

## INVITRO ANTIOXIDANT ACTIVITY IN ETHANOLIC EXTRACT OF VACCINIUM CYANOCOCCUS AND DETECTION OF FLAVONOIDS BENEATH HPLC, LC-MS

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### **ABSTRACT**

Plant research has been intensified worldwide in the recent times. a large number of medicinal plants and their purified constituents have immense therapeutic potentials and have been reported to exhibit antioxidant activity of ethanolic extracts of *Vaccinium cyanococcus* fruit. In-vitro antioxidant activity was investigated by using DPPH assay, total antioxidant and reducing power assay. The preliminary phytochemical analysis is also carried out. The results of the present study clearly depicted that ethanolic extract of *Vaccinium cyanococcus* fruit has potent antioxidant activity. From the HPLC, LC-MS studies it become to know that the *Vaccinium cyanococcus* contains rutin, quercetin which acts as a potent anti-oxidant and anti-inflammatory instrument.

**KEYWORDS:** DPPH, Reducing power assay, *Vaccinium cyanococcus*.

### **INTRODUCTION**

Phytopharmaceuticals form an important part of herbal drugs industry and so called allopathic system of medicine has also recognised their importance. Many of the drugs used in their system e.g. Sex and other hormones, anti-cancers and cardio-vascular drugs are derived from herbal source.

An important factor for a manufacture of herbal formulations like Tablets, Capsules, and Liquids etc., is about the dosage of the medicinal herbs used. In order to obtain a uniformity of the active principles in the formulation, it is advisable to use standardised herbal extracts.

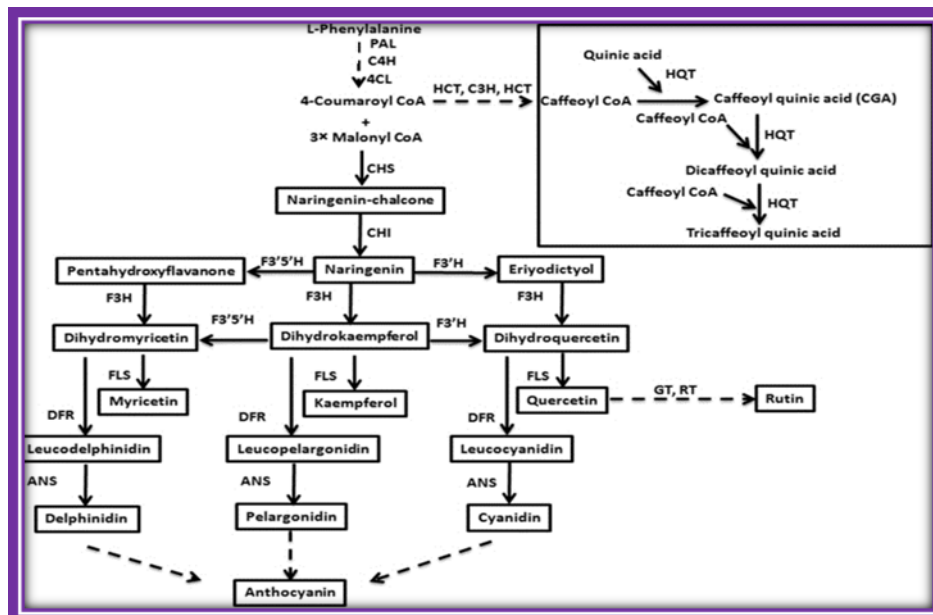
Unlike synthetic drugs, herbal drugs need to be extracted from medicinal herbs by various extraction processes involving the use of solvents like water, alcohol, organic solvents, oils, steam etc. Starting from the primitive use of copper maceration and distillation plants, extraction technology has made rapid progress in the last few decades with the result that medicinal herbs are now being processed in a scientific, economic and safe way with use of processing plants as good as any bulk drugs manufacturing plant for synthetic drug.

Flavonoids are important for human health because of their high pharmacological activities as radical scavengers Cook NC, (1996). Recent interest in these substances has been stimulated by the potential health benefits arising from the antioxidant activities of these polyphenolic compounds. As a dietary component, Flavonoids are thought to have health-promoting properties due to their high antioxidant capacity in both in vivo and in vitro systems Cook NC, *et al* (1996).

