8]

Therefore, antioxidants can be added to foods to prevent their taste, odor and color from disappearing.[6] This includes natural antioxidants vitamins such as A, C and E, And minerals such as copper, zinc, selenium, and Carotenoids such as beta-carotene and lycopene, polyphenols, and synthetic antioxidants Such as Butylated hydroxyanisole (BHA), Butylated hydroxytoluene (BHT) and Ethylenediaminetetraacetic acid (EDTA).[9]

There are studies has shown that synthetic antioxidants have toxic effects, so warnings of their use have been put in place, and for this reason studies have begun to focus on natural antioxidants derivatives for use instead of synthetic antioxidants.[6]

The use of artificial antioxidants (BHA-BHT) in food is not desirable due to its carcinogenic and unhealthy effect. Currently, plants containing natural antioxidants are receiving increasing attention such as plants containing phenols, flavonoids, carotenoids, and tocopherols.[8,10]

Polyphenols constitute a large field area of medicinal plants, due to the large number and variance of their structural structures,[11] More than 8000 phenolic compounds have been isolated and identified,[11] phenolic compounds are defined as secondary metabolites in plants,[12] the difference in the rings number and number and types of groups that associated with it makes it divide into several groups the most important of which are phenolic acids, flavonoids, tannins, and Flavonoids represent the largest part of them.[13]

Among the known roles of phenols are antifungal and antibacterial properties[14] also they are used in therapeutic indications for the treatment of many diseases as anti-inflammatory, antiviral, antibacterial, anti-arteriosclerotic, anti-allergic, and antioxidant.[15]

Numerous studies have demonstrated the effectiveness of fruits and leaves extracts of plants containing phenols, including eggplant, which has been used in the treatment of many diseases, including asthma, dermatitis and constipation, and various eggplant parts have been used to treat diabetes, gonorrhea, Cholera, bronchitis, dysuria, dysentery, debility, and these effects are attributed to the presence of active substances such as phenols and alkaloids.[16,17] Some studies also suggest that eggplant can help prevent cancer by inhibiting the process of feeding cancer cell with oxygen, and containing nasunin as antioxidant.[18]

Eggplant(Solanum melongena) is a plant belonging to the solanaceae family that contains 75 genera,[19] has been cultivated in India 4,000 years ago also it's very important crop in the tropics and subtropics area,[20] it is among the first ten vegetables that have the highest antioxidant activity.

Studies on anthocyanins and phenolic acids found in eggplant have been shown to be good metabolite and absorbed,[21,22] and it have a good antioxidant ability.[23,24]

Therefore, an important neglected part of it has been extracted which is the eggplant calyx to demonstrate their effectiveness and how to obtain the highest ratio of antioxidants in it, through calibration of the phenolic content and antioxidant activity of several extracts of it by different extraction methods with various solvents(ethanol 70%, distilled water, methanol).

MATERIALS AND METHODS

Devices and tools used

- High Resolution Camera, Evaporator (Germany, Heidolph, Laborata4000)
- Soxhlet extractor, ascender coolant extractor, UV compartment with lamp of 366nm
- Water bath extractor, UV compartment with lamp of 366 nm
- TLC Silica gel60GF254 Sheets, Merck, Darmstadt, Germany
- volumetric flasks sorted 1000 ml, 500 ml, 50 ml, sensitive electronic balance (type AX200, Shimadzu, japan).
- Spectrophotometer (Hitachi-U-1800USA type), frozen, micro-suckers of various capacities, micro-sucker heads.
- Spectro T80 + (UV –VIS SPECTROPHOTOMETER).

Materials

- Eggplant calyx

Eggplant calyx were collected from local markets, and then dried away from moisture, heat and light, and kept in sealed containers away from moisture.

- Distilled water
- Glacial Acetic Acid 99.7%- (Ethyl Acetate) – formic acid from(Sigma –Aldrich, USA).
- (Hydrochloric acid) from (sigma –Aldrich, USA).
- (Anhydrous sodium carbonate) from (panreac, spain).
- (Ferric choride hexahydrate) %99 from (panreac, spain).
- Vanillin, Aluminum chloride, sulfuric acid, Merck, Germany.
- absolute ethanol 99.5% from (Eurolab, UK).
- Methanol 99.8% (methanol) from (sigma –ldrich, usa).
- ANHYDROUS sodium carbonate (panreac, spain).
- Standard - gallic acid with 98% purity (Titan biotech, India).
- (Dpph) 2,2 –diphenyl 1-1- picrylhydrazyl (Santa cruz Biotechnology, united state of America).

