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Evaluation of Antioxidant Activity of *Benincasa hispida* Fruit Extracts

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ABSTRACT

The present study was to evaluate antioxidant activity of ethanolic and aqueous extract of *Benincasa hispida* (Thunb.) Cogn. fruit for their therapeutic potential. In vitro antioxidant activity was performed by 1, 1- diphenyl-2-picrylhydrazyl (DPPH) and Hydrogen peroxide (H_2O_2). For aqueous extract the scavenging activity of DPPH is 59.7% at the concentration of 200 µg/ml and the activity of H_2O_2 is 20.5% at concentration of 1000 µg/ml. For ethanolic extract The scavenging activity of DPPH is 77.4% at the concentration of 250 µg/ml and the activity of H_2O_2 is 21.3% at concentration of 1000 µg/ml. The method is compared to standard (ascorbic acid). Presence of phytochemicals like carbohydrates, proteins and amino acids, flavonoids, phenolic compounds might contribute to observed antioxidant activity. *Benincasa hispida* fruits are potential source of natural antioxidant compounds to replace synthetic antioxidants.

Keywords: *Benincasa hispida* (Thunb.) cogn. fruit, Cucurbitaceae, antioxidant activity, DPPH, H_2O_2, ascorbic acid.

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INTRODUCTION

The continuous formation of free radicals in humans' body can be controlled naturally by different beneficial compounds known as antioxidants. Oxidative stress can be caused in result of free radicals formation[23]. Aging and different chronic diseases including diabetes, cancer and cardiovascular diseases could be caused by oxidative stress. Free radicals are stabilized or deactivated by antioxidants before they attack cells. Antioxidants are important factor to maintain optimal cellular and human body health. Furthermore, fortification of food formulation by adding antioxidant compounds lead to prevention of oxidative reactions which adversely affect food quality attributes. Several epidemiologic studies revealed that consumption of foods containing high amount of antioxidant compound lowering the risk of human disease occurrence[1].

Oxidative stress depicts the existence of products called free radicals and reactive oxygen species (ROS), which are formed under normal physiological conditions but become deleterious when not being eliminated by the endogenous systems. In fact, oxidative stress results from an imbalance between the generation of reactive oxygen species and endogenous antioxidant systems. ROS are major sources of primary catalysts that initiate oxidation *in vivo* and *in vitro* and create oxidative stress which results in numerous diseases and disorders[12, 25] such as cancer[18], cardiovascular disease[29], neural disorders[28], Alzheimer's disease[30] mild congnitive impairment, Parkinsons disease[4], alcohol induced liver disease, ulcerative colitis[26], ageing, atherosclerosis[31].