### Biosynthesis of flavanoids

The biosynthesis of flavanoids are briefly explained in the schematic representation Table -1

**Table 1: Schematic Representation of Biosynthesis of Flavanoids.**



### Antioxidant Activity

Flavonoids may protect against cancer and anti-carcinogenesis through inhibition of oxidative damage. Flavonoids have been shown to have both antioxidant and pro-oxidant activities in vitro and in animal models. Flavonoids have a wide spectrum of pharmacological properties,

including anti-oxidative, anti-allergic, anti-inflammatory, anti-diabetic, hepato-and gastro-protective, antiviral and anti-neoplastic activities Zhang *et al.*, (2006).

### Medicinal Plants

Plants with medicinal properties “The gift of mother nature to mankind” are in use for centuries in the traditional system of medicine like Ayurvedha, Unani, Siddha etc., In India and other countries for the treatment of disease, they are considered to be effective and non-toxic (Sadique,1986). In India herbal medicines have been the basis of treatment and care for various diseases. Phytological conditions in traditional methods practiced such as ayurveda, Unani and Siddha Nadkarni (1908).

## MATERIALS AND METHODS

### Collection of Plant Material

The ethanol extract of fruit *Vaccinium cyanococcus* were collected from Singapore Hannifa.

### Preparation of Plant Extract

The fresh fruits were soaked in ethanol for about 3 days. After the duration period, the fruit pulp was filtrated by means of whatmann filter paper. The ethanol was kept in a boiling water bath to evaporate ethanol. The ethanol free extract was used for the preliminary phytochemicals analysis, *in vitro* anti-oxidant, HPLC, LC-MS.

### Total antioxidant capacity

To 1 ml of different concentration was treated with 1 ml of reagent solution (0.6M sulphuric acid, 28mM sodium phosphate and 4Mm ammonium molybdate) in eppendorf tube. Capped tubes were incubated in thermal block at 95°C for 90min cooling to room temperature; the absorbance was matured at 695nm against blank. The activity was capered with ascorbic acid standard.

#### Calculation: A control- A test

Percentage of total antioxidant capacity =  $\frac{x}{100}$

A Control

### DPPH Scavenging Activity

The DPPH Scavenging Activity was determined by (Blois, 1958).

**Procedure**

To 0.5 ml of DPPH radical solution, add 2 ml of the extract and the reaction mixture is vortexed for 10 s and allowed to stand at room temperature for 30 minutes. The absorbance was recorded at 517 nm the % of inhibition was calculated. Ascorbic acid was used standard.

**Calculation:**

Sample Absorbance

Percentage of DPPH scavenging activity =  $1 - \frac{\text{Sample Absorbance}}{\text{Blank Sample Absorbance}} \times 100$

Blank Sample Absorbance

**Reducing Power Assay (Oyaizu, 1986).**

1ml of varying concentration (1-5mg/ml) of plant extract was mixed with 2.5 ml phosphate buffer and 2.5ml of potassium ferricyanide. The mixture was incubated at 50° C for 20 min. Aliquots of 2.5 ml of trichloroacetic acid were added to the mixture, which was then centrifuged at 3000 rpm for 10min. the upper layer of the solution (2.5 ml) was mixed with equal volume of distilled water, to this 0.5ml of freshly prepared ferric chloride solution was added and the absorbance was measured at 700 nm. Increased absorbance of the reaction mixture indicates increase in reducing power

**Calculation**

$$\% \text{ increasing reducing power} = \frac{A_{\text{Test}}}{A_{\text{Blank}}} \times 100$$

**LC-MS****UHPLC Conditions**

UHPLC system (Shimadzu Corporation, Kyoto, Japan) equipped with two Shimadzu UHPLC: Nexera UHPLC system Column: Shim-pack XR-ODS III (100 x 2 mm, 2.2 µm particle size) Column temp. 40°C. The mobile phase consisted of (A) 0.1% formic acid in water and (B) Acetonitrile. Both mobile phases were filtered through a cellulose nitrate filter, diameter 47 mm, pore size 0.45 µm (Sartorius, Goettingen, Germany). After the gradient separation, the column was reequilibrated for 5 min using the initial solvent composition. The flow rate was set to 1 mL/min, the samples were kept in amber vials at 4°C in the

autosampler, and the injected volume was 5  $\mu$ L. The separation was performed at  $25.0 \pm 0.1^\circ\text{C}$ .

