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BIOACTIVE COMPONENTS OF *VACCINIUM MACROCARPON* AND ITS ANTIOXIDANT ACTIVITY: AN *IN-VITRO* STUDY

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Keywords:

Cranberry, *Vaccinium macrocarpon*, Phytochemical analysis, Phenol, Flavonoid, Antioxidant activity

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ABSTRACT: Cranberries (*Vaccinium macrocarpon*) contain many bioactive compounds and have some biological activities and beneficial health properties. This study aimed to screen phytochemicals of cranberry fruits from the different solvent, to estimate the total phenolic and flavonoid content of cranberry fruits and their antioxidant effect *in-vitro* by DPPH, superoxide and nitric oxide radical scavenging assay. Phytochemical screening of various extracts such as aqueous, ethanol, chloroform, acetone and petroleum ether of cranberry fruit extracts, revealed the presence of flavonoids, cardiac glycosides, phenols, coumarins, terpenoids, and betacyanin. The cranberry extracts were evaluated for phenol and flavonoid content with Gallic acid (GA) and Quercetin (Q) as standard. The optimum yield of phenol and flavonoid content were found in ethanol fruit extract 13.07 mg Gallic acid Equivalents (GAE)/g and 9.02 mg Quercetin Equivalents (QE)/g of cranberry. The cranberry extracts were evaluated for antioxidant activities by DPPH (1,1– diphenyl -2- picrylhydrazyl) radical scavenging assay. Among five different solvents used, maximum antioxidant activity was found in ethanolic fruit extract (81.4%) followed by others. The IC_{50} values of ethanolic cranberry extract in superoxide radical scavenging activity and Nitric oxide radical scavenging assay are 61.1 µg/ml and 54.7 µg/ml. The IC_{50} values showed a strong antioxidant activity of the extracts. The powerful antioxidant effect attributed to the greater amount of phenol and flavonoid compound in the ethanolic cranberry extract.

INTRODUCTION: Medicinal plants continue to be an important therapeutic aid for alleviating the ailments of humankind [1]. Today an abundant number of drugs are developed from plants which are active against several diseases. Majority of these involve the isolation of the active ingredients found medicinal plants and its subsequent modifications [2]. Phytochemical screening of various plants has been reported by many workers [3, 4].

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These studies revealed the presence of numerous chemicals including flavonoids, phenols alkaloids, steroids, glycosides, and saponins. The phenolic compounds are one of the largest and most ubiquitous groups of plant metabolites [5]. Polyphenolic compounds including flavonoids exhibit a wide range of biological effect including anti-oxidative, anti-inflammatory, anti-mutagenic and anti-carcinogenic, *etc.* [6] Free radicals and other reactive oxygen species are produced during aerobic metabolism in the body.

Oxidative stress, arising because of an imbalance between free radical production and antioxidant defenses, is associated with damage to a wide range of molecular species including lipids, proteins, and nucleic acid [7].

Oxidative stress induced by oxygen radicals is believed to be a primary factor in various degenerative diseases including atherosclerosis, ischemic heart disease, diabetes mellitus, cancer, immunosuppression as well as neurodegeneration said to be the normal process of aging. Antioxidants stabilize or deactivate free radicals, often before they attack targets in biological cells [8].

Vaccinium macrocarpon (Cranberry) belongs to the family Ericaceae; it is an evergreen groundcover native plant of North America. They are shrubs which grow about four meters having dark pink colored flowers and reddish black color berries. It is widespread throughout cool temperature northern hemisphere.

Cranberry fruit is a rich source of bioactive components with a broad spectrum of activities. Flavonoids, anthocyanins, proanthocyanidins, phenolic acids, and vitamin C are cranberry fruit compounds which are marked by high biological activity [9]. Cranberry is particularly a rich source of polyphenols, which have been associated *in-vitro* with anti-bacterial, anti-viral, anti-mutagenic, anti-carcinogenic, anti-tumorigenic, anti-angiogenic, anti-inflammatory, and anti-oxidant properties [10]. Cranberries are known that could prevent and treat an occurrence of urinary tract infections. This effect is achieved by proanthocyanidins contained in cranberries [11]. This study aimed to screen phytochemicals of cranberry fruits from the different solvent, to estimate the total phenolic and flavonoid content of cranberry fruits and their antioxidant effect *in-vitro* by DPPH, superoxide and nitric oxide radical scavenging assay.

