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EVALUATING THE CARDIOPROTECTIVE EFFECT OF VARIOUS PLANT EXTRACTS HAVING ANTIOXIDANT POTENTIAL IN MOUSE (*MUS MUSCULUS*)

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ABSTRACT

This study was conducted on the background knowledge that there is an association between diet - rich in fruits and vegetables and cardiovascular health. In this, we attempted to study the cardioprotective effect of various plant extracts of green leafy vegetables rich in antioxidants such as Spinach (*Spinacia oleracea*) and Broccoli (*Brassica oleracea*) in Swiss albino mice (*Mus musculus*). To determine the cardioprotective effect of these plant extracts, Mice can be divided into three groups. First and second group of mice was given broccoli and spinach leaf extract and third is the control group which did not receive any plant extract and both of them are maintained for three weeks. ISO is a known MI inducing agent and it was given subcutaneous injection in all the three groups of mice at a concentration of ISO (85mg/kg) for 2 days with an interval of 24 hrs. All the groups of mice were sacrificed and dissected out the heart. To check the expression of various myocardial infarction markers such as Aspartate transaminase and Creatine kinase in the heart were evaluated by RT - PCR. The expression of myocardial infarction markers were found to be considerably reduces in the mouse groups fed with Broccoli and spinach extract. When compared to the control group, there is high level expression of MI markers. Broccoli and Spinach were also tested for their antioxidant property by DPPH scavenging assay which shows that, Broccoli exhibits high antioxidant activity when compared to spinach and we have noticed a correlation of antioxidant activity and reduced expression of MI markers.

KEYWORDS

Spinacia oleracea, Brassica oleracea, Mus musculus, Isoproterenol, Myocardial infarction, MI Markers and DPPH.

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INTRODUCTION

Cardiovascular diseases (CVD) are the leading cause of global mortality and are a major contributor to reduced quality of life. Myocardial Infarction is a major ischemic heart disease characterized by an insufficient coronary blood supply to the heart muscles which leads to ischemia and myocardial death (Patel *et al*, 2010)[1]. As per

WHO report, it will be the leading cause of mortality worldwide by 2020 (Abubaker *et al*, 2012)[2]. It is a complex disease affecting the mechanical, electrical, structural and biochemical properties of the heart (Akhlaghi *et al*, 2009)[3]. Necrosis of the heart muscle in myocardial infarction occurs due to the imbalance between myocardial oxygen requirement and oxygen supply (Shaik *et al*, 2018)[4].

Recent epidemiological studies suggest that, cardioprotective action afforded specifically by green leafy vegetables (Lundberg *et al*, 2006)[5]. Natural antioxidant sources such as fresh fruits, vegetables or plants are always preferred over the synthetic ones owing to its efficacy and safety (Topliss *et al*, 2002)[6].

In this, we attempted to study the cardio protective effect of various plant extracts from green leafy vegetables rich in antioxidants such as Spinach and Broccoli in Swiss albino mice (*Mus musculus*). Spinach (*Spinacia oleracea*) is a leafy vegetable originated in central and western Asia belongs to the family Amaranthaceae. It is also one of the most important leafy vegetables with a high nutritional value (Yang *et al,* 2016)[7] which is rich in carotene, vitamin C, amino acids and iron. Broccoli (*Brassica oleracea*) belongs to the family *Brassicaceae* and its beneficial effects on human health have been associated with its phytochemical content. There are also reports on the presence of vitamin C, vitamin E and carotenoids (Rychlik *et al*, 2015)[8]. ISO is a chemical which cause the severe stress in the myocardium (Rona, 1985)[9] and it leads to myocardial infarction if administered in sub lethal doses (Rajadurai *et al*, 2007)[10].

The present study was carried out to evaluating the cardioprotective activities of various plants extracts having Antioxidant potential in Swiss albino mice (*Mus musculus*).

MATERIAL AND METHODS

Plant extracts given to them at a period of three weeks. After three weeks, subcutaneous injection of ISO was given in Swiss Albino Mice. After 3 weeks, all the three groups of mice were administered by ISO dissolved in (Myocardial infarction inducing agent) dissolved in physiological saline subcutaneously at a ratio of 85mg/kg body weight of the mice twice at an interval of 24h for two consecutive days in all the groups of mice. After the pre-treatment with these extracts orally for 3 weeks, animals of all the groups were sacrificed by cervical dislocation.

