INTERNATIONAL JOURNAL OF CURRENT RESEARCH IN CHEMISTRY AND PHARMACEUTICAL SCIENCES

(p-ISSN: 2348-5213: e-ISSN: 2348-5221)

www.ijcrcps.com

DOI: 10.22192/ijcrcps Coden: IJCROO(USA) Volume 6, Issue 3 - 2019

Research Article

DOI: http://dx.doi.org/10.22192/ijcrcps.2019.06.03.003

Antioxidant potential and Phytochemical analysis of fruit extract of *Cucurbita pepo*

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Abstract

Objectives: Free radical induced oxidative stress is involved in the pathogenesis of various diseases and disorders. Antioxidants play an important role against this oxidative stress to protect our body. The present study was carried out to evaluate the *in vitro* antioxidant properties of hydro alcoholic fruit extract of *Cucurbita pepo* (HFECP). **Methods**: HFECP was assayed on different *in vitro* free radical models like DPPH, FRAP, ABTS and total antioxidant assay models. Reductive ability of the extract was also tested by the complex formation with potassium ferricyanide. Further total phenolic and flavonoid contents of the crude fruit extract were also measured. Butylated hydroxy toluene was taken as standard. **Result**: The extract showed good dose dependent free radical scavenging activity in all the models. Reductive ability was also found to increase with increase in extract concentration. Determination of total phenolic and total flavonoids content showed that 1 gm of fruit extract contains 66.70±3.60 mg equivalent of rutin and 26.50±1.40 mg equivalent of gallic acid. **Conclusion**: All the results of the *in vitro* antioxidant assays revealed potent antioxidant and free radical scavenging activity of the fruit extract of *Cucurbita pepo*, equivalent to that of standard quercetin and this antioxidant property may be attributed to its high phenolic and flavonoid contents.

Keywords: Antioxidant, *Cucurbita pepo,* Phytochemical analysis, gallic acid, quercetin.

Introduction

Oxygen derived free radicals such as superoxide, hydrogen peroxide, hydroxyl radicals are collectively known as reactive oxygen species (ROS).During normal physiologic condition, ROS are continuously produced in the aerobic cells and removed by endogenous antioxidant defense mechanism of the cell. But, under pathologic condition the balance between ROS and antioxidant defense mechanism is lost. Overproduction of ROS and other free radicals then can damage cellular proteins, carbohydrates, lipids and DNA and may thus lead to oxidative stress which in turn results in a variety of diseases, such as liver cirrhosis, inflammation, atherosclerosis, diabetes, cancer, neurodegenerative disease, nephrotoxicity and also the aging process. Antioxidants have the potential to prevent these oxidative damages and thereby minimize the homeostatic disturbances.[1] The pumpkin is a vegetable crop belonging to the cucurbitaceae family. This family contains chemicals, including tetracyclic triterpenes, saponins, proteins, fibers, polysaccharides and minerals (iron, zinc, manganese, copper, etc) . The family is one of the

largest families in plant kingdom comprising of highest number of edible plant species. Seeds embedded in a bright-yellow fibrous endocarp are large, non endospermic and usually dark red in colour.[2]

Pharmacologically it is used for different activities like anti-hypercholesterolemia, anti-hypertensive, anti-inflammatory, anti-parasitic, anti-tumor, ant-oxidant, anti-diabetic, anti-carcinogenic, anti-bacterial, intestinal and anti-inflammation. Different categories of phyto-constituents contain in *Cucurbita pepo* such as linoleic acids, alkaloids, flavonoids and palmitic which may be responsible for its medicinal properties. *Cucurbita pepo* is one of the good supplement of protein, carbohydrate, minerals and fat. This coupled with high mineral content which is advantageous for human and animals [3].

However, because the proportion of each phytochemicals in the plant of same species vary in different regions, we standardized the pumpkin by measuring the amounts of its phenolic and polysaccharide compounds as well as antioxidant activity[4].

Materials and Methods

Collection and authentication of plant:

The fruit of *cucurbita pepo* were collected from the surrounding areas of Rasipuram, Tamilnadu, India during the month of July and authenticated by Botanical survey of India (BSI) southern circle, Coimbatore, Tamilnadu. The authentication certificate number is No.BSI/SRC/5/23/2018/Tech/1503.Soon after collection the fruit were cleaned, dried in shade and crushed to a coarse powder, stored in an air tight plastic container, until further use.