MATERIAL AND METHODS:

Collection of Plant Material: The healthy dried Cranberries were collected from Fieldfresh Food Private Limited, Haryana, India. The collected specimen was authenticated in National Institute of Siddha, Tambaram, Chennai, India (Authentication no: NISMB3102017).

Preparation of the Cranberries Extract: Preparation of the extracts was done according to a combination of the methods used by Pizzale *et al*., (2002) and Lu and Foo (2001). About 15g of dried Cranberries fruit fine powder was extracted with 150 ml acetone, ethanol (75%), chloroform, petroleum ether and aqueous extract for 1 min using an Ultra Turax mixer (13,000 rpm) and soaked overnight at room temperature. The sample was then filtered through Whatman no. 1 paper in a Buchner funnel. The filtered solution was evaporated under vacuum in a rotavator at 40 °C to a constant weight and then dissolved in respective solvents. The concentrated extracts were stored in airtight container in a refrigerator below 10 ºC.

Phytochemical Screening of Cranberry: The phytochemical screening of Cranberry extracts was assessed by standard methods [14, 15]. Phytochemical screening was carried out on the fruit extracts using different solvents to identify the major natural chemical groups such as tannins, saponins, flavonoids, phenols, terpenoids, alkaloids, glycosides, cardiac glycosides, coumarins, and steroids. General reactions in these analyses revealed the presence or absence of these compounds in the fruit extracts tested.

Estimation of Total Phenol Content in Cranberry: Total phenolic content in the ethanolic Cranberry extract was determined by Folin-ciocalteu colorimetric method [16]. For the analysis, 0.5 ml of dry powdered ethanolic fruit extract was added to 0.1 ml of Folin-ciocalteu reagent (0.5N), and the contents of the flask were mixed thoroughly. Later 2.5 ml of Sodium carbonate (Na_2CO_3) was added, and the mixture could stand for 30 min after mixing. The absorbance was measured at 760 nm in a UV-Visible Spectrophotometer. The total phenolic contents were expressed as mg gallic acid equivalents (GAE)/g extract.

Estimation of Total Flavonoid Content in Cranberry: Total flavonoids content in the ethanolic cranberry extract was determined by aluminium chloride colorimetric method [17]. 0.5 ml of cranberry extract at a concentration of 1mg/ ml was taken, and the volume was made up to 3 ml with methanol. Then 0.1ml $AlCl_3$ (10%), 0.1 ml of potassium acetate and 2.8 ml distilled water were added sequentially. The test solution was vigorously shaken. Absorbance was recorded at 415 nm after 30 min of incubation. A standard calibration plot was generated at 415 nm using known concentrations of quercetin. The concentrations of flavonoid in the test samples

were calculated from the calibration plot and expressed as mg quercetin equivalent/g of sample.

Qualitative Analysis of Antioxidant Activity of Cranberry: The antioxidant activity of cranberry was determined by following the method as described by George *et al*., (1996). 50 µL of cranberry extract was taken in the microtiter plate. 100 µL of 0.1% methanolic DPPH was added over the samples and incubated for 30 min in dark condition. The samples were then observed for discoloration; from purple to yellow and pale pink were considered as strong and weak positive respectively. The positive antioxidant samples were subjected for further quantitative analysis.

Quantitative Analysis of Free Radical Scavenging Activity of Cranberry: The antioxidant activities were determined using DPPH, (Sigma-Aldrich) as a free radical. 100 µl of cranberry extract was mixed with 2.7 ml of methanol, then 200 µl of 0.1% methanolic DPPH was added. The suspension was incubated for 30 minutes in dark condition. Initially, absorption of a blank sample containing the same amount of methanol and DPPH solution was prepared and measured as a control [19]. Subsequently, at every 5 min interval, the absorption maxima of the solution were measured using a UV double beam spectra scan (Chemito, India) at 517 nm. The antioxidant activity of the sample was compared with known synthetic standard of (0.16%) of Butylated Hydroxy Toluene (BHT). The experiment was carried out in triplicate.

