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Research Article

EVALUATION OF TOTAL PHENOL, FLAVONOID CONTENTS AND *INVITRO* ANTIOXIDANT ACTIVITY OF *BENINCASA HISPIDA* FRUIT EXTRACTS

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ABSTRACT

Oxygen is an element obligatory for life. Biological combustion produces harmful intermediates called free radicals. Free radicals causes damage to the cells, inspite of the body possessing defense mechanisms like enzyme and antioxidants. Antioxidants scavenge the free radicals that are produced in the body and their levels are depleted. Hence there is a need for new antioxidants from plant sources. So the present study was designed to investigate phytochemicals quantitatively and antioxidant activity of *Benincasa hispida* fruit extracts. Ethyl acetate, ethanol and aqueous extract of *Benincasa hispida* fruit was subjected to quantitative analysis for total phenols, total flavonoids, total antioxidant and free radical scavenging activity by various methods like DPPH, Nitric oxide, Superoxide, Hydroxyl radicals and Hydrogen peroxide scavenging activity and reducing capacity. All the extracts showed significant activities in all the assays compared to the standard antioxidant BHT in a dose dependent manner. IC_{50} value for each radical was calculated. The results showed that ethanolic extract of *Benincasa hispida* had high amounts of phenols, flavonoids and had better scavenging capacity. *Benincasa hispida* fruit extracts significantly inhibited super oxide anion radicals compared to other radicals. The IC_{50} value for super oxide anion radical for ethanol extract was 128±1.91µg/ml, ethyl acetate extract was 184±1.29µg/ml and for aqueous extract was found to be 271±1.89 µg/ml. So *Benincasa hispida* fruit was found to be a good source of many natural antioxidant compounds. Hence it could be considered as alternate antioxidant.

Keywords: *Benincasa hispida*, DPPH, Butylated hydroxy toluene, Nitric oxide.

INTRODUCTION

People of India have been using thousands of medicinal plants for curing various diseases and about 8000 herbal remedies have been identified in Ayurveda. The medicinal plants and their parts like leaves, flowers, fruits, seeds, bark and heartwood have been used as an excellent source in treating various diseases as they possess active constituents that are used in the treatment of many human diseases[1]. Reactive oxygen species[ROS], otherwise known as active oxygen species in some cases which are in various forms of activated oxygen, include free radicals such as super oxide ions(O_2-) and as well as non free radical species such as hydrogen peroxide(H_2o_2)[2]. Free radicals cause pathological conditions such as cancer, Ischaemia, Asthma, arthritis, inflammation and neurodegenerative diseases like Parkinson's, Mongolism, ageing process and perhaps dementias[3]. Antioxidants have capacity to scavenge the free radicals and prevent the damage caused by free radicals. They can reduce the oxidants by neutralizing the free radicals before they can attack the cell and prevent damage to lipids, proteins, enzymes, carbohydrates and DNA[4]. Synthetic antioxidant compounds such as Butylated hydroxy toluene which is used in food processing may cause side effects[5]. Antioxidant rich plant extracts are used

as antioxidant scavengers as they possess fewer side effects. *Benincasa hispida* belongs to cucurbitaceae family. It is a tendril climber which is cultivated throughout India. Fruit of this plant are traditionally used to treat Cardiotonic, renal diseases, epilepsy, jaundice, dyspepsia, fever and menstrual disorders.The methanolic extract of *Benincasa hispida* fruit is reported to possess antiulcer, anti-inflammatory and antidepressant activities[6]. Hence the present study was designed to estimate the total phenols and flavonoids contents quantitatively and antioxidant potential of ethyl acetate, ethanol and aqueous extracts of *Benincasa hispida* using different methods.

MATERIALS AND METHODS

Collection of plant materials

Plant specimen for the proposed study was collected from medicinal plant vendor. Care was taken to select healthy fruits. The *Benincasa hispida* fruit was authenticated by Dr. P. Jayaraman, Director of National Institute of Herbal science, Plant Anatomy Research Centre, Chennai.