### MS/MS Conditions

LC-MS/MS System (Make: Shimadzu Corporation, Kyoto, Japan, Model: LCMS 8040, Triple Quadrupole) Ionization: ESI (Positive / Negative), Ion spray voltage: +4.5 kV / -3.5 kV, MRM :: 427 MRM transitions (2 MRMs / compound) Dwell time 5 msec. / Pause time 1 msec Ambient CDL Temperature :  $250^\circ\text{C}$  Block Temperature :  $400^\circ\text{C}$  Detector voltage : 1.3kv Nebulizer Gas flow: 1.5 l/min Drying gas : 10 L/min Detection : External Standard: Quercetin – 339. The mobile phase was filtered through a  $0.22\ \mu$  membrane and degassed using ultrasonicator. LC-MS

### HPLC

#### Shimadzu CLASS-VP V6.13 SP2 Area % Report

Method Name: C:\CLASS-VP\Data\2013\Commercial\Flavonoids\flavonoids.met

Data Name: C:\CLASS-VP\Data\2016\commercial\commercial 690 23.02001.2016

User: System

Acquired: 3/2/2010 5.52 PM

Printed: 2/25/2016 5:44:43 PM

Standards: Gallic acid, caffeic acid, Rutin, Quercetrin and Ferulic acid

Sample: *Vaccinium cyanococcus*

### General Method Parameters

- ❖ Colum: C18
- ❖ Mobile Phase
- ❖ Solvent A - Water - Acetic acid (25:1)
- ❖ Solvent B - ethanol
- ❖ Pumps(Binary Gradient)
- ❖ T. Flow: 1.000 mL/min
- ❖ B. Conc: 0.0
- ❖ B. Curve: 0.0
- ❖ P. Max: 400.0 kgf/cm<sup>2</sup>
- ❖ P. Min: 0.0 kgf/cm<sup>2</sup>
- ❖ CTO-10ASvp
- ❖ Temperature:  $40^\circ\text{C}$

- ❖ SPD-10Avp(Det.A)
- ❖ Lamp: D2
- ❖ Polarity: +
- ❖ Wavelength Ch 1: 280 nm

## RESULTS AND DISCUSSION

The analysis of various concentrations of *Vaccinium cyanococcus* with numerous activities such as preliminary phytochemical analysis, *in-vitro* antioxidant activity, HPLC, LC-MS results were shown below.

### Preliminary Phytochemical Analysis

Table 1 depicts the result of preliminary phytochemical analysis of ethanolic extract of *Vaccinium cyanococcus* fruits. It shows the presence of Saponins, Tannins, Flavonoids, Terpenoids, Coumarin Quinones Lignins, Proteins and Phenols.

### *In vitro* Antioxidant Assay

Table 2, 2a, 3, 4 shows the result of *in-vitro* antioxidant activity of the ethanol extract of *Vaccinium cyanococcus* fruits DPPH Assay, standard ascorbic acid, total antioxidant activity, reducing power assay. The antioxidant activity of the extract was compared with standard Ascorbic acid.