Free radical scavenging activity was calculated by the following formula:

Percentage of DPPH radical-scavenging = [(Absorbance of control - Absorbance of test Sample) / (Absorbance of control)] × 100

Superoxide Radical Scavenging Activity of Cranberry: The assay was based on the capacity of the fruit extracts to inhibit nitro blue tetrazolium (NBT) up to 50% in the presence of riboflavin-light-NBT system. The reaction medium contains 50 mM phosphate buffer pH 7.6, 20 µg riboflavin, 12 mM EDTA, different concentrations of cranberry ethanolic extract (25-400 µg/ml), NBT 0.1 mg / 3 ml and BHT was taken in a different test tube and the same reagents were added. The reaction was initiated by illuminating the sample cuvette at regular intervals of 30 sec and increases in absorbance were measured at 590 nm up to 2.5 min. The superoxide radical scavenging activity was calculated using the formula:

Percentage of inhibition of superoxide radical = OD (extract absent) - OD (extract present) / OD (extract absent)

Nitric Oxide Radical Scavenging Assay of Cranberry: Sodium nitroprusside (SNP) in aqueous solution at physiological pH spontaneously generate iNO which interacts with oxygen to produce nitrite ion that can be estimated using Griess reagent. The reaction mixture with 2 ml of the extract at different concentrations (25-400 µg/ml) and 50 mM SNP (0.5 ml) in 10 mM PBS was incubated at 37 °C for 60 min. An aliquot (0.5 ml) of the incubation solution was pipetted out and diluted with 0.5 ml of Griess reagent (1% sulfanilamide in 5% H_3PO_4 and 0.1% N-(1-naphthyl) ethylenediamine dihydrochloride (NED).

The absorbance of the chromophore that formed during diazotization of nitrite with sulfanilamide and subsequent coupling with NED was immediately recorded at 540 nm. The absorbance from various concentrations of sodium nitrite salt treated the same way with Griess reagent was plotted for a standard curve. The capability to scavenge iNO radicals was calculated using the following equation:

Scavenging activity (%) = [1 - (A1 - A2) /A0] × 100%

Where A0 was the absorbance of the control (the reaction mixture without the extract), A1 was the absorbance in the presence of the extract and A2 was the absorbance without Griess reagent. BHT was used as a standard.

RESULTS: Phytochemical analysis revealed the presence or absence of the compounds in the cranberry extract is summarized in **Table 1**.

In the present study, phytochemical screening was performed with ethanol, chloroform, petroleum ether, acetone and aqueous fruit extracts of Cranberry. The ethanolic extract of Cranberry was rich in flavonoids, quinones, cardiac glycosides, terpenoids, phenol, betacyanin and coumarins **Table 1**.

TABLE 1: PHYTOCHEMICAL SCREENING OF CRANBERRY EXTRACTS

Phytochemicals Tested	Cranberry (*Vaccinium macrocarpon*) extracts				
	Ethanol	Aqueous	Chloroform	Petroleum ether	Acetone
Tannins	+/-	+/-	-	-	-
Saponins	-	+	-	-	-
Flavonoids	++	++	+	+	++
Quinones	++	+	+	+	++
Glycosides	+	+	-	-	-
Cardiac glycosides	++	+	+	-	+
Terpenoids	++	+/-	-	-	+
Phenol	++	++	+	+	++
Coumarins	++	++	+	+	+
Steroids	+	+	+	+	+
Alkaloids	-	-	-	-	-
Anthocyanin	-	-	-	-	-
Betacyanin	++	+	-	-	+/-

Key: + = positive, ++ = strong positive, - = negative, +/- = semi positive

In our study, total phenol and flavonoid content of cranberry extract were estimated by using Folin-ciocalteu method and represented regarding gallic acid equivalent (GAE) and the total flavonoid contents as measured by aluminium chloride method and represented concerning Quercetin Equivalents (QE) respectively. The optimum yield of phenol and flavonoid contents found in ethanol the fruit extract were 13.07 mg Gallic Acid Equivalents (GAE)/g and 9.02 mg Quercetin Equivalents (QE)/g of cranberry **Table 2**. Phenolic compounds are effective hydrogen donors that make them good antioxidants [20], which exhibited considerable scavenging activity against free radicals.

TABLE 2: DETERMINATION OF PHENOL AND FLAVONOID CONTENT FROM CRANBERRY EXTRACT

Sample	Total phenol content (mg GAE/g)	Total flavonoid content (mg QE/g)
Cranberry extract	13.07	9.02