Preparation of extract

The *Benincasa hispida* fruit (pumpkin) skin was peeled off and seeds were removed. The fruit plup were taken cut into pieces, dried and then ground to powder form. The dried powder was extracted with ethyl acetate, ethanol and water using soxhlet apparatus. The extract was stored in a glass bottle in refrigerated condition throughout the period of experiment.

Estimation of Total phenols

Total phenolic content was estimated using the Folin-Ciocalteu method of Yu et al.,[7]. Extract (100µL) was mixed thoroughly with 2 ml of 2% Na2CO3. After 2 minutes 100µl of Folin-Ciocalteu reagent was added to the mixture. The resulting mixture was allowed to stand at room temperature for 30 min and the absorbance was measured at 743 nm against the blank. A calibration curve was established using varying concentration of gallic acid. The values were expressed in mg/g of sample.

Estimation of Total Flavonoids

The determination of flavonoids was performed according to the colorimetric assay of Chang et al., [8]**.** To 1ml of extract, 3 ml of methanol, 0.2ml of 1 M potassium acetate, 0.2ml of 10% aluminium chloride and 5.6ml of distilled water was added and left at room temperature for 30 minutes. Absorbance of the mixture was read at 415 nm using UV spectrophotometer. Calibration curve was prepared using quercetin as standard.

Determination of Total Antioxidant Capacity

The assay is based on the reduction of molybdenum (VI) to molybdenum (V) by the extract and the subsequent formation of a green phosphate Mo (V) complex at acid pH Preito et al.,[9]. An aliquot of sample solution (100 µg/ml was combined with reagent solution (0.6 M Sulfuric acid, 28mM Sodium Phosphate and 4mM Ammonium molybdate). The tubes were capped and incubated in a boiling water bath at 95^0C for 60-90 min. The samples were then cooled at room temperature and the absorbance was measured at 695 nm against the blank in spectrophotometer. The values were expressed as equivalents of BHT.

DPPH Radical scavenging activity

DPPH scavenging activity of the plant extract was carried out according to the method of Koleva et al.,[10] and Mathiesen et al [11]. 0.2 of plant extract samples at different concentration (50,100,250,500 and 1000µg /ml) was mixed with 0.8 ml of Tris Hcl buffer (100Mm, pH 7.4). One ml DPPH (500 M in ethanol) solution was added to above mixture. The mixture was shaken vigorously and incubated for 30min in room temperature. Absorbance of the resulting solution was measured at 517nm UV Visible Spectrophotometer. BHT was used as standard. Percentage of DPPH scavenging activity was determined. IC_{50} value was calculated. Decrease in absorbance of the reaction mixture indicates stronger DPPH radical scavenging activity.

Determination of reducing power

Reducing power of *Benincasa hispida* was determined by method of Oyaizu[12]. 1ml of *Benincasa hispida* extract (concentrations 20,40,60,80 and 100µg/ml) were mixed with 2.5ml phosphate buffer (0.2 M, pH 7.6) and 2.5 ml potassium ferricyanide [K3 Fe(CN)6] (1%, w/v), and then the mixture was incubated at 50°C for 30 min. After incubation 2.5 ml of trichloroacetic acid (10%, w/v) was added to the mixture, which was then centrifuged at 3000 rpm for 10 min. Finally, 2.5 ml of upper-layer was mixed with 2.5 ml distilled water and 0.5 ml FeCl3 (0.1%) was added. The absorbance was measured at 700nm. BHT was used as standard antioxidant. Increased absorbance of the reaction mixture indicated increased reducing power.