Extraction of fruit material:

Coarsely powdered fruit of *cucurbita pepo* were defatted by using petroleum ether(60–80°C) and then extracted with hydoalcohol using Soxhlet apparatus for about 72 h at 40°C.After that the sediment was filtered with Whatman no.1 filter paper (Whatman Ltd, England). The fruit extract was further concentrated under vacuum using rotary vacuum evaporator (Buchan R-V120, Switzerland) at 40°C. The obtained crude extract was weighed and stored at 4 ° C for the further analysis [5].

Preliminary phytochemical analysis

The hydroalcoholic fruit extract of *Cucurbita pepo* was subjected to phytochemical evaluation and identified the various plant constituents present in the test sample both qualitatively and quantitatively.

The following studies were carried out in phytochemical analysis.

Preparation of sample

A small quantity of the extract was dissolved in 5ml of distilled water and then filtered. The filtrate was tested to detect the presence of different phytochemical constituents in the sample.

Detection of carbohydrate:

a. Molisch's test:

1 ml of filtrate was treated with 2-3drops of 1% alcoholic α-napthol solution and 2 ml of conc. sulphuric acid was added along the sides of the test tube. Appearance of brown to violet ring, indicate the presence of carbohydrate.

b. Fehling's test:

To the Fehling solution A and B extract was added and boiled. The formation of brick red precipitate indicates the presence of reducing sugar.

c. Benedict's test:

1 ml of extract was added to 5ml of Benedict's reagent, was added and boiled for 2 mins and cool. Formation of a red precipitate shows the presence of sugars.

Test for glycosides:

a. Legal's test:

The filtrate was hydrolyzed with dilute hydrochloric acid and heated on water bath. Then added 1ml of pyridine and few drops of sodium nitroprusside solution, made alkaline with sodium hydroxide solution. Appearance of pink to red colour shows the presence of glycosides.

b. Baljet test:

1ml of extract was added to 1ml of sodium picrate solution and the yellow to orange colour shows the presence of glycosides.

c. Keller killiani test:

To 2ml of extract, add glacial acetic acid, trace quantity of ferric chloride and add 2 to 3 drops of concentrated sulphuric acid. reddish brown colour appears at the junction of two liquid indicates the presence of cardiac glycosides.

Detection of alkaloids:

Small quantity of extract was treated with few drops of dilute hydrochloric acid and filtered it. The filtrate was collected and subjected for tests with following reagents.

a. Mayer's reagent:

To the filtrate potassium mercuric iodide was added. The formation of cream colour precipitate, it shows the presence of alkaloids.

b. Dragendroff's reagent:

To the filtrate potassium bismuth iodide was added. If it shows reddish brown precipitate, indicates the presence of alkaloids.

c. Wagner's reagent:

To the filtrate iodine in potassium iodide solution was added. If it shows reddish brown precipitate, indicates the presence of alkaloids.

d. Hager's reagent:

To the filtrate saturated aqueous solution of picric acid was added. Formation of yellow precipitate indicates the presence of alkaloids.

Detection of phytosterol and steroids:

Small quantity of extract was dissolved in 5ml of chloroform and then subjected to the following tests.

a. Salkowski test:

To the above solution 1 ml chloroform and few drops of concentrated sulphuric acid was added. The test tube was shaken for few minutes. The development of red colour in chloroform layer indicates the presence of steroids.

b. Liebermann- Burchard reaction:

To the above solution 1ml of chloroform add few drops of concentrated sulphuric acid and 1-2 ml of acetic anhydride. Development of red colour first, then blue and finally green colour, indicates the presence of steroids.

Detection of proteins and aminoacids

Small quantity of the extract was dissolved in few ml of water and filtered. The collected filtrate was used for following tests.

a. Biuret test:

Filtrate was treated with 5% sodium hydroxide and few drops of 1% copper sulphate solution. Formation of violet or pink colour indicates the presence of proteins.

b. Ninhydrin test:

To the filtrate Ninhydrin reagent was added. Development of violet or purple colour indicates the presence of amino acids.

Detection of tannins:

a. Lead acetate test:

To 5 ml of aqueous extract was treated with 1 ml of 10% lead acetate solution. Yellow colour precipitation, indicates the presence of tannins.

b. Vanillin hydrochloride test:

1ml of extract was added with vanillin hydrochloride. Formation of purplish red colour indicates the presence of tannins.