Oxygen derived free radicals such as superoxide anions, hydroxyl radicals and hydrogen peroxide are cytotoxic and give rise to tissue injuries[16]. Excessive amount of ROS is harmful because they initiate bimolecular oxidation which leads to cell death and creates oxidative stress. In addition, oxidative stress causes inadvertent enzyme activation and oxidative damage to cellular system[33]. Free radical is a chemical compound which contains an unpaired electron spinning on the peripheral layer around the nucleus. The family of free radicals generated from the oxygen is called ROS which cause damage to other molecules by extracting electrons from them in order to attain stability. ROS are ions, atoms or molecules that have the ability to oxidize reduced molecules. ROS are various forms of activated oxygen, which include free radicals such as superoxide anion radicals (.O2-) and hydroxyl radicals (OH·), as well as non-free radicals (H_2O_2) and singlet oxygen[13]. In the body, free radicals are derived from two sources: endogenous sources, e.g. nutrient metabolism, ageing process etc and exogenous sources e.g. tobacco smoke, ionizing radiation, air pollution, organic solvents, pesticides, etc[5]. When oxygen traps single electron, it becomes unstable and thus very reactive, since it generates harmful chain reactions against many biological molecules. The extreme toxicity of oxygen is related to its high capability of generating free radicals and in turn destroying many major biological molecules. They can attack on lipids and proteins and destroy membranes. ROS can damage DNA and lead to mutation and chromosomal damage. Oxidized cellular thiols abstract hydrogen atoms from unsaturated fatty acids to initiate the peroxidation of membrane lipids[32]. ROS can attack various substrates in body and contribute to development of chronic diseases. For example, oxidatively modified LDL has been hypothesized to be a causative agent in the development of cardiovascular diseases. Exogenous chemicals and endogenous metabolic processes in human body produce free radicals, especially oxygen derived radicals, which are capable of oxidizing biomolecules, resulting in cell death. Superoxide anion radicals increase under stress conditions such as heavy exercise, certain drugs, infection and various disease states. During normal metabolic processes, human body generates more than 2 Kg of .O2 - per year. Cells are equipped with different kinds of mechanisms to fight against ROS and to maintain the redox homeostasis of cell. For example, antioxidant enzymes such as superoxide dismutase (SOD), catalase (CAT) and glutathione peroxidase (GPx) play important roles in scavenging the free radicals and preventing cell injury[2]. Molecules such as vitamin C and E inhibit lipid peroxidation in cell. When the mechanism of antioxidant protection becomes unbalanced in human body, antioxidant supplement may be used to help reduce oxidative damage. Several degradation reactions, which may occur on heating or during long term storage, deteriorate fats and oils and the lipid constituents of foods. Oxidation reactions and the decomposition of oxidation products are the main processes which result in decreased nutritional value and sensory quality[9]. Research has implicated oxidative and free-radical-mediated reactions in degenerative processes related to ageing and diseases such as cancer, coronary heart disease and neurodegenerative disorders such as Alzheimer's disease. The prevention or retardation of these oxidation processes is essential for the food producer and almost everyone involved in the entire food chain from “farm to fork”. Various methodologies may be employed to inhibit oxidization, including prevention of oxygen access, use of lower temperature, inactivation of enzymes catalyzing oxidation, reduction of oxygen pressure and the use of suitable packaging[34]. Another common method of protection against oxidation is to use specific additives which inhibit or retard oxidation. These oxidation inhibitors are generally known as antioxidants[15].

The fruit of *Benincasa hispida* (Thunb.) Cogn. Commonly called as ash guard, belonging to cucurbitaceae family is employed as a main ingredient in kusmanda lehyam, in Ayurvedic system of medicine. The fruit is used as rejuvenating agent and also numerous nervous disorders. For centuries it has been used in many empirical applications in India for various ailments such as GIT problems like dyspepsia and burning sensation, heart disease, vermifuge, diabetes and urinary disease[24].

However, some scientific studies carried out reveal its anti-inflammatory[10], diureti[7], anti-Alzheimer's[6], antidiarrheal[22], antiulcer[20], anorectic[21], antihistaminic activities[19]. It is also used as an antioxidant. The major constituents of this fruits are triterpenoids, flavanoids, phenolic compounds, tannins, proteins and amino acids, carbohydrates, glycosides, saccharides, carotenes, vitamins,β-sitosterol and uronic acid.

MATERIALS AND METHODS

Collection, identification and authentification of plant material

The fresh fruit of *Benincasa hispida* (Thunb.) Cogn. was collected from the local vegetable market of Solan, Himachal Pradesh. The authentification of the plant was done in the Forest Research Institute, Dehradun, Uttarakhand, India No. III-1/Dean (A)/2012/981.

Preparation of extract

The fruit were air dir dried, seeds were separated and then dried mass was powdered using mixture. 100 gm of dried powder was extracted with ethanol (500ml × 5time) using Soxhlet apparatus at 60˚C temperature. Then ethanolic extract was separate out using vacuum evaporator. Total yield of ethanolic extract was 2.5 gm. Ethanolic extract was further extracted with distilled water to give aqueous extract (500ml × 6times). Both of the extracts were carried by separate name as AEBH for aqueous extract and EEBH for ethanolic extract. All the extracted material were stored in desiccators throughout the period of experiment.

Reagent and chemicals

The ascorbic acid as the standard drug and all the reagents used in the study were of analytical grade and were procured from S D Fine Chemicals Limited. 1, 1-diphenyl-2-picrylhydrazyl was from Hi Media Pvt. Ltd., and hydrogen peroxide was from Merck Pvt. Ltd.

Preliminary phytochemical screening

The ethanolic and aqueous extract of *Benincasa hispida* was subjected to qualitative phytochemical analysis for presence of various constituents like Alkaloids, Carbohydrate, Glycosides, Terpanoids, Protein and Amino acids, Phenolic and Tannins, Flavanoids, Oils and

Fats, Saponins etc.