Inhibition of Nitric oxide Generation

The nitric oxide generation was studied using Griess reagent method as explained by Green et

al.,[13]. 3 ml of reaction mixture containing sodium nitroprusside in PBS and extracts (concentration 50, 100, 250, 500 and 1000µg/ml) was incubated at 25°C for 150 minutes. Controls were kept without sample in an identical manner. After incubation, 0.5 ml of Griess reagent was added. The absorbance of the chromophore formed was read at 546nm. The percentage inhibition of nitric oxide generation was measured by comparing the absorbance values of control and test and IC50 value was calculated.

Determination of Hydroxyl radical scavenging activity

Deoxy ribose method[14] was used to determine the hydroxyl radical scavenging activity. The reaction mixture which contained sample (concentration 50,100,250,500 and1000µg/ml) Deoxy ribose (3.75mM), H2o2(1mM), potassium phosphate buffer(20mM, pH=7.4), Fecl3(0.1mM), EDTA(0.1mM) and ascorbic acid(0.1mM), was incubated in a water bath at 100°C for 20 minutes. The absorbance of the resulting solution was measured in UV- Visible spectrophotometer at 532nm.

Hydrogen Peroxide Scavenging Activity

The ability of the extracts to scavenge hydrogen peroxide radical was determined by measuring the decrease in absorbance at 230nm spectrophotometrically, as explained by Ruch et al., [15]. The solution of H_2o_2 (4 mM) was prepared in Phosphate buffer (pH 7.4). The extract concentration (50,100,250,500 and 750 µg/ml) is added to 0.6ml of H_2o_2 solution. The absorption of H_2o_2 is determined at 230nm after 10 minutes against a blank solution containing phosphate buffer without H_2o_2. This was compared with BHT. The percentage of H_2O_2 scavenged by the extract was calculated.

Superoxide anion radical scavenging assay

This assay was carried out according to the method of Liu et al., [16] Superoxide anion radical was generated in PMS-NADH system by oxidation of NADH and assayed by the reduction of NBT to blue formazan. To one ml of (156 µM) nitroblueT, add 1 ml of (468 µM) of nicotinamide adenine dineucleotide and 0.5 ml of varying concentration (50,100,250,500 and 1000µg/ml) of extract. To this mixture 100µl of phenazine methosulphate was added and incubated at 25°C for 5 minutes. Absorbance was read at 560 nm.

RESULTS

The Table1 shows the quantitative analysis of phytochemicals such as phenols, flavonoids and Total antioxidant capacity of *Benincasa hispida* fruit extracts.

Total Phenols

Phenols in different extracts of *Benincasa hispida* fruit was shown in Table 1 Hence ethyl acetate shows 23.66±1.77mg/g, ethanol has 91.75±0.92 mg/g and aqueous extract shows 80.71±0.94 mg/g.

Total Flavonoids

Ethyl acetate extract shows 16.76±1.46 mg/g, Ethanol extract shows 70.46±0.80 mg/g and aqueous extract shows 65.65±0.94 mg/g.

The total antioxidant capacity

The total antioxidant capacity was shown in Table 1. The total antioxidant capacity was measured as equivalent of BHT (mg/g of extract). The Ethyl acetate extract has 120±1.78, Ethanol extract 140.16±1.47 mg/g and aqueous extract 113.33±1.63 mg/g.

Table 2 shows IC_{50} value calculated for all the free radicals**.**

DPPH radical scavenging activity

IC_{50} value is 454±4.65µg for ethyl acetate extract, ethanolic extract was found to be 228±5.68 µg and aqueous extract was 254±7.63 µg respectively. IC_{50} value of BHT was found to be 197±6.11 µg. The results were shown in Figure 1.

Reducing activity

The ethyl acetate, ethanol and aqueous extract of *Benincasa hispida* were found to possess concentration dependent reducing power and the result are given in Figure 2. *Benincasa hispida* exhibited good reducing power compared with BHT at concentration between 20-100µg/ml. High absorbance indicates high reducing power.