- (Folin-Ciocalteu) from(Fluka Germany)

Experimental work

1- Preparing plant extracts

Three types of extracts were prepared: ethanol (70%), aqueous and methanol.

1-1 - Methanolic exract

The methanolic extract was prepared with the soxhlet apparatus where 30 g of the plant sample was extracted by 250 ml of methanol 99% for four hours after which the resulting extract was collected and dried using a rotary evaporator.[25]

1-2- Ethanolic extract

1. The ethanolic extract is prepared with a soxhlet apparatus where 30 g of the plant sample was extracted by 300 ml of ethanol 70% for a period of four hours after which the resulting extracts are collected and dried using a rotary evaporator device.[25]
2. The ethanolic extract is prepared by soaking with 70% ethanol for 48 hours, stirring.

1-3 - Aqueous extract

1. 30 g of calyx powder is placed in the extraction beaker with 200 ml of distilled water and heated under an ascending cooler for an hour and a half of the resulting extract is filtered and then evaporated using a rotary evaporator until dryness.
2. The aqueous extract is prepared by soaking with distilled water for 48 hours with stirring.[26]

2-Thin Layer chromatography (TLC)

Among the benefits of thin layer chromatography is the short separation period, and the possibility of conducting the experiment on several samples at the same time.[27]

Chromatography on the thin layer helps to perform qualitative analysis and separation of different compounds involved in the composition of the examined material according to its polarity, the liquid of relay used and its general structure can be identified by using different detection methods such as examining the plate under ultraviolet wavelengths of 246 and 365 nanometers and spraying the plate with different reagents that help to identify some of the chemical functions expected to be present in the studied extracts.[28]

A variety of different solvent have been used to separate flavonoids using TLC.

Chromatography is carried out on the substrate layer on the two transport medium, one of which is non-polar (chloroform-methanol) (1:15) and the other is polar (ethyl acetate - formic acid - Glacial Acetic Acid- water (100-11-11-26).

Appearance reagents: iron chloride, sulfuric acid, vanillin, aluminum chloride, sulfuric acid expanded with methanol.

3-Determination total phenolic content (TP)

The total phenolic content (TP) was determined using the FolinCiocalteu reagent method.[29] Where phenols return phosphorus molybdenum tungsten acid in an alkaline medium resulting in a blue solution whose absorption is measured at a wavelength of 760 where a series of redaction reactions occur with the transfer of an electron or two of the phenols that lead to blue complex formation.

1 ml of sample are placed into, 4.8 ml of distilled water removed from electrolytes and 4 ml of Na 2CO 3 (2 %w/v), 200 µl (0.2 ml) of Folin-Ciocalteu reagent.

It remain in dark at room temperature for 60 minutes, then absorption was measured at 760 nm.

A calibration curve of Gallic acid solutions were prepared in ethanol 70% (0-150 mg l-1); the total phenolic compounds were determined according to the following formula (y = 0.0044x-0.01) slope = 0.0041, and R2 = 0.9991) Obtained from the standard graph of Gallic acid where Y is the absorbance at 760 nm and X is total phenolic content in extracts.

The results were expressed as mg of Gallic acid equivalents per 1 g of dry plant powder.

4-Determination of anti-oxidant activity - test (DPPH): (2.2-diphenyl-1-picryl hydrazyl) Principle: The plant extracts antioxidant ability was measured by measuring its ability to remove the free radicals of the fixed root DPPH based on the method of (Yu et al).[31,30]

The ability to inhibit oxidation is evaluated by the IC50 standard

It is the concentration of the extract that achieves 50% free radical inhibition and is calculated graphically from the extracts concentration series graph. The low IC50 values indicate the high ability of the extract to inhibit the DPPH free radicals.[32]

Preparation of extracts for DPPH[33]

A series of concentrations were prepared for each of the extracts: ethanol, aqueous, methanol, at concentrations of 200-400-800-1600 g / ml. Then 100 µl of extracts was placed in each tube with 2.5 ml of DPPH solution (0.0024g in 50 ml of ethanol) after that the tubes are placed in the dark at room temperature for 30 minutes, then the decreasing intensity of purple color of DPPH free radicals is measured at a wavelength of 517 nm using the spectrophotometer, the measurement is repeated three times and the arithmetic average is taken.

$$\%DPPH = (A_{blank} - A_{sample}/A_{blank}) \times 100$$

Where: A_{blank}: is the pure absorption that contains all reagents except the examined extract.