Detection of flavonoids

a. Zinc hydrochloride reduction test:

Treat extract with mixture of zinc dust and concentrated hydrochloric acid. Formation of red colour indicates the presence of flavonoids.

b. Alkaline reagent test:

To the extract, add few drops of sodium hydroxide solution. Formation of an intense yellow colour, which turns to colorless on addition of few drops of dilute hydrochloric acid, indicates the presence of flavonoids.

Detection of saponins

a. Foam test:

The extract was diluted with distilled water to 20 ml and shaken in a graduated cylinder for 15 minutes. Development of stable foam suggests the presence of saponins.

b. Froth test:

To 5 ml of test sample add few drops of sodium bicarbonate. Shake the mixture vigorously and keep it for 3 minutes. A honey comb like froth formation indicates the presence of saponins.

Detection of triterpenoids

a. Libermann-Burchard test:

To the extract add few drops of acetic anhydride, followed by few drops of concentrated sulphuric acid. A brown ring forms at the junction of two layers and the upper layer turn green colour, infers the presence of phytosterols and formation of deep red colour indicates the presence of triterpenoids.

b. Salkowski Test:

5ml of extract was mixed in 2ml of chloroform and concentrated sulphuric acid was carefully added to form a layer. Formation of reddish brown coloration of the interface indicates the presence of triterpenoids.

Test for phenols:

a. Ferric chloride test:

To 1 ml of extract, add 2 ml of distilled water followed by few drops of 10% ferric chloride. Formation of blue or green colour indicates the presence of phenols.

b. Lead acetate test:

Dilute 1 ml of extract with 5 ml of distilled water and to this add few drops of 1% lead acetate solution. Formation of yellow colour precipitate indicates the presence of phenols.

Quantification of total phenolics and flavonoids

Estimation of total phenolics

Reagents

Folin-Ciocalteu's reagent
Gallic acid (1mg/ml)
20% sodium carbonate

Preparation of standard

Standard solution was prepared by adding 10 mg of accurately weighed Gallic acid in 10 ml of distilled water.

Preparation of sample

10 mg of the accurately weighed extract was dissolved in 10 ml water and used for the estimation.

Procedure

The total phenolic content of the extract was determined by Folin-Ciocalteau assay method. To an aliquot 100 µl of extract (1mg/ml) or standard solution of Gallic acid (10, 20, 40, 60, 80, 100 µg/ml) added 50 µl of Folin-Ciocalteau reagent followed by 860 µl of distilled water and the mixture is incubated for 5 min at room temperature. 100 µl of 20% sodium carbonate and 890 µl of distilled water were added to make the final solution to 2 ml. It was incubated for 30 min in dark to complete the reaction. The absorbance of the mixture was measured at 725 nm against blank. Distilled water was used as reagent blank. The tests were performed in triplicate to get mean the values. The total phenolic content was found out from the calibration curve of Gallic acid. And it was expressed as milligrams of Gallic acid equivalents (GAE) per gram of extract[6].

Estimation of total flavonoids

Reagents

Methanol
10% aluminium chloride
1M Potassium acetate

Preparation of standard

Standard solution was prepared by adding 10mg of accurately weighed quercetin in 10 ml of ethanol.

Preparation of sample

10mg of the accurately weighed extract was dissolved in 10ml ethanol and used for the estimation.

Procedure

The total flavonoid content of the HFECP (Hydroalcoholic fruit extract of *Cucurbita pepo*) was determined by using Aluminium chloride colorimetric method. To an aliquot of 100µl of extract or standard solutions of Quercetin (10, 20, 40, 60, 80, 100µg/ml) ethanol was added separately to make up the solution upto 2ml. The resulting mixture was treated with 0.1 ml of 10% aluminium chloride, 0.1 ml of 1M potassium acetate and 2.8ml of distilled water. Shaken well and incubated at room temperature for 30 minutes. The absorbance was measured at 415nm against blank, where a solution of 2ml ethanol, 0.1ml potassium acetate, 2.8ml distilled water and 0.1ml of aluminium chloride serve as blank solution. The total flavonoid content was determined from the standard Quercetin calibration curve and it was expressed as milligrams of Quercetin equivalents (QE) per gram of extract[7].