Homogenization

First and second group (Table No.1) of mice was fed with Broccoli and Spinach extract at a dose of 150mg/kg body weight/day and also control group was maintained for standard diet with adlibitum for a period of 3 weeks.

SWISS ALBINO MICE

↓

MYOCARDIAL INFARCTION/INJURY

↓

EXPRESSION OF CARDIAC MARKERS (AST and CK)

Plant Material

Green leafy vegetables such as Spinach (*Spinacia oleracea*) and Broccoli (*Brassica oleracea*) were purchased from the fresh Supermarket Pattom, Trivandrum, Kerala.

Preparation of the extract

Collected plant materials were washed with water to remove any dirt. Weighing 1.8g of plant materials are grinded and crushed using mortar and pestle and made into paste form. It was then mixed with 100ml of water and filtered using whatmann No.40 filter paper to make them aqueous extract.

Animal

Swiss albino strain of mouse (*Mus musculus*) is the most commonly used mammalian research model. Mice of age groups, two weeks to four months old were maintained at a temperature (27+°C) and humidity controlled condition under 14hr. light: 10hr. dark and provided with food and water. All experiments were carried out in female swiss albino mice obtained from Animal House of Life Science (Zoology) in the University of Kerala. The present study was approved by University institutional animal ethical committee.

Experimental Protocol

For the study, Swiss albino mice weighing 150-180g were divided into three groups.

Antioxidant assay

Heart was dissected out and homogenizes the tissue samples with the help of homogenizer. To clean the probe before using, run the homogenizer for 30sec with DEPC treated water, then with ethanol absolute and last with 2ml of trizol in a 5ml tube. After homogenization, samples can be used for the RNA extraction. Dissected out the heart from these three animals and transferred into a watch glass. The required materials are under the DEPC Treatment. The heart of each mouse can be separated and placed into watch glass. Using scissors to cut the heart tissues and adding 1M Tris - Hcl and stored in -20^{o}C. Homogenization can be done by using Homogenizer.

TRIzol RNA Extraction

Total RNA was extracted using TRIzol reagent from the heart tissue of all the groups of animals of test group and control (Chomczynski, 1993)[11] following the manufacturer's instructions (Sigma Chemical Company, Milwaukee, WI, USA). 5µg of the total RNA was used for reverse transcription to synthesize cDNA. Primers (Table No.2) to PCR, amplify partial coding sequence of myocardial infarction markers such as Creatine kinase and Aspartate Transaminase were designed using Primer 3 Software (http://bioinfo.ut.ee/primer3-0.4.0/) and synthesized by Sigma-Aldrich Corporation, Bangalore, India.

PCR was performed with the cDNA prepared as template in reactions containing 50mM KCl, 10mM Tris-HCl, pH 8.3, 1.5mM MgCl2, 0.25mM each dNTP and 2pM forward and reverse primers. The reaction conditions for the PCR consisted of initial denaturation of 94°C for 2 min, followed by 35 cycles of denaturation at 94°C for 30s, annealing at 60°C for 30s and extension at 72°C for 1min and 30s, with the last cycle followed by a 10-min final extension at 72°C. PCR amplification of b-actin (ACTB), used as an endogenous control, was performed at an annealing temperature of 58°C, with other conditions remaining the same.

The PCR products were run on 1.0% Agarose gel containing running buffers are TAE (40mM Tris-acetate, 1mM EDTA) and TBE (45mM Tris-borate, 1mM EDTA). The gel is made by dissolving agarose powder in boiling buffer solution. The concentration of agarose in a gel depends on the sizes of the DNA fragments to be separated, with most gels ranging between 0.5%-2%. The solution is then cooled to approximately **55°C** and poured into a casting tray which serves as a mold. A well-former template (often called a comb) is placed across the end of the casting tray to form wells when the gel solution solidifies.

After the gel solidifies, the gel is submerged in a buffer-filled electrophoresis chamber which contains a positive electrode (anode) at one end, and a negative electrode (cathode) at the other. The volume of the buffer should not be greater than 1/3 of the electrophoresis chamber.

Samples are prepared for electrophoresis by mixing them with loading dyes. Gel loading dye is typically made at 6X concentration (0.25% bromphenol blue, 0.25% xylene cyanol, 30% glycerol). The bands were visualized and images documented using Versa Doc (Bio-Rad) equipped with Quantity One Software (Bio-Rad). Band intensities were quantified using Phoretix Gel Analysis Software version 2003.02 (Phoretix International) and band intensities of Creatine kinase and Aspartate transaminase were normalized to that of beta actin.