A_{sample}: absorption of the tube containing the tested extract in addition to the reagent.

- The graph curve for each concentrations series plotted then Calculated from it the extract concentration Which inhibits the free radicals of DPPH by 50% (IC50)

Then Repeat the measurement three times, and take the average.

RESULTS AND DISCUSSION

1-Results of TLC thin layer separation

TLC was performed on the two transport medium, one of which is nonpolar (Chloroform-methanol) (1:15) and the other is polar (ethyl acetate – formic acid - Glacial Acetic Acid - water (100-11-11-26), respectively.

The spots appeared clearly in the aqueous extract while their appearance was limited and to a lesser number in the methanol and ethanolic extracts at a concentration of 1: 20 ml, and Figure (1) shows the appearance of the spots in the aqueous extract and the calculation of the (retention factor) for each spot.

The blue spot appeared with iron chlorine at a height of 2.4 / 5.6 = 80.42 = RF of the aqueous extract.

With sulfur acid and vanillin a spot appeared 3.5 / 5.9 = 0.59 RF = for the 70% ethanol and methanol extracts.

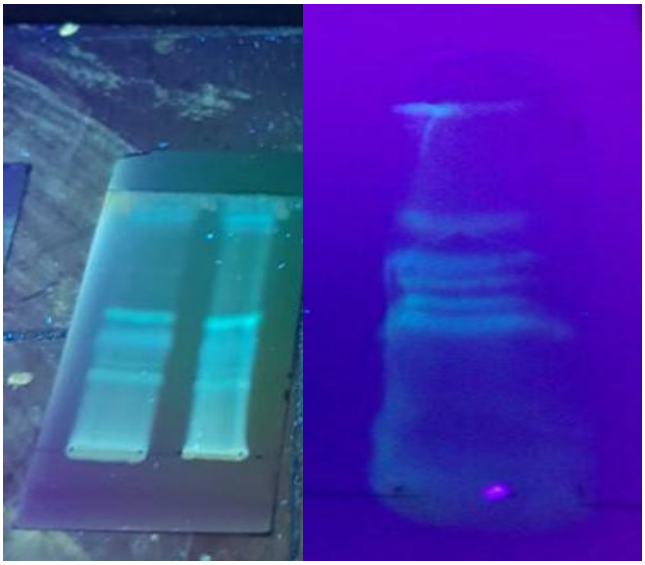


Figure (1): thin layer Chromatography plate on the aqueous layer of the aqueous extract.

The spots appeared on the first day after spraying them with clear aluminum chlorine and with extended sulfuric acid, but with methanol, they never gave results.

On the second day, the experiment was repeated to confirm the results and stains were sprayed with aluminum chlorine and the following stains appeared in the first layer) for the ethanolic extract of 70% and the metatolic extract) yellow - green - blue (RF =1.5 / 6- RF = 2.9 / 6 RF = 5.6 / 6) That is, the RF values were, respectively:

(RF = 0.25 -RF = 0.48 -RF = 0.93) The numbers of these patches are listed in Table (1) according to their related extracts.

As for the second layer (of the aqueous extract), it showed the stains in the following colors: Yellow - Yellow - Yellow - Yellow - Purple - Blue - Yellow (RF=0.8 / 4.8 RF = -1.8 / 4.8 RF = -2.1 / 4.8 RF = -2.5 / 4.8 RF = -3 / 4.8 RF = -4.6 / 4.8), meaning that the RF values were respectively:

(RF = 0.16 –RF= 0.375- RF =0.43- RF =0.52 -RF =0.62- RF= 0.95) The numbers of these patches are listed in Table(1)as the results of the aqueous extract and are also shown in Figure (1),

Table (1): spots distribution of extracts due to TLC thin layer separation.

RF	0	0.16	0.25	0.375	0.43	0.48	0.52	0.59	0.62	0.93	0.95
Aqueous extract	+	+		+	+		+		+		+
Ethanolic extract70%	+		+			+		+		+	
Methanolic extract	+		+			+		+		+	

RF: retention factor

As for the flavonoids expected to be present in the studied extracts (aqueous, ethanol 70%, methanol), Table (2) shows them according to the RF of this comparison with the results of the RF of the previously studied and well-known flavonoids and phenolic compounds.

Table (2): The flavonoids compounds expected to be present in the studied extracts.