In vitro anti-oxidant study

Various methods were used to investigate the antioxidant property of samples. In the present study the antioxidant properties of various extracts were evaluated by *in vitro* methods. The antioxidant

properties could not be concluded based on the single antioxidant test method. It is in practice that generally several in vitro test procedure are carried out to conclude the antioxidant properties of the sample. Among various free radical scavenging methods DPPH, ABTS, FRAP and Total antioxidant assays was carried out in the present study.

DPPH Free radical scavenging assay

Principle

The molecule of 1, 1-diphenyl-2-picrylhydrazyl (α, α-diphenyl-β-picryl hydrazyl; DPPH) is characterized as a stable free radical by virtue of the delocalization of the spare electron over the molecule as a whole, so that the molecules do not dimerise, as would be the case with most other free radicals. The delocalization also gives rise to the deep violet color, characterized by an absorption band in ethanol/methanol solution centred at about 520 nm. When a solution of DPPH is mixed with that of a substance that can donate a hydrogen atom, then this gives rise to the reduced form with the loss of this violet color (although there would be expected to be a residual pale yellow color from the picryl group still present). Representing the DPPH radical by Z• and the donor molecule by AH, the primary reaction is Z• + AH = ZH + A•.

Procedure

A solution of 0.135 mM DPPH in methanol was prepared and 1.0 ml of this solution was mixed with 1.0 ml of extract in methanol containing 0.02–0.1 mg of the extract. The reaction mixture left in the dark at room temperature for 30 min. The absorbance of the mixture was measured spectrophotometrically at 517 nm. Ascorbic acid and BHT were used as references. The ability to scavenge DPPH radical was calculated by the following equation: DPPH radical scavenging activity (%) = [(Abs Control - Abs Sample)]/ (Abs Control)] x 100 where Abs Control is the absorbance of DPPH radical + methanol; Abs Sample is the absorbance of DPPH radical + sample extract /standard[8] .

ABTS Free radical scavenging assay

Principle

In ABTS decolorization assay, the peroxidase substrate 2, 2'- azinobis (3- ethylbenzothiazoline- 6-sulfonic acid) (ABTS), forms a relatively stable radical (ABTS +)upon one electron oxidation. This assay is based on the scavenging of light by ABTS radicals. An antioxidant with an ability to donate a hydrogen atom will quench the stable free radical and inhibits the absorption of the radical cation which has characteristic long- wavelength absorption spectrum showing maxima at 660, 734, and 820 nm.

The relatively stable ABTS radical has a green color and is quantified spectrometrically at 734 nm.

Procedure

To determine ABTS radical scavenging assay, The stock solutions included 7 mM ABTS solution and 2.4 mM potassium persulfate solution. The working solution was then prepared by mixing the two stock solutions in equal quantities and allowing them to react for 12 h at room temperature in the dark. The solution was then diluted by mixing 1 ml ABTS solution with 60 ml methanol to obtain an absorbance of 0.706 ± 0.001 units at 734 nm using the spectrophotometer. Fresh ABTS solution was prepared for each assay. Plant extracts (1 ml) were allowed to react with 1 ml of the ABTS solution and the absorbance was taken at 734 nm after 7 min using the spectrophotometer. The ABTS scavenging capacity of the extract was compared with that of BHT and percentage inhibition calculated as ABTS radical scavenging activity(%) = [(AbsControl- AbsSample)]/(AbsControl) x 100 where Abs Control is the absorbance of ABTS radical + methanol; AbsSample is the absorbance of ABTS radical + sample extract /standard percentage inhibition (I %) = (Abs control- Abs sample /Abs control) X 100. Different sample concentrations were used in order to obtain calibration curves and to calculate the IC50 values. (IC50 - concentration required to obtain a 50% radical scavenging activity) [8][9].

Ferric Reducing Anti-Oxidant Power Assay (FRAP)

Reagents

Acetate buffer (pH. 3.6)
20mMol TPTZ (2,4,6, - tripyridyl-5-triazine solution)
20mMol Ferric chloride
Vitamin-C (10mg/10ml)

Principle

The basic principle involved in the assay is the reduction of the ferric complex of Fe(TPTZ) 3+ i.e: tripyridyltriazine (a ferroin analogue) to Fe(TPTZ) 2+ complex (intensely blue in color) in the presence of anti-oxidant in an acidic pH. There will be an increase in absorbance value at 593nm.