Nitric oxide radical scavenging assay

Ethyl acetate extract of *Benincasa hispida* decreased the generation of Nitric oxide significantly *in vitro* condition compared to all the extracts. This can be compared with standard BHT. The results are given in Figure 3. The IC_{50} value for Nitric oxide radical of ethyl acetate, ethanol and aqueous extracts of *Benincasa hispida* were found to be 331±4.04µg, 338±3.44 µg and 394±1.44 µg respectively. IC_{50} value of standard BHT was found to be 250±0.85 µg.

Hydroxyl scavenging activity

The effect of *Benincasa hispida* fruit extract and standard BHT on hydroxyl radical was

compared and shown in Figure 4. The IC_{50} value for hydroxyl radical of ethyl acetate, ethanol and aqueous extracts of *Benincasa hispida* were found to be 314±1.00µg, 254±1.91 µg and 284±2.38 µg respectively. Hence The IC_{50} value of standard BHT was found to be 246±1.73 µg. The results are given in Table 2.

Hydrogen peroxide radical scavenging activity

Both *Benincasa hispida* and BHT significantly inhibited hydrogen peroxide radical activity in a dose dependent manner. The IC_{50} value for hydrogen peroxide radical of ethyl acetate, ethanol and aqueous extracts of *Benincasa hispida* are found to be 248±4.76µg, 243±1.52 µg and 285±3.51 µg respectively. While IC_{50} value of standard BHT was 144±2.00 µg and this was shown in Figure 5 and Table 2.

Superoxide anion radical scavenging assay

Benincasa hispida and BHT significantly inhibited superoxide radicals in a dose dependent manner and the result are given in figure 6. The IC_{50} value for super oxide radical of ethyl acetate, ethanol and aqueous extracts of *Benincasa hispida* were found to be 184±1.29µg, 128±1.91 and 271±1.89 respectively. IC_{50} value of BHT was found to be 74.4±1.80 µg. The results are shown in Table 2 and Figure 6.

DISCUSSION

The quantitative phenol content of *Benincasa hispida* extracts were ranging 23.66±1.77 to 91.75±0.92mg/gm. The ethanolic extracts showed higher phenol content is 91.75±0.92 compared to ethyl acetate and aqueous extracts. Phenolic compounds are considered to be the most important antioxidants of plant materials. They constitute one of the major groups of compounds acting as primary antioxidants few radical terminators. Antioxidant activity of phenolic compounds is based on their ability to donate hydrogen atoms to free radicals. In addition, they possess structural properties for free radical scavenging[17]. Phenolic compounds are known as powerful chain breaking antioxidants. Phenols are very important plant constituents because of their hydroxyl groups and may contribute directly to antioxidative action. It is suggested that polyphenolic compounds have inhibitory effects on mutagenesis and carcinogenesis in humans[18 19]. Phenolics have antioxidative, antidiabetic, anticarcinogenic, antimicrobial, antiallergic, antimutagenic and antiinflammatory activities[20 21]. Flavonoid content of *Benincasa hispida* extracts were ranging from 16.76±1.46 to 70.46±0.80mg/gm of extacts. Compared with other extract ethanolic extract has higher quantity is about 70.46±0.80. Flavonoids possesss anti-inflammatory, antioxidant, anti-allergic, hepatoprotective,anti-thrombic, antiviral and anti-carcinogenic activity [21 22]. Flavonoids, on the other hand, are potent water soluble antioxidants and free radical scavengers, which prevent oxidative cell damage and have strong anticancer activity[24 25]. The total antioxidant also increased in ethanolic extracts is 140.16±1.47 compared with ethyl acetate and aqueous extracts. Hence the extract possessed significant antioxidant activity, which was capable of reducing DPPH radical to its corresponding hydrazine hence the change in colour depends on the number of electrons [26]. Ethyl acetate, ethanol and aqueous extract of *Benincasa hispida* exhibited good reducing power. The reducing ability of a compound generally depends on the presence of reductants which have been exhibited by antioxidative potential by breaking the free radical chain,donating a hydrogen atom [27].The hydroxyl radical is the most reactive of the reactive oxygen species, and it induces severe damage to adjacent biomolecules. The hydroxyl radical can cause oxidative damage to DNA, lipids and proteins[28]. H2o2 itself is not very reactive, but it can sometimes be toxic to cell because it may give rise to hydroxyl radical in the cells[14] Superoxide anion is produced from molecular oxygen due to oxidative enzymes[29]. *Benincasa hispida* fruit extracts scavenge all free radicals. The reducing capacity, super anion radical and Hydrogen peroxide radical scavenging capacity of *Benincasa hispida* fruit extracts is a significant indicator of its antioxidant potential. The order of radical scavenging activity is in following manner BHT>ethanol>aqueous>ethyl acetate. So that ethanol extract has better scavenging capacity compared to ethyl acetate and ethanol.