RF	Result
0	flavonoids
0.16-0.25	Flavonoids double bond
0.37-0.42-0.43	Phenolic bond
0.48-0.52-0.59	Flavonoids double bond
0.93-0.95	Phenolic double bond

2-Results of phenolic titration

The phenols titration result were shown in the three studied extracts (aqueous, 70% ethanol, methanol), which exposed to heat by soxhlet apparatus for the ethanolic 70 % and methanolic extracts, and in water bath method of the aqueous extract, and Table (3) shows these results.

Table (3): Results of phenol titration in the three extracts.

Extract type	Gramic equivalent of dry Gallic acid mg / 100 g
Ethanolic extract 70 %	^{b}12.83± 1220
Aqueous extract	^{c}21.85 ± 1125
methanolic extract	^{a}16.67 ±995

Similar letters indicate that the statistical differences are insignificant, while the different letters indicate that the statistical differences are significant, and it was relied upon considering the value of P <0.05 to indicate the statistically significant difference.

Here we find that the highest phenolic content was for the ethanol extract 70%, followed by the aqueous extract with a slight difference Then methanol extract, and Figure (2) show the mean values of the phenolic content of the three extracts (aqueous, ethanol 70%, methanol).

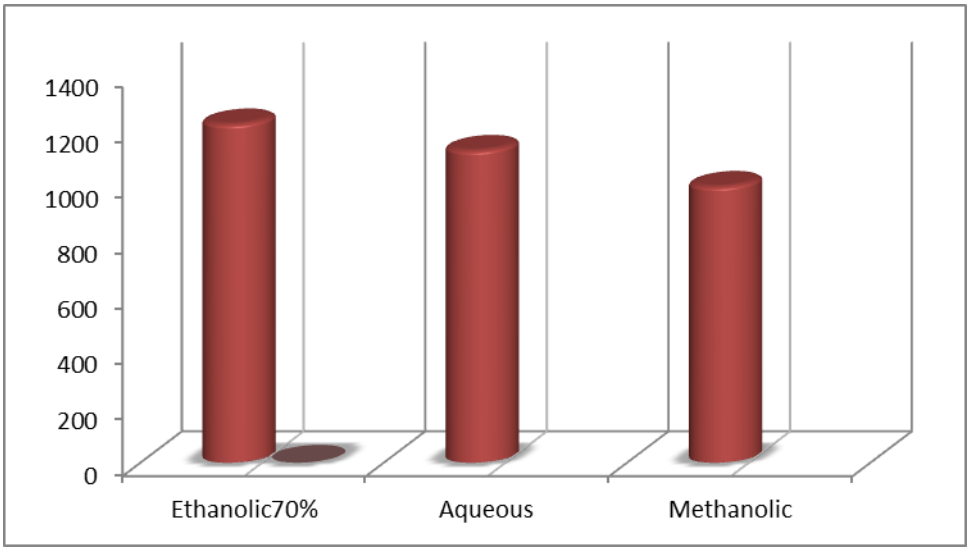


Figure (2): a graph showing the mean values of the phenolic content of the three extracts (aqueous, methanol, ethanol 70%).

These averages were calculated from the equation y = 0.0044x-0.01 of the graph line drawn according to the average readings of the standard series of Gallic acid, and Figure (3) shows the calibration graphical curve of Gallic acid, while table (4) shows Average readings for the standard series of Gallic acid.