Procedure

The determination of the total antioxidant activity (FRAP assay) in the extract is a modified method of Benzie and Strain. The stock solutions included 300 mM acetate buffer (3.1 g $C_2H_3NaO_2 \cdot 3H_2O$ and 16 ml $C_2H_4O_2$), pH 3.6, 10 mM TPTZ (2, 4, 6-tripyridyl-s-triazine) solution in 40 mMHCl, and 20 mM $FeCl_3 \cdot 6H_2O$ solution. The fresh working solution was

prepared by mixing 25 ml acetate buffer, 2.5 ml TPTZ, and 2.5 ml FeCl3·6H2O. The temperature of the solution was raised to 37 °C before use. Plant extracts (150 µL) were allowed to react with 2850 µl of the FRAP solution for 30 min in the dark condition. Readings of the colored product (ferrous tripyridyltriazine complex) were taken at 593 nm. The standard curve was linear between 200 and 1000 µM FeSO4. Results are expressed in µM Fe (II)/g dry mass and compared with that of quercetin[10].

Total antioxidant activity

Plant extracts were dissolved in methanol to obtain a concentration of 500 µg/ml. 3 ml of extract was placed in a test tube, 0.3 ml of reagent solution (0.6 M Sulphuric Acid, 28mM Sodium Phosphate, 4mM Ammonium molybdate) was then added and the resulting mixture was incubated at 950C for 90 minutes. After the mixture was cooled to room temperature, the absorbance of the each solution was measured by using UV-Visible spectrophotometer at 695nm against blank. The experiment was performed in triplicate. A calibration curve was constructed, using ascorbic acid (100-500 µg/ml) as standard and total antioxidant activity of extract (µg/ml) expressed as ascorbic acid equivalents. Total phenolic content Total phenolic content were quantified and expressed as Gallic acid equivalent [11].

Results

Preliminary phytochemical analysis

Table 1: Qualitative Chemical Tests

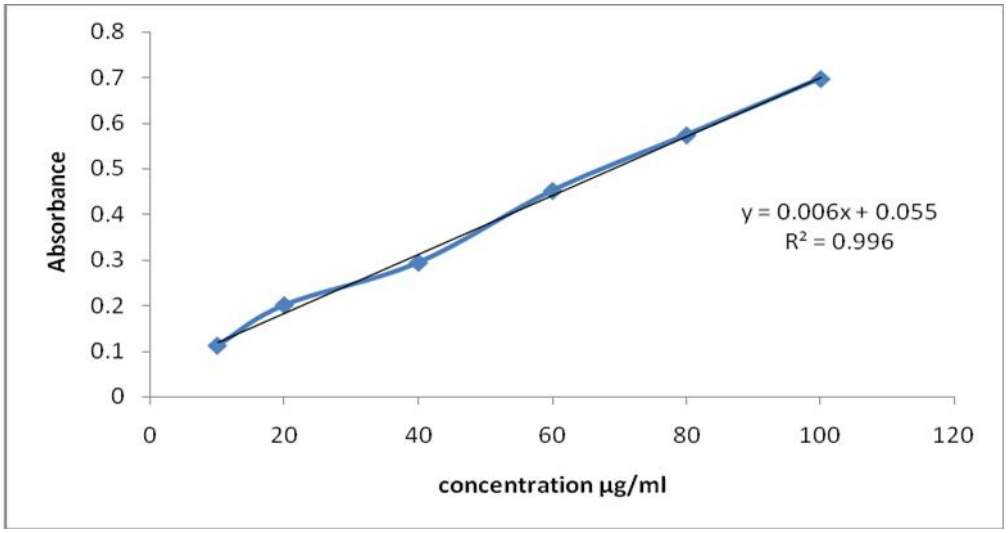
S.No	Phytochemical analysis	Hydroalcoholic extract of *Cucurbita pepo.*
1	Alkaloids	Positive
2	Glycosides	Positive
3	Flavonoids	Positive
4	Terpenoids	Positive
5	Tannins	Positive
6	Carbohydrates	Positive
7	Steroids	Positive
8	Protein and aminoacid	Positive
9	Phenols	Positive
10	Saponin	Negative