Table 1: Total phenol, Flavonoid and Antioxidant level of *Benincasa hispida* fruit extracts

S. No.	Parameter	Concentration mg/g of extract		
		Ethyl acetate	Ethanol	Aqueous
1	Total Phenol(gallic acid/gm of extract)	23.66±1.77	91.75±0.92	80.71±0.94
2	Total Flavonoid(Quercetin/gm of extract)	16.76±1.46	70.46±0.80	65.65±0.94
3	Total Antioxidant(BHT/gm of extract)	120±1.78	140.16±1.47	113.33±1.63

Table 2: IC_{50} value of different extracts of *Benincasa hispida* fruit

S. No.	Free radical scavenging method	50% Inhibition Concentration(IC_{50}) in µg			
		Ethyl acetate	Ethanol	Aqueous	BHT Standard
1	DPPH	454±4.65	228±5.68	254±7.63	197±6.11
2	Nitric oxide	331±4.04	338±3.44	394±1.44	250±0.85
3	Hydroxyl	314±1.00	254±1.91	284±2.38	246±1.73
4	Hydrogen peroxide	248±4.76	243±1.52	285±3.51	144±2.00
5	Super oxide	184±1.29	128±1.91	271±1.89	74.40±1.80

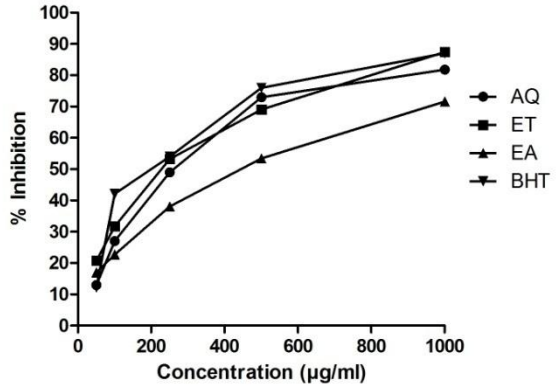

Fig. 1: DPPH radical scavenging activity

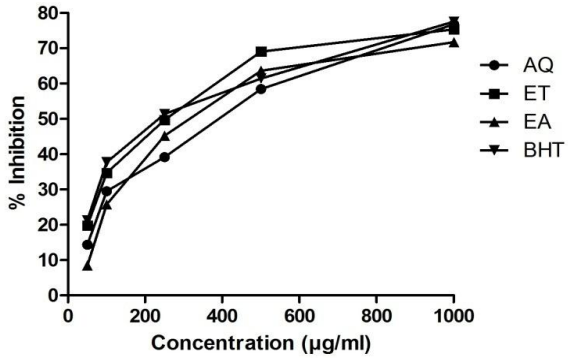

Fig. 4: Hydroxyl radical scavenging activity

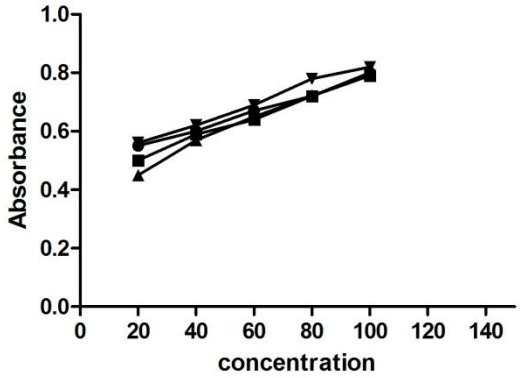

Fig. 2: Reducing activity

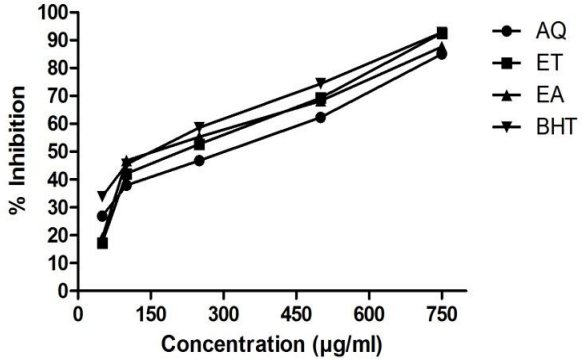

Fig. 5: H_2O_2 Peroxide radical scavenging activity

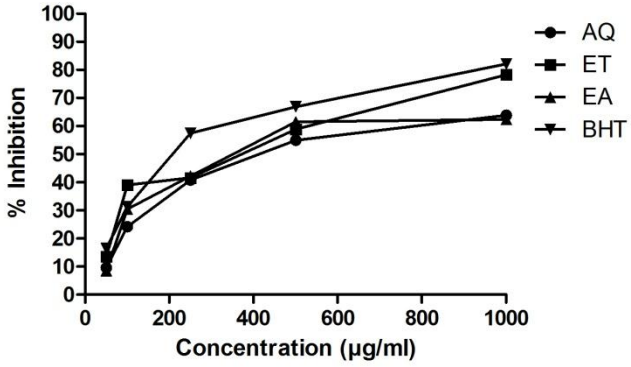