Antioxidant assay – DPPH

Antioxidant activity

Antioxidant activity was determined by the DPPH radical scavenging method of Zhang and Hamauzu (2004)[12]. 0.1mM solution of DPPH in methanol was prepared and 4ml of this solution was treated with 0.2ml of the diluted extract. Concentration representing 5% of original fresh plant Crushed and grinded these vegetables with the help of water. Finally, the supernatant was transferred into a bottle. Ascorbic acid was used as the standard control (Figure No.4). 1ml of plant extract + 4ml of methanolic solution of DPPH + mix well. Incubation of room Temperature at 30 minutes.

Record the absorbance at 521nm. Inhibition ratio was calculated using the formula.

$$\% \text{ Inhibition} = \frac{(A - A)}{A} \times 100$$

RESULTS

ISO Induced Myocardial Infarction

Plant extracts given to them at a period of three weeks. After three weeks, subcutaneous injection of ISO was given in Swiss Albino Mice. The control mice received ISO injection died after one hour while the spinach treated died after three hours and Broccoli treated mice did not die until three hours and we sacrificed the animal, dissected out the heart from these three animals and transferred into a watch glass.

The required materials are under the DEPC Treatment. The heart of each mouse can be separated and placed into watch glass. Using scissors to cut the heart tissues and adding 1M Tris - Hcl and stored in -20 C. Homogenization can be done by using Homogenizer.

After homogenization, samples can be used for the RNA extraction.RNA isolation was done by using TRIzol RNA Extraction.

Quantification of RNA by Spectrophotometer

To check the concentration of RNA in different samples using Spectrophotometer (Table No.3).

Expression of cardiac markers

Reverse Transcriptase PCR analysis of GAPDH in the Heart tissue of control, Broccoli and Spinach treated mice

PCR can be done by GAPDH primers are used to check the expressions of GAPDH (Figure No.1) or housekeeping gene. The expression of GAPDH is visible in the heart tissue of control, broccoli and spinach treated mice.

Reverse Transcriptase PCR analysis of the expression of Cardiac Markers (Aspartate Transaminase)

Reverse Transcriptase PCR analysis of the expression of Cardiac Markers (Aspartate Transaminase) in the heart tissue of control, broccoli and spinach treated mice. The expressions of Aspartate transaminase (Figure No.2) have some similarities at the level of broccoli and spinach. In control mice have high concentrations of Aspartate transaminase. The level of expression of the gene was calculated from the band intensity values (Table No.4) obtained for the target genes such as Aspartate Transaminase calculated using the phoretix software:

Level of expression of the target genes (Aspartate Transaminase) normalized to control

$$= \frac{\text{band intensity of Aspartate transaminse}}{\text{GAPDH}} \times 100$$

Reverse Transcriptase PCR analysis of the expression of Cardiac Markers (Creatine kinase)

Reverse Transcriptase PCR analysis of the expression of Cardiac Markers (Creatine kinase) in the heart tissue of control, broccoli and spinach treated mice (Figure No.3). The expression of Creatine kinase is low in broccoli than control and spinach. The level of expression of the gene was calculated from the band intensity values (Table No.5) obtained for the target genes such as Creatine kinase calculated using the phoretix software:

Level of expression of the target genes (Creatine kinase) normalized to control

$$= \frac{\text{band intensity of Creatine kinase}}{\text{GAPDH}} \times 100$$

Figure No.1 and Figure No.2 shows that Reverse transcriptase PCR analysis of the expression of Aspartate transaminase and Creatine kinase. Both of these are the cardiac markers expressed in our heart tissue. The concentrations of cardiac markers will change depending on the condition of the heart whether it is healthy or diseased condition. Myocardial muscle Creatine kinase (CK- MB) is found mainly in the heart. Aspartate transaminase is an enzyme which is present in both mitochondria and cytoplasm released into blood when certain organs or tissues, particularly liver and heart are injured. The determination of Aspartate Transaminase (AST) was used to diagnose acute myocardial infarction. The level of cardiac markers is very low it helps to keep our healthy heart condition. Comparing to spinach and broccoli; the cardiac markers are low in broccoli than spinach.

Antioxidant assay – DPPH

The concentration of antioxidants in our samples like Spinach and Broccoli extracts can be determined using Antioxidant Assay - DPPH.