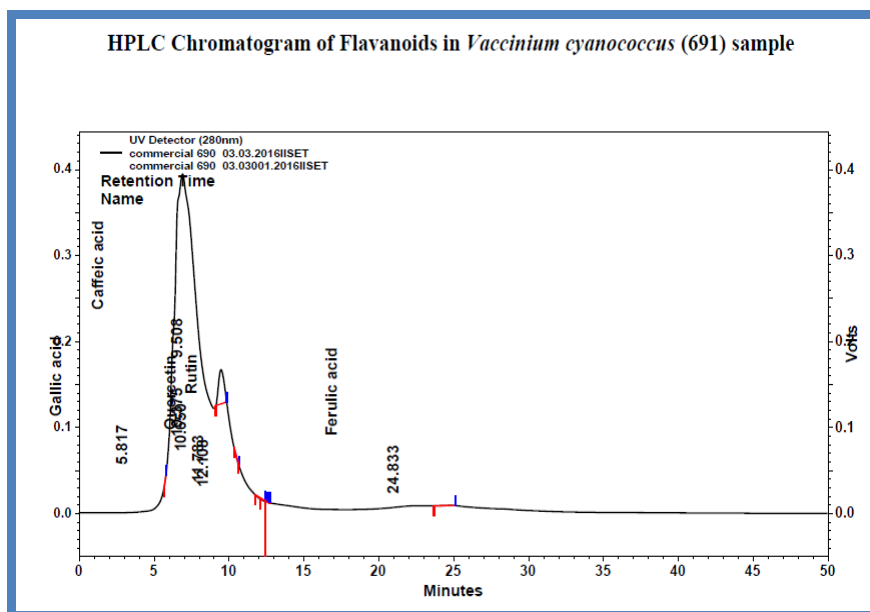
**Table I: Preliminary Phytochemical Analysis.**

S.No.	Name of the compound	Result
1	Saponins	+
2	Tannins	+
3	Steroids	-
4	Flavanoids	+
5	Coumarins	-
6	Alkaloids	-
7	Phenols	+

+ = Presence; - = Absence

### HPLC

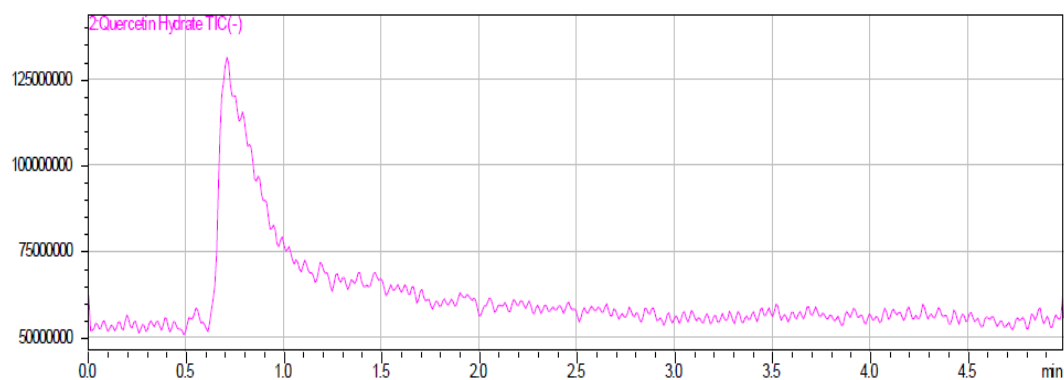
HPLC Results of analysis are enclosed herewith.

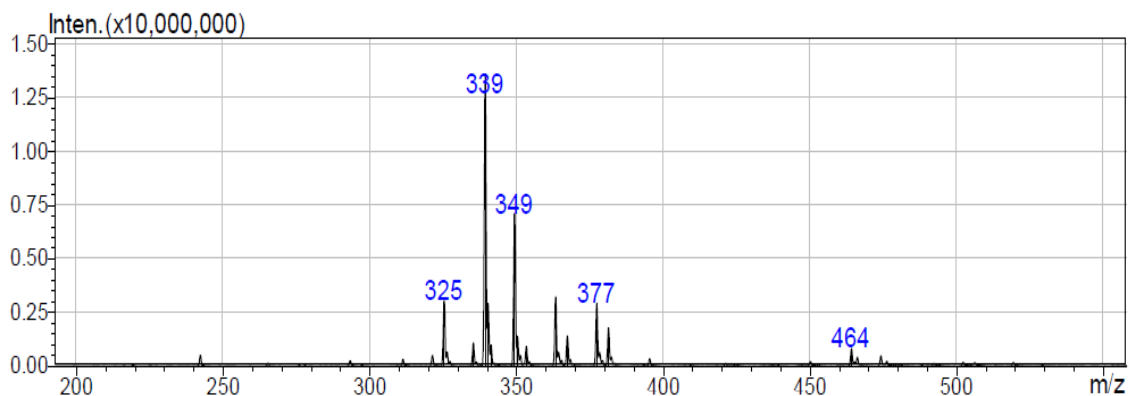


UV Detector (280nm)				
Retention Time	Area	Height	Concentration (mg/ml of sample)	Name*
5.817	3300	182	0.01	Gallic acid
9.508	989758	39635	0.567	Caffeic acid
10.650	3027551	642	0.702	Rutin
12.108	22346	1337	0.17	Quercetin
24.833	12433	384	0.07	Ferulic acid