Determination of total phenol

Table 2: Concentration and Absorbance of Standard Gallic acid and Sample extracts

Sample	Concentration (µg/ml)	Absorbance
Standard (Gallic acid)	10	0.1124
	20	0.2014
	40	0.2945
	60	0.4521
	80	0.5741
	100	0.6978
Sample	100	0.4765

Figure 1: Standard graph for Gallic acid for the estimation of total phenolic content



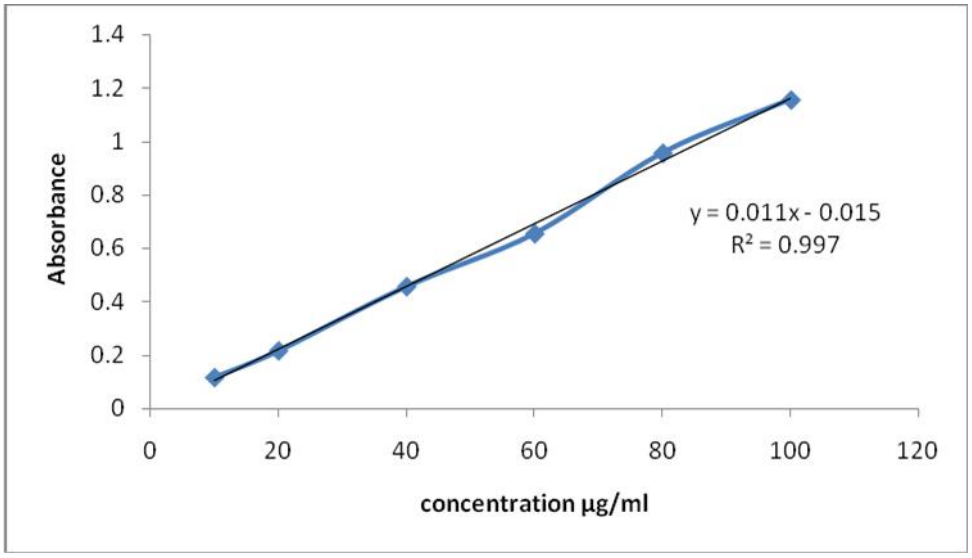
Total Phenol content present in HFECP was found to be 70.25 mg/g of extract calculated as Gallic acid equivalent.

Estimation of total flavonoid content of HFECP

Table 3: Concentration and Absorbance of Standard Quercetin and Sample extracts

Sample	Concentration (µg/ml)	Absorbance
Standard (Quercetin)	10	0.1151
	20	0.2154
	40	0.4563
	60	0.6547
	80	0.9571
	100	1.1569
Sample	100	0.3014

Figure 2: Standard graph of Quercetin for the estimation of total flavonoid content



Total flavonoids content present in HFECP was found to be 26.03 mg /g extract calculated as quercetin equivalent.

In vitro Anti oxidant study

DPPH Radical scavenging activity

Table 4: Percentage inhibition and IC_{50} value of DPPH radical by quercetin and HFECP

Treatment	Concentration(µg/ml)	% of inhibtion	IC_{50} Value (µg/ml)
Standard (Quercetin)	20	49.20	5.981
	40	59.14	
	60	70.36	
	80	85.33	
	100	97.87	
Sample (HFECP)	20	35.68	33.74
	40	47.86	
	60	68.54	
	80	89.45	
	100	94.74	