Concentration of antioxidants in standard and percentage of DPPH scavenging activity

Ascorbic acid is used in the standard or control. The concentrations of antioxidants in standard and the percentage of DPPH scavenging activity (Table No.6). Graph shows that Concentrations of antioxidants (Vitamin C) µg/ml or standard and DPPH scavenging activity in percentage (Figure No.4).

Concentrations of Antioxidants in Broccoli and Spinach

Concentrations of antioxidants in spinach and broccoli using DPPH assay (Table No.7). Graph shows that the concentration of antioxidants in selected samples (Figure No.5).

From the graph shows that, X- axis contains our selected samples and Y- axis contain different concentrations of antioxidants found in Broccoli and Spinach. Table No.6 show that concentration of antioxidants in standard. Vitamin C or Ascorbic acid is used for standard and Table No.7 shows that how much antioxidants present in our selected samples. Results found that, Broccoli contain more concentrations of antioxidants comparing to spinach. So, Broccoli is good for our heart health because, the more antioxidant contents found in broccoli than spinach. The concentration of antioxidants in our samples like Spinach and Broccoli extracts; comparing to them, Broccoli contain more antioxidants than Spinach. Broccoli included in the cruciferous vegetable family.

DISCUSSION

Myocardial infarction is an ischemic heart disease (IHD), characterized by the sudden and persistent curtailment of the myocardial blood supply which leads to myocardial necrosis (Tao *et al*, 2007)[13].

Recent studies show that spinach may help to protect people against inflammatory problems, oxidative stress-related problems, cardiovascular problems, and cancers (Song *et al*, 2010)[14]. Broccoli contains high levels of glucosinolates, carotenoids and total phenols, which are related with the prevention of chronic diseases (Bazzano *et al*, 2003)[15].

In our study was aimed at "Evaluating the cardio protective effect of various plant extracts having antioxidant potential in mouse (*Mus musculus*)". To determine the antioxidant potential activity in plant extracts the effects of expressions of genes and cardiac markers. There is a correlation between the concentration of antioxidants and the expression of cardiac markers. The high concentrations of antioxidants decrease the expression of various cardiac markers. We choose two cardiac markers like Aspartate transaminase and Creatine kinase. Comparing to them the Broccoli has low cardiac markers and increasing concentration of antioxidants. Broccoli contains more antioxidant concentrations comparing to Spinach and our study discussed about, evaluating the cardio protective efficiency of Broccoli in ISO induced myocardial infarction in Swiss Albino mice.

Previous studies found that leafy vegetables contain more numbers of antioxidant concentrations. In our study, determine the expressions of Myocardial Infarction markers like Aspartate transaminase and Creatine kinase and the expression is low. That means, Broccoli and Spinach are included in normal diet. Evidence suggests that regular consumption of fruit and vegetables minimizes the harmful effects, which has been somewhat accredited to the presence of compounds possessing antioxidant properties (Podsedek, 2007)[16]. The major antioxidants present in fruit and vegetables are: Vitamin C, Vitamin E, carotenoids and polyphenols, especially flavonoids, which all provide protection against free radicals (Monero *et al*, 2010)[17]. The quality and quantity of these antioxidant components are major attributes to the health benefits of fruit and vegetables that are associated with reduced risk of chronic disease (Roy *et al*, 2009)[18]. Higher cruciferous vegetables intake is associated with reduced risk of all-cause mortality (Mori *et al*, 2018)[19]. To increase the consumption of vegetables, particularly cruciferous vegetables and fruits to promote cardiovascular health and overall longevity (Zhang *et al*, 2011)[20]. Higher cruciferous vegetables intake is associated with reduced risk of all-cause mortality. Broccoli included in the cruciferous family (Mori *et al*, 2018)[19].

In our study we select two Green leafy vegetables like Spinach and Broccoli. As one of most important leafy vegetables, spinach has a high nutritional value, and is rich in carotene, Vitamin C, amino acids and iron, among others (Koh *et al*, 2012)[21]. Recent studies show that spinach may help to protect people against inflammatory problems, oxidative stress-related problems, cardiovascular problems, and cancers (Song *et al*, 2010)[14]. As such, spinach leaves play an important role in health and have been used in the treatment of human diseases since ancient times (Ammar *et al*, 2017)[22].