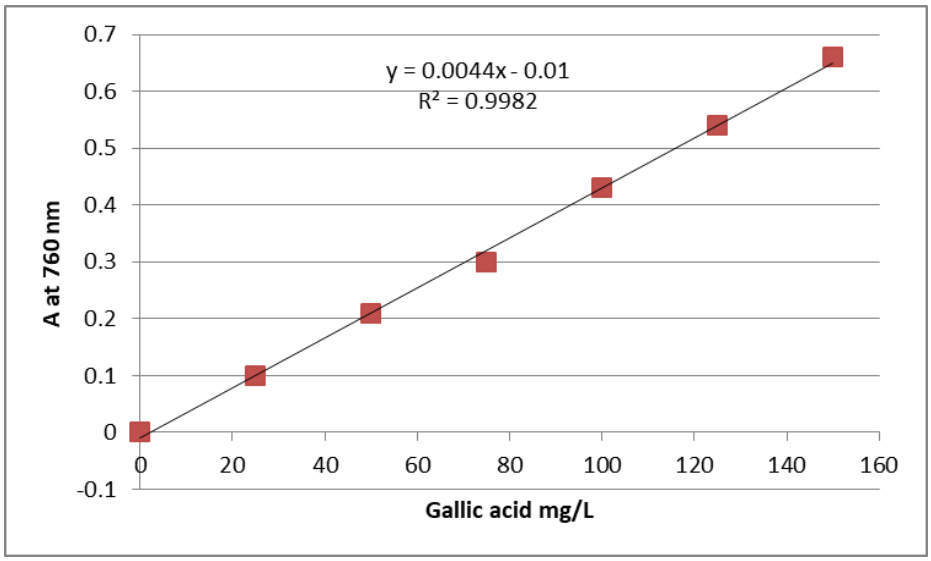


Fig.(3): Standard curve of Gallic acid to determine the total content of phenols TP.

Table (4): Average readings for the standard series of Gallic acid.

The concentration mg / l	0	25	50	75	100	125	150
Average absorption	0	0.1	0.21	0,3	0.43	0.54	0.66

In comparison between the current study and previous studies, we find that the phenolic content of the three extracts within the specific field that was studied in 33 varieties of eggplant of different origins found its value ranges from 740 to 1430 mg Gallic acid 100 / g.[34] dry matter in the (San José et al 2013) study However, this study was on fruit and not on calyx.

In another study of eggplant (Diab et al 2011)[35,36,37,38,39], studied the phenolic content in calyx shows a significant difference in the study, the value of its aqueous extract is greater (2869 mg / 100 g), and the probability of the difference is due to the extraction method.

The phenolic content of the ethanolic extract in this study is (1220 ± 12.83 mg / 100 g), and in a (Diab et al 2011) study the result was (826 mg / 100 g), the ratio in this study was higher, with the difference in the way of preparation.

3- Results of measuring antioxidant activity in eggplant calyx prepared extracts

The current study was conducted to calculate the antioxidant capacity on the six extracts, the first three of which were according to their phenolic content (EH, AH, M) and the two extracts (EB, AB) were for two ethanolic70% and aqueous properties prepared by soaking method for a 48-hour with stirring, while the extract(AA) was for an aqueous extract prepared in a water bath method and preserved in the refrigerator at -20 C for a period of three months, and Table (5) shows the values of the antioxidant capacity of the six extracts from the eggplant calyx, mentioned above. The graph represents the ratio of the antioxidant capacity values of the six prepared extracts from eggplant calyx, Figure (4) illustrates it.

Table (5): Antioxidant capacity values of the six prepared extracts from eggplant calyx.

Extract type	SD ± IC 50 µg/ ml
EH	263±4.9
EB	328±4.3
AH	277±6.7
AB	1644±2.8
AA	997±3.4
M	401±8.4

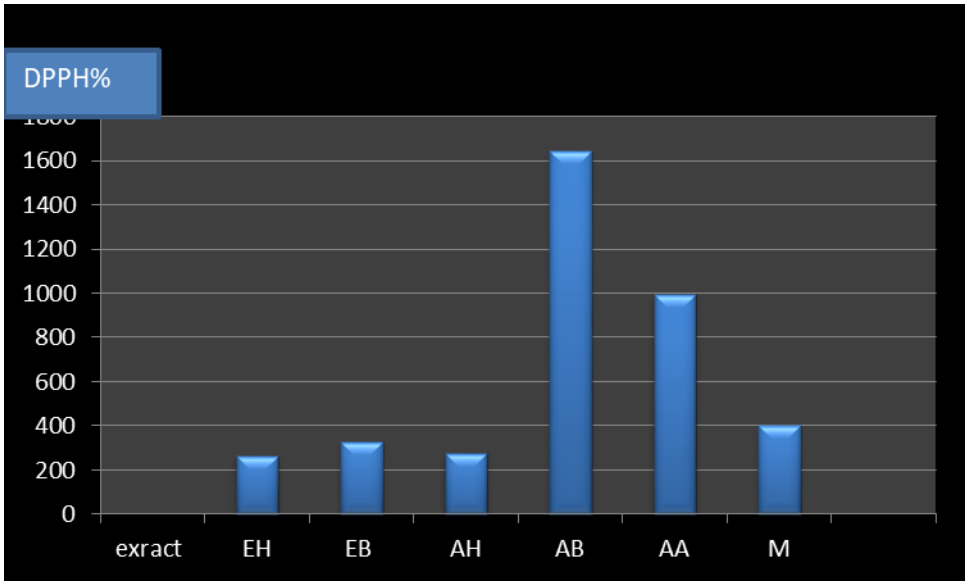


Figure (4): is a graph representing the averages of the antioxidant activity values of the six prepared extracts from eggplant calyx.