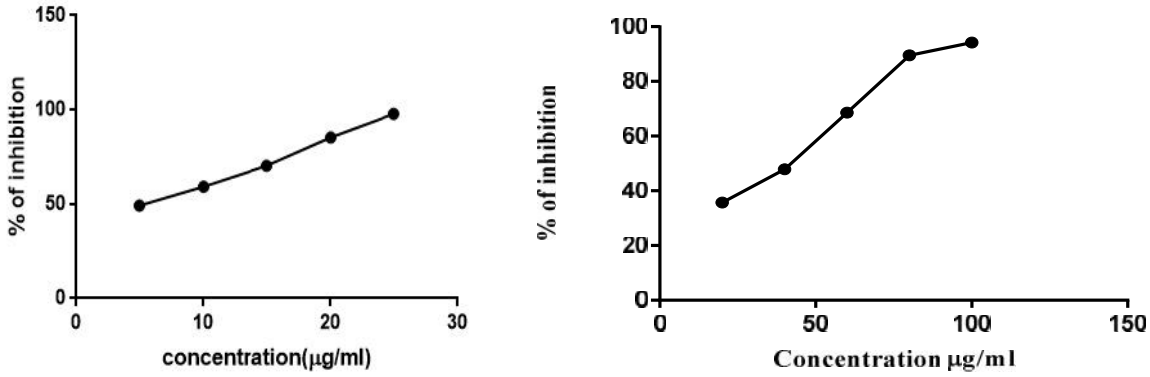


Figure 3: DPPH radical scavenging activity

ABTS Radical scavenging activity

Table 5: Percentage inhibition and IC_{50} value of ABTS radical by quercetin and HFECP

Treatment	Concentration (µg/ml)	% of inhibtion	IC_{50} Value (µg/ml)
Standard (Quercetin)	20	54.34	0.2013
	40	65.55	
	60	72.31	
	80	75.38	
	100	80.25	
Sample (HFECP)	20	56.71	13.44
	40	67.26	
	60	72.31	
	80	75..38	
	100	80.25	

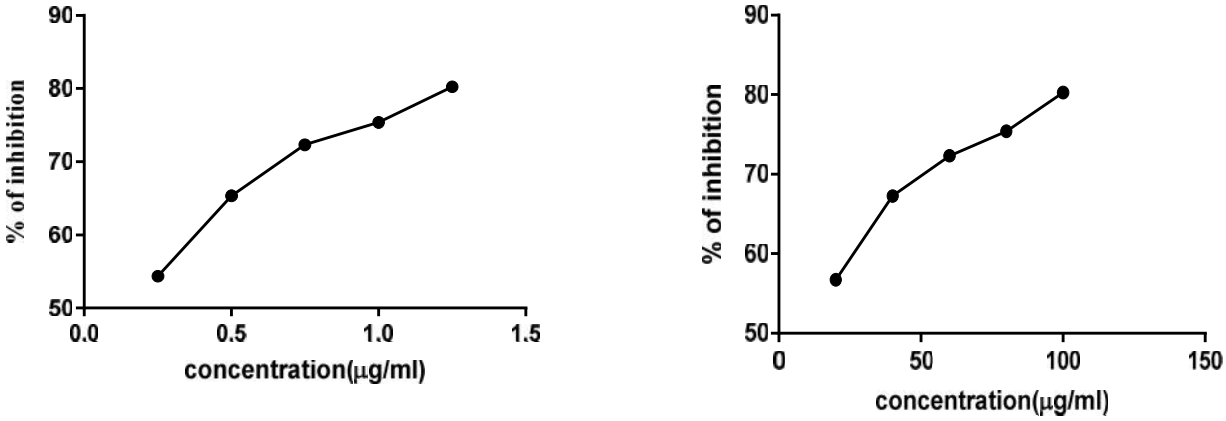


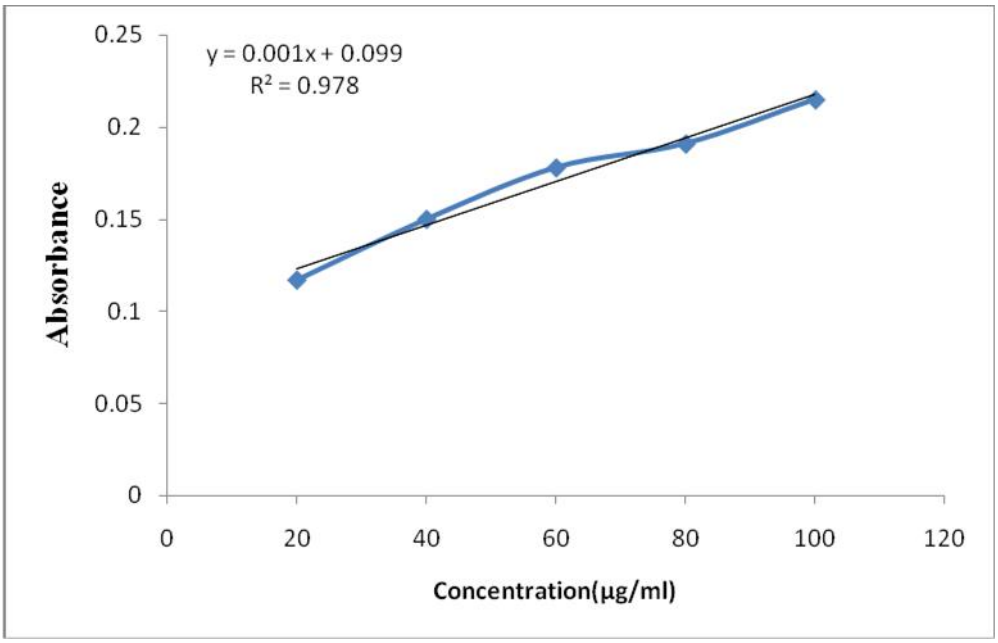
Figure 4 : ABTS radical scavenging activity