Broccoli belongs to the Brassica genus (Podsedek, 2007)[16] and is rich in both nutritional antioxidants; vitamins C and E, and non-nutritional antioxidants; carotenoids, and phenolic compounds, particularly flavanoids (Lin *et al*, 2005)[23] and also rich in polyphenols, a large group of phytochemicals that are often considered the most abundant antioxidants in the diet (Faller *et al*, 2009)[24]. Broccoli has been reported to contain both flavonol and hydroxycinnamoyl derivatives (Vasanthi *et al*, 2009)[25]. Few studies have investigated anthocyanins in broccoli which are the most prominent group of plant pigments among the coloured flavonoids and possess high antioxidant activity (AA) (Monero *et al*, 2010)[17]. Another major health-promoting compound present in broccoli is Vitamin C (Munyaka *et al*, 2010)[26].

Antioxidants play an important role in inhibiting and scavenging free radicals, thus, providing protection to human against infection and degenerative diseases. Now the modern research is directed towards “Natural antioxidants” from the herbal plants due to safe therapeutic. Current research activities found that, how to prevent cardiovascular diseases using a natural products. Natural products are used in daily diet to protect our heart. In our childhood, eat only healthy food items; avoid fatty food items and any other harmful activities like smoking, tobacco use, drinking etc. Previous studies found that the many drugs are used to treat cardiovascular diseases. In latest studies conduct only natural products because, drugs used for this purpose affect our system functions. Natural products are daily used food items, so they cannot damage tissues. In our study, we choose the broccoli and spinach. These are leafy vegetables and contain more numbers of antioxidant concentrations. These antioxidants help to protect our tissues. 75% peoples were used to eat fast foods and fatty food items. To avoid that items and vegetables and leafy vegetables are choose them in our diet. Leafy vegetables, vegetables and fruits contain more antioxidant concentrations.

Table No.1: Different group of mice and their treatments

S.No	Groups	Treatment
1	Group 1	Broccoli Extract
2	Group 11	Spinach Extract
3	Group 111	Control (Standard diet with adlibitum)

Table No.2: Primers used in RT – PCR

S.No	Primer Name	Primer Sequence
1	GAPDH F	AAGCCCATCACCATCTTCCA
2	GAPDH R	CCTGCTTCACCACCTTCTTG
3	CTK F	ACCTCCGAAAGCACAACAAC
4	CTK R	GTTGCCGCCTTTCTCCATAG
5	ATA F	GACCTCCAGATCCCATCCTG
6	ATA R	CAGACATTGATGCCCTGCTC

Table No.3: Conc. of RNA in different samples

S.No	Sample	Con. of RNA
1	Control	0.108µg/µl
2	Broccoli	0.764µg/µl
3	Spinach	0.768µg/µl

Table No.4: Level of expression of Aspartate transaminase Normalized to control (GAPDH)

S.No	Sample	Level of expression of Aspartate transaminase Normalised to control (GAPDH)
1	Control	118
2	Broccoli	78
3	Spinach	88

Table No.5: Level of expression Creatine kinase Normalized to control (GAPDH)

S.No	Sample	Level of expression of Creatine Kinase Normalised to control (GAPDH)
1	Control	78
2	Broccoli	32
3	Spinach	65

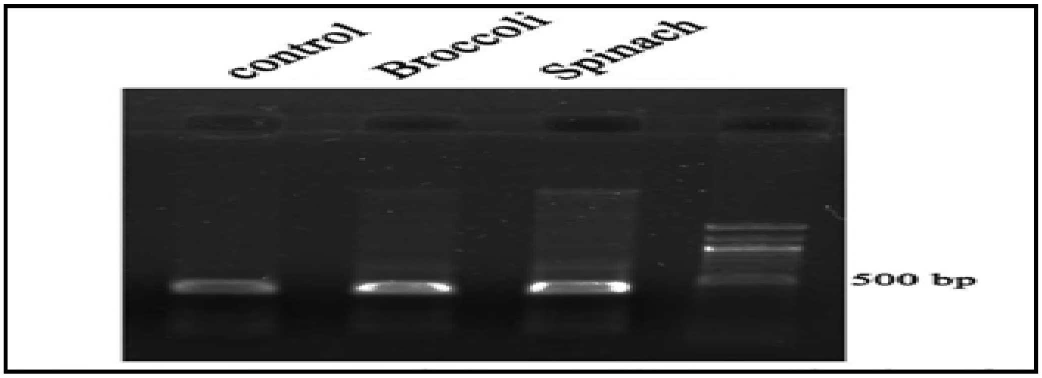
Antioxidant assay – DPPH

Table No.6: Concentration of antioxidants (Vitamin C) and percentage of DPPH scavenging activity