EH: ethanol extract with heat (soxhlet extract).

M: Methanol extract with heat (soxhlet extract).

EB: ethanol extract with maceration for 48 hours.

AH: aqueous extract with heat (water bath extraction).

AB: aqueous extract with maceration for 48 h.

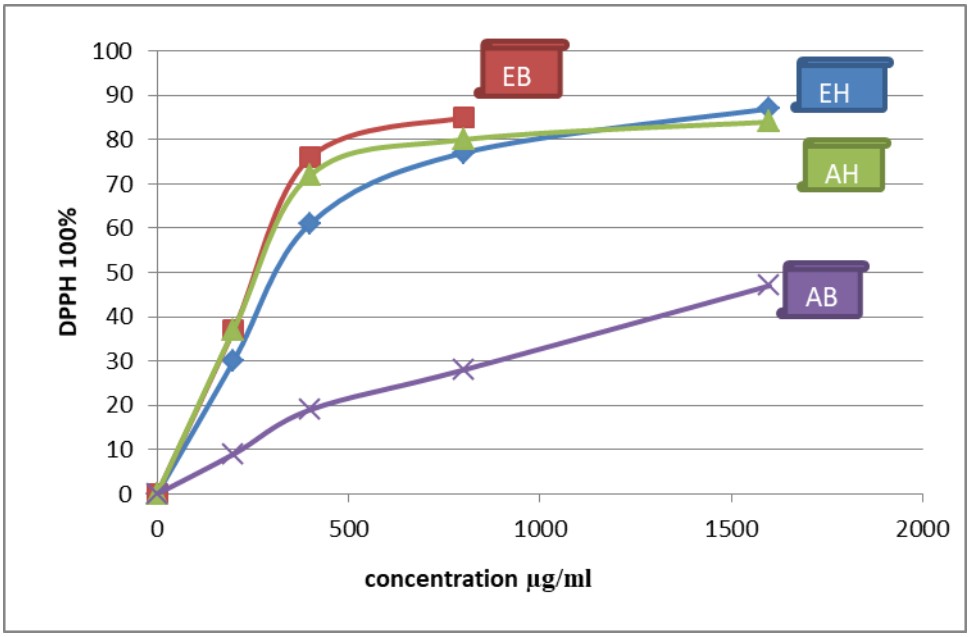
AA: Water extract with heat (water bath extraction) kept in freezer in -20 degree for three months.

Table (6) shows different concentrations of antioxidant activity values in of the six prepared extracts from eggplant calyx in different ways with multiple solvents (aqueous, ethanol 70%, methanol).

Table (6): Antioxidant activity values in of six different extracts concentrations prepared from eggplant cones.

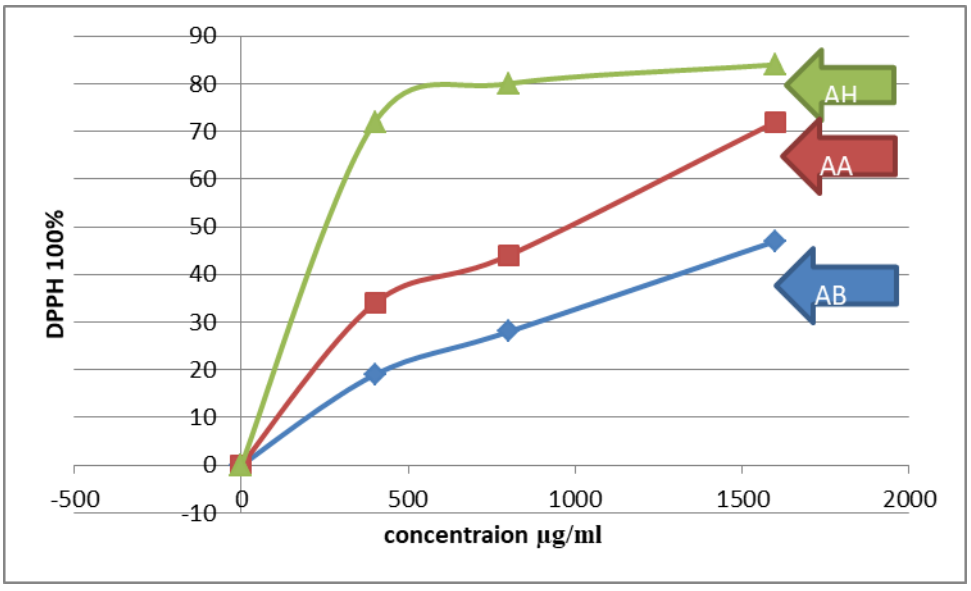
Extracts	EB	EH	AH	AB	AA	M
Concentration µg/ml	DPPH 100%					
0	0	0	0	0	0	0
200	30 ±0.4	37±0.55				30±0.7
400	61±2.1	76±1.1	72±2.1	19±0.4	34±1.1	59±1.2
800	77±1.2	85±0.9	80±1.3	28±1.2	44±2.6	90.4±0.5
1600	87±0.5		84±0.8	47±1.5	72±0.7	