Fig. 3: Nitric oxide radical scavenging activity

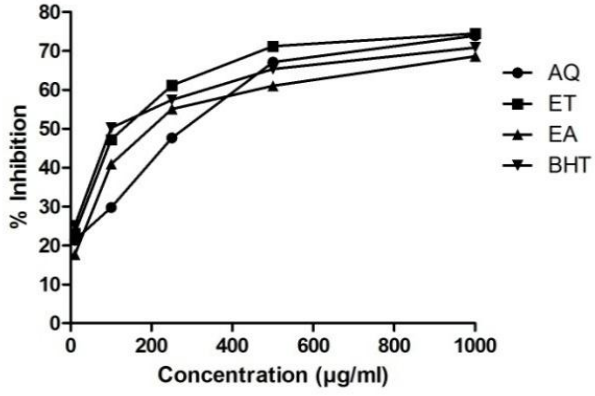

Fig. 6: Super oxide radical scavenging activity

Legends: AQ-Aqueous extract, **ET**-Ethanolic extract, **EA**-Ethyl acetate extract, **BHT**- Butylated hydroxy toluene.

CONCLUSION

The present study reveals that the ethanol extract of *Benincasa hispida* showed potent antioxidant activity compared to the other extracts. From the results, ethanolic extracts showed strong antioxidant activity towards Hydroxyl radical, hydrogen peroxide, DPPH, superoxide radicals, Nitric oxide and reducing activity. *Benincasa hispida* fruit extracts significantly inhibited super oxide anion radicals compared to other radicals. The IC_{50} value for super oxide anion radical for ethanol extract was 128±1.91µg/ml, ethyl acetate extract was 184±1.29µg/ml and for aqueous extract was found to be 271±1.89 µg/ml. So *Benincasa hispida* fruit was found to be a good source of many natural antioxidant compounds So this extract can be exploited in future for medicinal use. However further studies are to be carried out to evaluate the antioxidant potential of the extract in an *in vivo* system using Wistar rats as animal model.

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