## LC-MS

### LC-MS Chromatogram of Quercetin in *Vaccinium cyanococcus*



Parameters for Quercetin analysis in *Vaccinium cyanococcus* plant sample (691)

Analyte	Molecular ion (M-H) <sup>-</sup>	Cone Voltage (V)	Collision energy (eV)
Quercetin Dihydrate	339	15	-35

Table II: Effect of DPPH free radical scavenging activity of ethanolic extract of *Vaccinium cyanococcus*.

S.No	Concentration	DPPH (%)
		Ethanol
1.	50	42.7±0.5
2.	100	49.3±0.3
3.	150	57.7±1.2
4.	200	66.06±0.6
5.	250	74.7±0.7

Values are expressed as Mean± SD for 3 samples in each group

Students “t” test is followed \*\*\*P<0.05

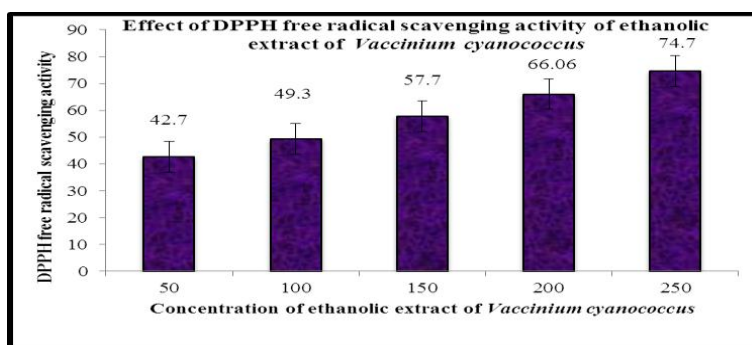
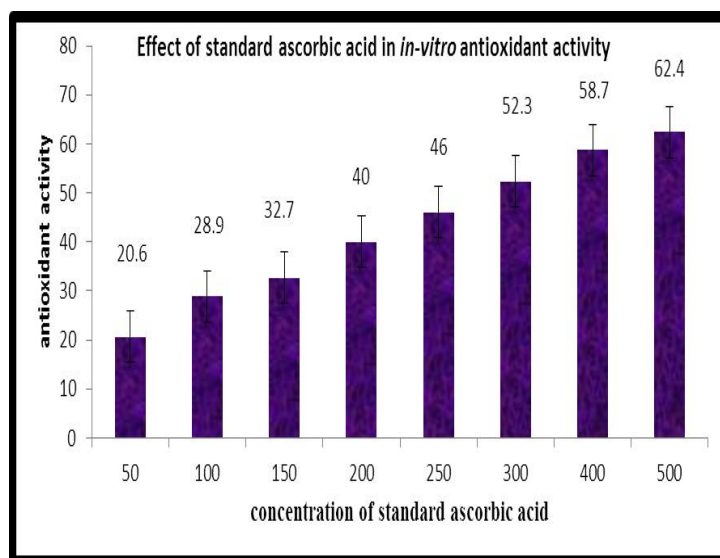
Figure 1: Effect of DPPH free radical scavenging activity of ethanolic extract of *vaccinium cyanococcus*.



Table II: Effect of standard ascorbic acid in *in-vitro* antioxidant activity.