FRAP Radical scavenging activity

Table 6: Percentage inhibition and IC_{50} value of FRAP radical by quercetin and HFECP

Sample	Concentration (µg/ml)	Absorbance at 593nm
Standard (Vitamin-C)	20	0.129
	40	0.325
	60	0.457
	80	0.735
	100	0.952
Sample (HFECP)	100	0.874

Figure 5: FRAP radical scavenging activity



The total antioxidant activity of FRAP assay 77.7 µg/ml equivalent to Vitamin- C

Total antioxidant activity

Table 7: Percentage inhibition and IC_{50} value of Total antioxidant radical by Vitamin-C and HFECP

Sample	Concentration (µg/ml)	Absorbance at 695nm
Standard (Vitamin-C)	20	0.117
	40	0.150
	60	0.178
	80	0.191
	100	0.215
Sample (HFECP)	100	0.184

Discussion

Free radicals are chemical entities that can exist separately with one or more unpaired electrons. The propagation of free radical can brings about many adverse reactions leading to extensive tissue damage. Lipid proteins are all susceptible to attack by free radical. Many plant species with antioxidant activities act as protective agents against these radicals. In the present investigation potent antioxidant activity of hydroalcoholic fruit extract of *Cucurbita pepo* was observed using different methods. However the efficacy of extract to scavenge the different radicals differed in each method depending upon the mechanism of free radical scavenging and assay methodology. The result of DPPH scavenging activity assay in this study indicated that the plant was potently active. This suggested that the fruit extract did contain compounds that could be capable of donating hydrogen to a free radical in order to remove the odd electron which is responsible for the radical's reactivity[12].

FRAP assay directly measured antioxidant or reductants in a sample that react with ferric tripyridyltriazine (Fe3+ TPTZ) complex and produce colored ferrous tripyridyltriazine (Fe2+ TPTZ). The antioxidant ability of hydroalcoholic fruit extract of *Cucurbita pepo* showed higher FRAP antioxidant activity. The phenolic compounds exhibited reduction properties by acting as reducing agents, hydrogen donators and singlet oxygen quenchers[13].

The ABTS assay is based on the inhibition of the absorbance of radical cation, $ABTS^{+}$, which has a characteristic wavelength at 734 nm, by antioxidants. The principle behind the technique involves the reaction between ABTS and potassium per sulphate to produce the ABTS radical cation ($ABTS^{+}$) which is a bluegreen chromogen. In the presence of antioxidant reductant, the coloured radical is converted back to colourless ABTS. The order of ABTS radical scavenging activity of the fruit extract was almost similar to that observed for DPPH.

Conclusion

The results obtained in the present study indicated that *Cucurbita pepo* fruit extract exhibited free radical scavenging activity against hydrogen peroxide and DPPH. The overall antioxidant activity of *Cucurbita pepo* might be attributed to its polyphenolic content and other phytochemical constituents. The findings of the present study suggested that *Cucurbita pepo* could be a potential source of natural antioxidant that would have great importance as therapeutic agents in preventing or slowing the progress of reactive oxygen species and associated oxidative stress related degenerative diseases.

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DOI: 10.22192/ijcrcps.2019.06.03.003	

How to cite this article:
Srividhya V, Sengottuvel Thangavel, Gopala Satheeskumar K, Kanupriya J, Arihara Sivakumar G. (2019). Antioxidant potential and Phytochemical analysis of fruit extract of *Cucurbita pepo*. Int. J. Curr. Res. Chem. Pharm. Sci. 6(3): 22-32.
DOI: http://dx.doi.org/10.22192/ijcrcps.2019.06.03.003