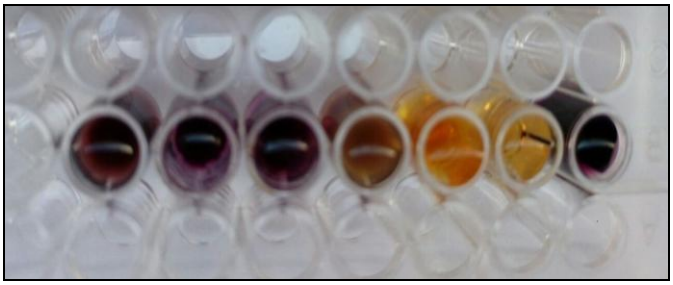

FIG. 1: QUALITATIVE ANTIOXIDANT ACTIVITY OF CRANBERRY EXTRACT

The fruit extracts of *Vaccinium macrocarpon* were used for different antioxidant studies. Analysis of different extractions of acetone, ethanol, petroleum ether, chloroform, and aqueous extract showed the presence of anti-oxidants. 100 µl of cranberry extracts were estimated for free radical scavenging activity using 1, 1-Diphenyl-2-picrylhydrazyl (DPPH) assay. The samples were observed for the color change from purple to yellow and pale pink were considered as strong positive and weak positive respectively **Fig. 1** and **Table 3**.

TABLE 3: QUALITATIVE ANTIOXIDANT ACTIVITY OF CRANBERRY EXTRACT

S. no.	Extractions	Cranberry
	BHT (standard)	++
S1	Ethanol	++
S2	Aqueous	+
S3	Chloroform	-
S4	Petroleum ether	-
S5	Acetone	Semi-positive

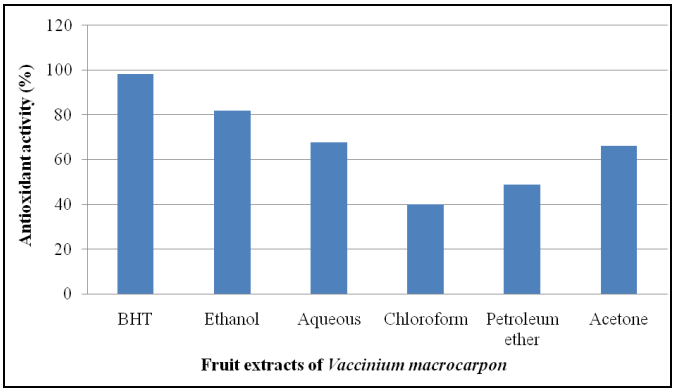

FIG. 2: QUANTITATIVE ANTIOXIDANT ACTIVITY OF CRANBERRY EXTRACT

Among five different solvent of cranberry extract, the ethanolic cranberry extract recorded the most effective DPPH radical scavenging activity (81.4%) followed by followed by aqueous, acetone, chloroform and petroleum ether extracts **Fig. 2**. Cranberry value being very close to synthetic anti-oxidant (BHT) as a positive control (98.4%).

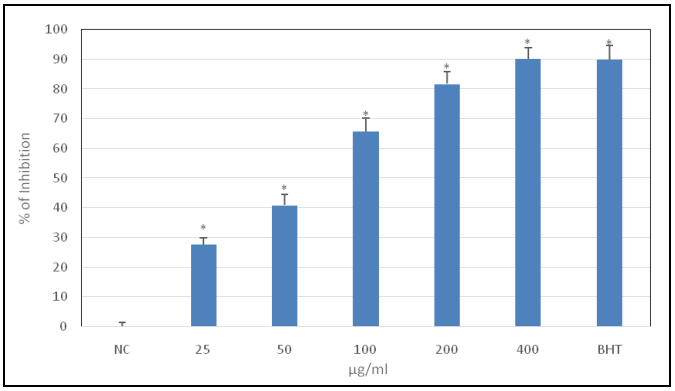

FIG. 3: SUPEROXIDE RADICAL SCAVENGING ACTIVITY OF CRANBERRY ETHANOLIC EXTRACT

The relative amount of ROS (%) is shown as mean + SD of triplicate measurements. The amount of effective concentration of the extract needed to inhibit free radicals by 50%, IC_{50} was estimated from the regression analysis between scavenging activities (%) versus various concentration of the extract. The IC_{50} of the extract is 61.1 µg/ml. 400 mg of cranberry ethanolic extract recorded the most effective superoxide radical scavenging activity **Fig. 3**.