Evaluation of antioxidant activity of extracts

Antioxidant activity of fruit extracts of *Benincasa hispida* under different conditions were assessed using Hydogen Peroxide[27], and 1, 1-diphenyl-2-picrylhydrazyl (DPPH)[36].

DPPH radical scavenging activity determination

This assay was carried out according to[36] with some modifications in which increase in absorbance due to reduction of the DPPH• radical determined spectrophotometrically at 515 nm for 60 min based on preliminary experiments. In this method 50mg of each of ethanolic and aqueous extract were weighed separately and dissolved in 100ml of methanol to get 500µg/ml stock solution. Ascorbic acid is used as an synthetic antioxidant. Mix 1ml of DPPH in methanol was mixed with equal volume of the extract solution, mixed well and kept in dark for 20 min. The absorbance of DPPH at 517nm was monitored in presence of different concentrations (25, 50 - 250µg/ml) of extract. Blank was also carried out to determine the absorbance of DPPH before reaction with the extract. The percentage inhibition is calculated by following equation[8]:

$\%\ \text{inhibition} = 100 \times A_0 - A_1 / A_0$

Where; A_0 is the absorbance of control and A_1 is the absorbance of test.

Hydrogen peroxide radical scavenging (H_2O_2) assay

This assay was carried out according to[27] with some modifications. A solution of hydrogen peroxide (40 mM) is prepared in phosphate buffer (7.4). The concentration of hydrogen peroxide is determined by absorption at 230 nm using a spectrophotometer. Extract (200 - 1000 µg/ml) in methanol is added to hydrogen peroxide and absorbance at 230 nm is determined after 10 min against a blank solution containing phosphate buffer without hydrogen peroxide. The percentage of Hydrogen Peroxide scavenging is calculated as follows:

$\%\ \text{scavenged}\ (H_2O_2) = (A_0 - A_1 / A_0) \times 100$

Where; A_0 is the absorbance of control and A_1 is the absorbance of test. Ascorbic acid, rutin BHA[17], α-tocopherol[11] or quercetin[27] can be used as a positive control.

Statistical analysis

The data were analysed using one way analysis of variance (ANOVA) followed by Dunnett test. p values were considered significant.

RESULTS AND DISCUSSION

Phytochemical screening reveals that the major constituents of *Benincasa hispida* fruit extracts are phenolic compound, glycosides, carbohydrates, proteins and amino acids, tannins, alkaloid, and flavanoids. Phenolic compounds may be responsible for the antioxidant activity.

DPPH radical scavenging activity

The reduction capability of DPPH was determined by the decrease in its absorbance at 517 nm induced by antioxidants. The AEBH and EEBH showed DPPH radical scavenging activity. The highest DPPH radical scavenging activity of AEBH the highest DPPH scavenging activity was found to be 61.5% at concentration of 250 μg/ml and EEBH was found to be 77.4% at the same concentration.

Table 1: % Scavenging activity

Concentration	% scavenging of DPPH		
	Ascorbic acid	Aqueous extract	Ethanolic extract
25	68.3	49.4	61.6
50	72.3	51.3	62.7
100	78.5	54.2	63.2
150	83.8	57.7	65.6
200	92.6	59.1	70.0
250	97.3	61.5	77.4