To clarify the differences between these results, we made a comparison between the results of ethanol 70% and aqueous extracts, with the two preparation methods for each of it. Figure (5) illustrates this.



(Figure 5): a comparison of antioxidant activity of ethanolic 70% and aqueous extracts (EB-EH –AH-AA).

As for the comparison between the results of the aqueous extracts with different preparation methods, Figure (6) illustrates this.



(Figure 6): A comparison of antioxidant activity of aqueous extracts (AH-AA-AB).

Figure (7) shows the comparison between the antioxidant capacity of aqueous ethanolic (70%) and methanolic extracts.

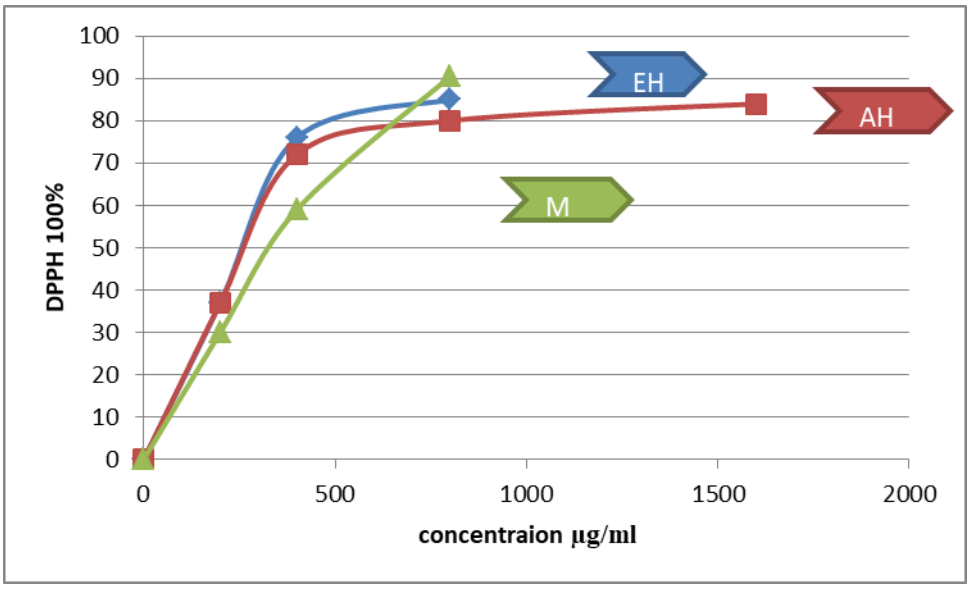
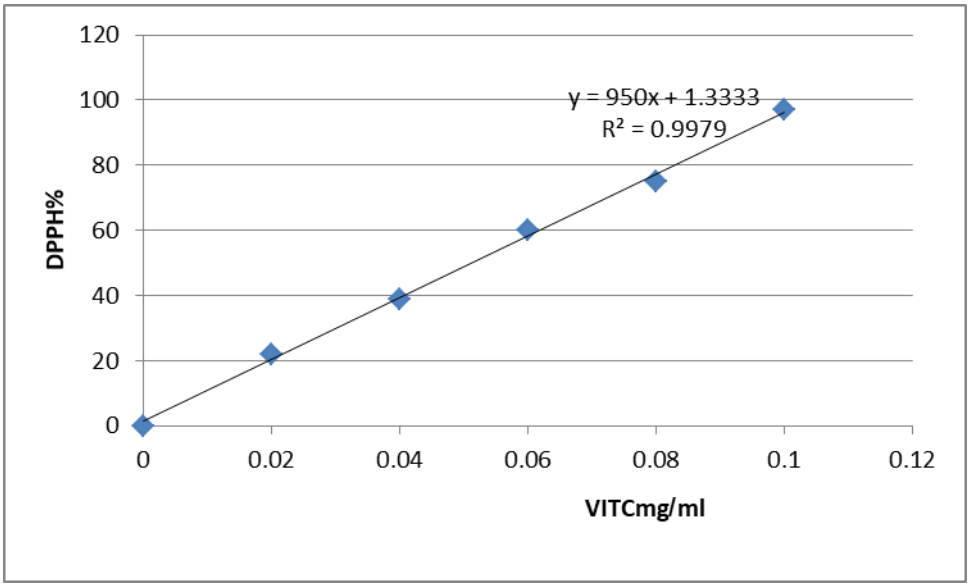