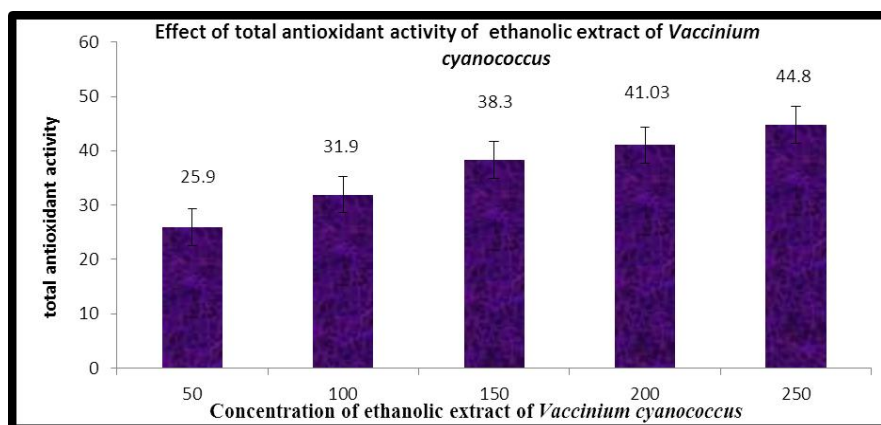
S. No	Concentration	% of antioxidant activity
1.	50	20.6±0.8
2.	100	28.9±0.3
3.	150	32.7±1.2
4.	200	40±2.3
5.	250	46±0.1
6.	300	52.3±0.8
7.	400	58.7±0.4
8.	500	62.4±0.5

Figure 2: Effect of standard ascorbic acid *in-vitro* antioxidant activity.Table II a: Effect of total antioxidant activity of ethanolic extract of *Vaccinium cyanococcus*

S. No	Concentration	TAC
1.	50	25.9±0.45
2.	100	31.9±1.4
3.	150	38.3±1.6
4.	200	41.03±1.1
5.	250	44.8±1.1

Values are expressed as Mean± SD for 3 samples in each group

Students "t" test is followed \*\*\*P<0.05



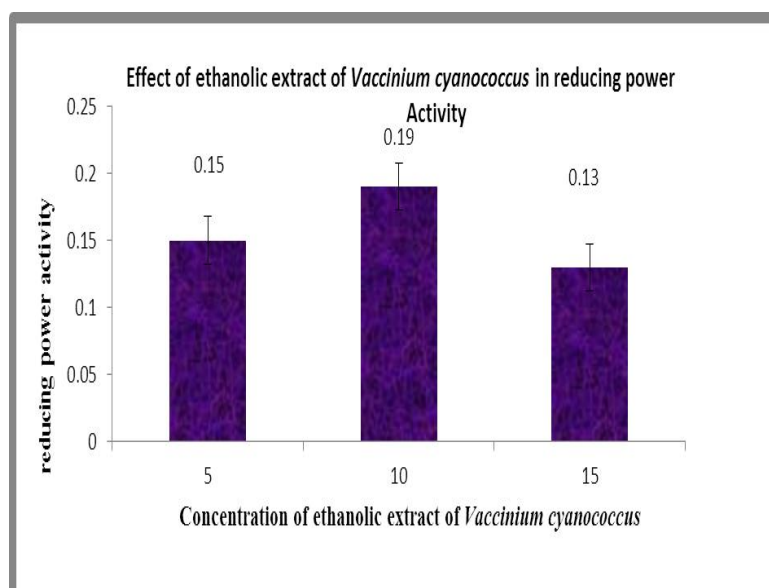
**Figure 3:** Effect of total antioxidant activity of ethanolic extract of *Vaccinium cyanococcus*.

**Table IV:** Effect of ethanolic extract of *Vaccinium cyanococcus* in reducing power Activity

S. No.	Concentration	Reducing power
		Ethanol
1	5	0.15± 0.055
2	10	0.19± 0.020
3	15	0.13±0.01

Values are expressed as Mean± SD for 3 samples in each group

Students “t” test is followed \*\*\*P<0.05



**Figure 4:** Effect of ethanolic extract of *Vaccinium cyanococcus* in reducing power Activity.

### Quercetin

Quercetin has been reported to inhibit the oxidation of other molecules and hence is classified as an antioxidant (Williams RJ, 2014). Quercetin contains a polyphenolic chemical substructure that stops oxidation by acting as a scavenger of free radicals that are responsible for oxidative chain reactions (Murakami A, Ashida H, Terao J (2008).