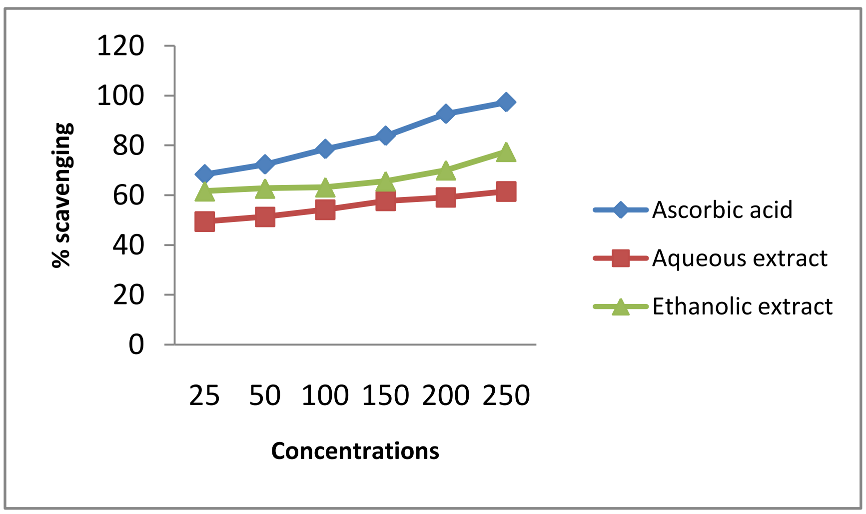

Figure 1: Comparative effect of AEBH, EEBH and Ascorbic acid on DPPH assay

Table 2: Statistical analysis of DPPH assay

Sr. N.	Conc.(μg/ml)	Absorbance (Mean ± SEM)		
		Ascorbic acid	AEBH	EEBH
1.	25	0.2300 ± 0.0007	0.3512 ± 0.0003**	0.2322 ± 0.0004*
2.	50	0.1944 ± 0.001	0.3386 ± 0.0004**	0.2240 ± 0.0004**
3.	100	0.1500 ± 0.0007	0.3188 ± 0.0003**	0.2546 ± 0.0002**
4.	150	0.1092 ± 0.0008	0.2944 ± 0.0005**	0.2396 ± 0.0004**
5.	200	0.0668 ± 0.0009	0.2848 ± 0.0003**	0.2086 ± 0.0004**
6.	250	0.0220 ± 0.0007	0.3068 ± 0.0003**	0.1570 ± 0.0005**

***-** $p<0.05$ (significant), **- $p<0.01$ (significant), Values are expressed in Mean ± SEM

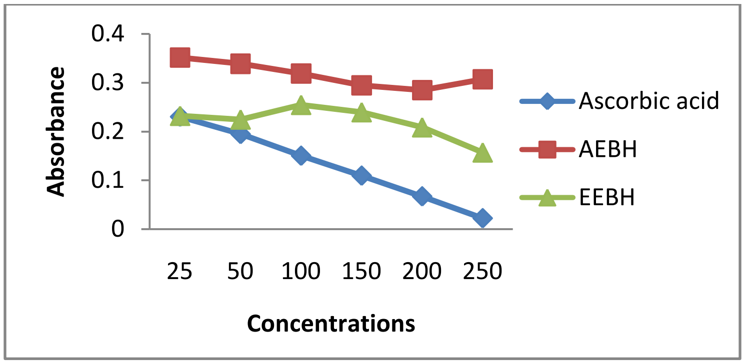

Figure 2: Statistical analysis of DPPH assay

H_2O_2 scavenging activity

The hydrogen peroxide scavenging activity of ethanolic and aqueous extract of *Benincasa hispida* is shown in Table 3, Figure 3. The maximum hydrogen peroxide scavenging activity of EEBH was found to be 21.3% at concentration of 1000 µg/ml and in AEBH the maximum activity was found to be 20.5% at 1000 µg/ml concentration.

Table 3: % scavenging activity

Drug conc. (µg/ml)	% scavenging activity of H_2O_2		
	Ascorbic acid	EEBH	AEBH
200	4.2	11.5	11.1
400	6.2	14.2	13.8
600	9.1	17.3	16.8
800	9.8	19.9	19.5
1000	10.4	21.3	20.5