Figure (7): A comparison of antioxidant activity of ethanolic (70%), aqueous and methanolic extracts.

The results has shown that the ethanolic and aqueous extracts with heat-based extraction methods had a higher antioxidant activity than the methanolic extract and from the rest of the extracts, Knowing that positive witness's antioxidant activity of (ascorbic acid) was = 50 µg / ml Figure(8) shows the graphic curve of the antioxidant capacity of vitamin C.

However, in the current study, the antioxidant activity of ethanolic extract (70%) was 0.32 mg / mL and 1.64 mg / mL in the aqueous extract. In contrast, in previous studies (Jung, EJ, et al)[40], it was estimated as 3.45 mg / ml in the ethanolic extract(70%), and 0.49 mg / ml in the aqueous extract.

Thus, the antioxidant activity result of ethanolic extract (70%) was better and greater in the current study. As for the aqueous extract, the result is better in previous study and this comparison was made by the same preparation method, and if we wanted to compare with the other preparation methods. The result is better and greater in both extracts in the present study. The differences are due to the variety of the preparation method.



(Figure 8): Curved graph of the antioxidant capacity of vitamin C.

4- The correlation between the phenolic content and the antioxidant ability of extracts

The R2 correlation coefficient of the total phenolic polymers of the three extract (prepared by heating).

Aqueous, ethanolic (70%) and methanolic extracts, was compared with their anti-oxidant method using DPPH, and the results were as follow (correlation coefficient -0.86 at O.OO3 significance) this value is significant because it is Within the limits (-1, + 1).

DISCUSSION

The current study focused on studying some active substances in eggplant cones and extracting phenols from it,using three solvent (water, ethanol (70%), methanol), the antioxidant activity of these extracts was measure. The results of the research can be summarized as follows:

Calibration results for phenolic substances showed that ethanol extract (70%) possesses the highest phenolic content, followed by aqueous extract and methanol extract.

- When separating with a thin layer chromatography TLC by two transfer medium, one is non-polar and the other is polar. The spots appeared(which indicate the flavonoids content) was clearly in the aqueous extract, while their appearance was limited and in a smaller number in methanol and ethanol extracts at a concentration of 20: 1 ml.

The ethanol extract (70%) and the aqueous extract with heat-based extraction methods showed higher antioxidant activity than the methanol extract and other soaking extracts.

Aqueous extract preserved in the refrigerator at -20 ° showed a decrease in the antioxidant activity.

The statistical study demonstrated a strong to very strong negative correlation between the phenolic content and the value of the antioxidant capacity, which indicates that the antioxidant capacity is mainly due to its polyphenol content.

CONCLUSIONS

This study has showed the importance of eggplant calyx as a rich source of beneficial active substances and cheap renewable natural antioxidants, due to the high total phenolic contents, in addition to their DPPH inhibition ability, And the most important result in this study is that the preparing method of the aqueous extract with exposure to heat gave much better results than aqueous extract preparing by soaking method, and this gives us an indication that cooking the cones by keeping it with the rest of the eggplant and exposing it to heat during cooking will not affect the ratio of phenolic substances and the anti-oxidant activity in it, On the contrary, it will increase all of it and gives the prepared food a greater nutritional and therapeutic benefit, with the attention that keeping this extract in the refrigerator for three months will make it lose its anti-oxidant ability, therefore, preserving food from this type in the refrigerator is not useful, especially if the period of preservation is prolonged and is better to eat it directly, with the advice not to give up on eggplant calyx because of its high nutritional and therapeutics benefit on human body.

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