### Rutin

Rutin (quercetin rutinoside), like quercitrin, is a glycoside of the flavonoid quercetin. As such, the chemical structures of both are very similar, with the difference existing in the hydroxyl functional group. Both quercetin and rutin are used in many countries as medications for blood vessel protection, and are ingredients of numerous multivitamin preparations and herbal remedies. It can combine with cations, supplying nutrients from the soil to the cells in plants. In humans, it attaches to the iron ion  $\text{Fe}^{2+}$ , preventing it from binding to hydrogen peroxide, which would otherwise create a highly reactive free radical that may damage cells. It is also an antioxidant.

### DPPH

It is a rapid, simple, inexpensive and widely used method to measure the ability of compounds to act as free radical scavengers or hydrogen donors, and to evaluate antioxidant activity of foods. It can also be used to quantify antioxidants in complex biological systems, for solid or liquid samples. This method is easy and applies to measure the overall antioxidant capacity (Prakash 2001) and the free radical scavenging activity of fruit and vegetable juices (Sendra et al. 2006). The method is unique in carrying out the reaction of the sample with DPPH in methanol/water, which facilitates the extraction of antioxidant compounds from the sample.

### Total antioxidants

ROS/RNS interfere with DNA and leads to oxidative damage. DNA is highly susceptible to damage by the free radicals such as  $\bullet\text{OH}$ . These can react with DNA by addition or loss of hydrogen atoms from the sugar moiety. In particular, the C4-C5 double bond of pyrimidine is very sensitive to attack by  $\bullet\text{OH}$ , which results generation of a spectrum of oxidative pyrimidine damage products, such as thymine glycol, uracil glycol, urea residue, 5-hydroxydeoxyuridine, 5-hydroxydeoxycytidine, hydantoin and others. Likewise, purines are susceptible to attack by  $\bullet\text{OH}$  which leads to the generation of 8-hydroxydeoxyguanosine (8-OHdG), 8-hydroxydeoxyadenosine, formamidopyrimidines and other less characterized

purine oxidative products. Free radical attack also causes the activation of the poly (ADP-ribose) synthetase enzyme which can lead to fragmentation of DNA and programmed cell death. This process depletes the cellular level of NAD<sup>+</sup> levels thereby disrupting electron transport chain functions (Devasagayam, 2004).

### Reducing power assay

Reducing power is associated with antioxidant activity and may serve as a significant reflection of the antioxidant activity. Oktay M, 2003. Compounds with reducing power indicate that they are electron donors and can reduce the oxidized intermediates of lipid peroxidation processes, so that they can act as primary and secondary antioxidants. In this assay, the yellow colour of the test solution changes to various shades of green and blue depending on the reducing power of each compound. Presence of reducers causes the conversion of the Fe<sup>3+</sup>/ferricyanide complex used in this method. This may be due to the decrease in the reducers which would have converted the Fe<sup>3+</sup>/ferricyanide complex to the ferrous form within a short time. The reducers are no longer available for the conversion of the ferricyanide complex.

### SUMMARY AND CONCLUSION

Free radical generate oxidative stress which is a deleterious process that can damage cell structures, lipids, proteins and produce degenerative ailments such as cancer. Recently there is increasing interest in finding antioxidant phytochemicals because they can effectively inhibit propagation of these radicals and protect human body from disease.

The use of medicinal plants as a source of relief from illness can be traced back over 15 millennia. In the recent years, herbal medicine and herbal supplements are considered for better than synthetic drugs. Higher plants as a source of medicine for various human ailments are still unexplored. *Vaccinium cyanococcus* which belongs to the family is very well known for its therapeutic values. In view of its high medicinal potential and previous findings, the present study is designed to evaluate the *invitro* antioxidant and anticancer activities of *Vaccinium cyanococcus* fruits.

*In-vitro* antioxidant activity was investigated by using DPPH assay, total antioxidant, reducing power assay, ascorbic acid. The preliminary phytochemical analysis is also carried out. The results of the present study clearly depicted that ethanolic extract of *Vaccinium cyanococcus* fruit has potent antioxidant activity.

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