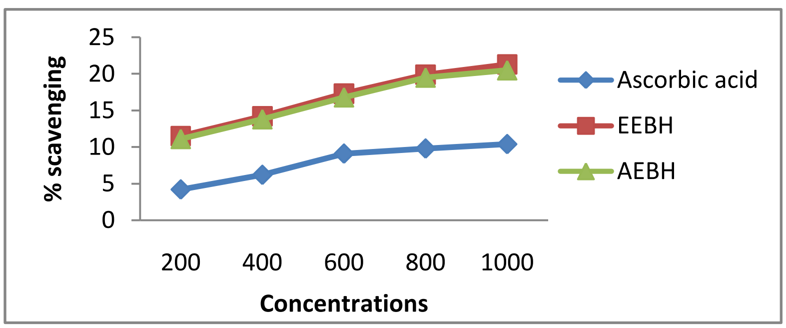

Figure 3: Comparative effect of AEBH, EEBH and Ascorbic acid on H_2O_2 assay

Table 4: Statistical analysis of H_2O_2 assay

Sr. No.	Conc.(µg/ml)	Absorbance (Mean ± SEM)		
		Ascobic acid	AEBH	EEBH
1.	200	3.400 ± 0.08	3.348 ± 0.04	3.329 ± 0.03
2.	400	3.551 ± 0.06	3.252 ± 0.06*	3.257 ± 0.04*
3.	600	3.452 ± 0.08	3.350 ± 0.04	3.304 ± 0.04
4.	800	3.561 ± 0.1	3.256 ± 0.04*	3.254 ± 0.05*
5.	1000	3.638 ± 0.09	3.376 ± 0.08	3.362 ± 0.04

*- p<0.05 (significant),**- p<0.01 (significant),Values are expressed in Mean ± SEM

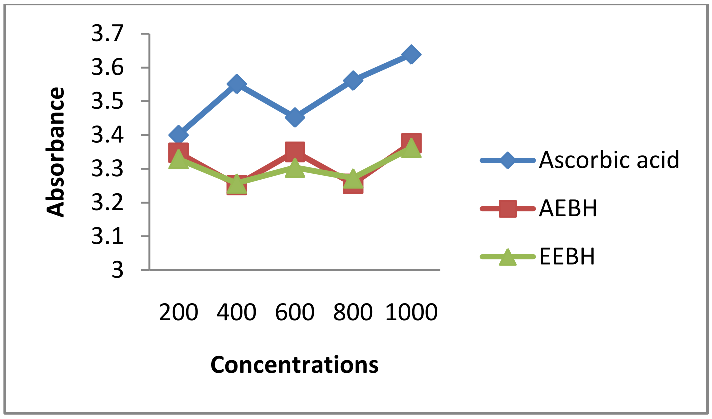

Figure 4: Statistical analysis of H_2O_2 assay

Some phytoconstiuents are beneficial and some of them are acting as natural antioxidants[3]. Flavonoids are a group of more than 4000 polyphenolic compounds that occur naturally in foods of plant origin. Flavonoids are a large class of phytochemicals which are omnipresent in human diets, found for example in fruit, vegetables, tea, chocolate, and wine, and to which a number of beneficial effects on human health, such as antioxidant, anti-inflammatory, antiallergic, antiviral, and anticarcinogenic activities[35].

Phenolic compounds are known as powerful chain breaking antioxidants may contribute directly to antioxidative action. These compounds are very important constituents of plants and their radical scavenging ability is due to their hydroxyl groups[14].

Moreover, high correlations were observed in antioxidant capacities and phenolic and flavonoid content of the selected plants. Thus, the antioxidant activities most probably might be contributed by polyphenols contents in the plant extracts.

In the extracts study were suggestive of the potential of solvent extracts in scavenging free radical. According to our study, the presence of phytoconstituents such as carbohydrates, glycosides, tannins & polyphenols, proteins and amino acids, flavonoids and phenolic compounds in different extracts i.e. ethanolic and aqueous extract. Further, *in vitro* antioxidant activity of the extract was evaluated by DPPH and H_2O_2 method. The quantitative method was measured from the decrease in absorbance at 517 nm owing to rapid hydrogen donating ability of DPPH. Scavenging activity by H_2O_2 may be due to donation of electrons to hydrogen peroxide and thus neutralise it to water and it measured at the absorbance of 230 nm. The DPPH radical scavenging activity of EEBH was found to be 77.4% at concentration of 250 µg/ml and in AEBH was found to be 59.1% at concentration of 200 µg/ml. The H_2O_2 scavenging activity of EEBH was found to be 21.3% at concentration of 1000 µg/ml and in AEBH the activity was found to be 20.5% at 1000 µg/ml concentration.

CONCLUSION

In the current study, the two extracts i.e. ethanolic and aqueous extracts were used for extraction of fruit of *Benincasa hispida*. In EEBH the result is (77.4%) for DPPH activity and (21.3%) for H_2O_2 scavenging activity and in AEBH (59.1%) of DPPH activity and (20.5%) of H_2O_2. It was found that higher amount antioxidant activity was obtained in the ethanol (77.4%) & (21.3%). Thus, ethanolic extract of *Benincasa hispida* (Thunb.) Cogn. fruit can be used to treat diseases caused by free radicals and oxidative stress.

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