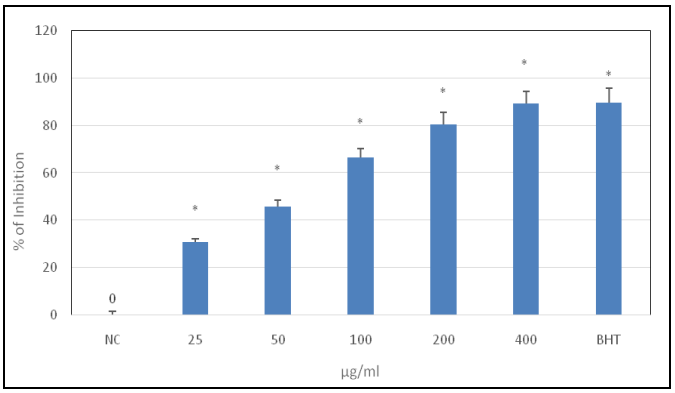

FIG. 4: NITRIC OXIDE RADICAL SCAVENGING ASSAY OF CRANBERRY ETHANOLIC EXTRACT

Values are expressed as mean + SD of triplicate measurements. The amount of effective concentration of the extract needed to inhibit free radicals by 50%, IC_{50} was estimated from the regression analysis between scavenging activities (%) versus various concentration of the extract. The IC_{50} of the extract is 54.7 µg/ml. Among different concentration of cranberry ethanolic extract 400 mg recorded the most effective nitric-oxide radical scavenging activity **Fig. 4**.

DISCUSSION: Preliminary screening of phytochemicals may be useful to discover and develop novel therapeutic agents with improved efficacy. In the present study phytochemical screening of various extracts such as aqueous, ethanol, chloroform, acetone and petroleum ether of cranberry extracts were done. According to the result of this study, the ethanolic cranberry extract was rich in flavonoids, quinones, cardiac glycosides, terpenoids, phenol, and betacyanin. Cranberry has a complex and rich phytochemical composition, particularly flavan-3-ols, A-type procyanidins (PACs), anthocyanins, benzoic acid, and ursolic acid. (−)-Epicatechin is the predominant constitutive unit in cranberry PACs.

Anthocyanins are cyanidin, peonidin, malvidin, pelargonidin, delphinidin, and petunias Cranberry also contains phenolic acids, including hydroxybenzoic and hydroxycinnamic acids [10]. These secondary metabolites contribute significantly towards the biological activities of medicinal plants such as anti-diabetic, anti-oxidant, anti-microbial, anti-inflammatory and anti-carcinogenic activities. Flavonoids are potent water-soluble anti-oxidants and free radical scavengers, which prevents oxidative cell damage and have strong anticancer activity [3].

Phenolic compounds are a class of antioxidant agents which act as free radical terminators [21]. Flavonoids regarded as one of the most widespread groups of natural constituents found in plants. The values of flavonoid content varied from plants. It has been recognized that flavonoids show antioxidant activity and their effects on human nutrition and health are considerable. The mechanisms of action of flavonoids are through scavenging or chelating process [22].

In our study, total phenol and flavonoid content of cranberry extract were estimated by using Folin-ciocalteau method and aluminium chloride method. The optimum yield of phenol and flavonoid contents was found in ethanolic cranberry extract, 13.07 mg Gallic Acid Equivalents (GAE)/g and 9.02 mg Quercetin Equivalents (QE)/g of cranberry extract.

In a study by Mustarichie R *et al.*, [23] the total phenol content of methanolic extract of cranberry was 17.1mg/100g. Neto CC [24] has reported that total flavonol content of cranberry fruit usually falls in the range of 20-30 mg / 100 g fresh fruit

weight, with; 75% of the flavonols being quercetin glycosides. According to Kalin P *et al.,* [25] the content of phenolic compounds in the lyophilized aqueous extract (LAEC) was 26.0 µg GAE/g (LAEC). On the other hand, 7.06 µg QE/g (LAEC) was measured in the same LAEC sample. Among five different solvents of cranberry extract, the ethanolic cranberry extract recorded the most effective DPPH radical scavenging activity (81.4 %). The IC_{50} values of ethanolic cranberry extract in Superoxide radical scavenging activity and nitric oxide radical scavenging assay were 61.1 µg/ml and 54.7 µg/ml. The IC_{50} values showed a strong antioxidant activity of the extracts.

CONCLUSION: Our results showed that ethanolic extract of cranberry had more phytochemical constituents and phenolic compounds. The phenolic compounds present in cranberry are responsible for its antioxidant and antiradical activities. The antioxidant activity, total phenol and total flavonoid content of medicinal plants are very important in identifying new sources of therapeutically and industrially important compounds. It is essential to initiate an imperative step for a screening of plants for secondary metabolites. The present research work showed that the cranberry contains significant amounts of active antioxidant compounds, which may regard cranberry as a functional food to improve the health status of people and to use in nutraceutical products of commercial importance.

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CONFLICT OF INTEREST: The authors have no conflict of interest.

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