S.No	Con. of antioxidant (Vitamin C)µg/ml	DPPH Scavenging activity (%)
1	2	42.6
2	4	73.1
3	6	96.8

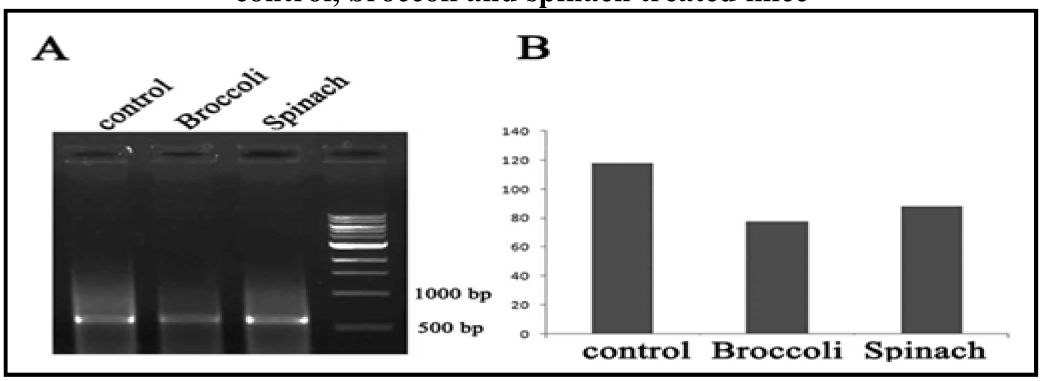
Table No.7: Selected samples like spinach, broccoli and its antioxidant concentrations

S.No	Sample	Antioxidants (µg/G)
1	Broccoli	73
2	Spinach	43.3



Reverse Transcriptase PCR analysis of GAPDH in the heart tissue of control, broccoli treated and spinach treated mice

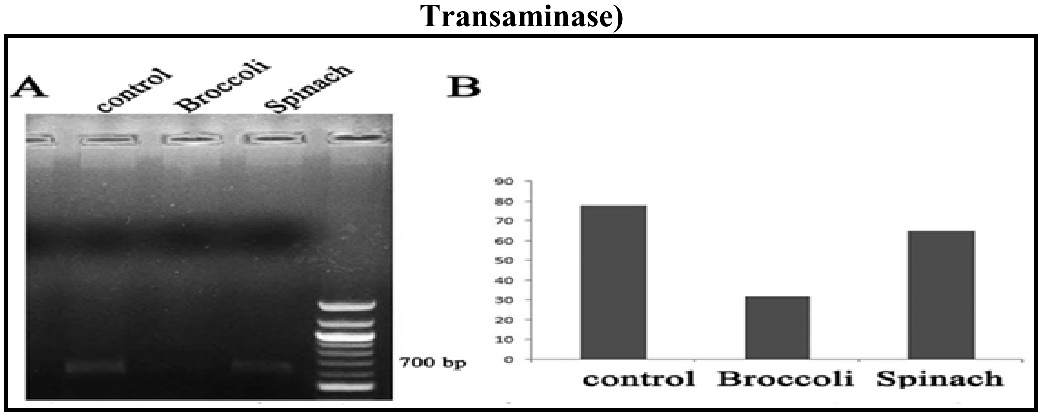
Figure No.1: Reverse transcriptase PCR analysis of the expression of GAPDH in the heart tissue of control, broccoli and spinach treated mice



(A) Reverse transcriptase PCR analysis of the expression of Aspartate Transaminase in the heart tissue of control, broccoli treated and spinach treated mice

(B) Quantitative analysis of the expression of Aspartate Transaminase done from the band intensity of Aspartate Transaminase normalized to GAPDH (Control)

Figure No.2: Reverse transcriptase PCR analysis of the expression of Cardiac Markers (Aspartate Transaminase)



(A) Reverse transcriptase PCR analysis of the expression of Creatine kinase in the heart tissue of control, broccoli treated and spinach treated mice

(B) Quantitative analysis of the expression of Creatine kinase done from the band intensity of Creatine kinase normalized to GAPDH (Control)

Figure No.3: Reverse transcriptase PCR analysis of the expression of Cardiac Markers (Creatine kinase)

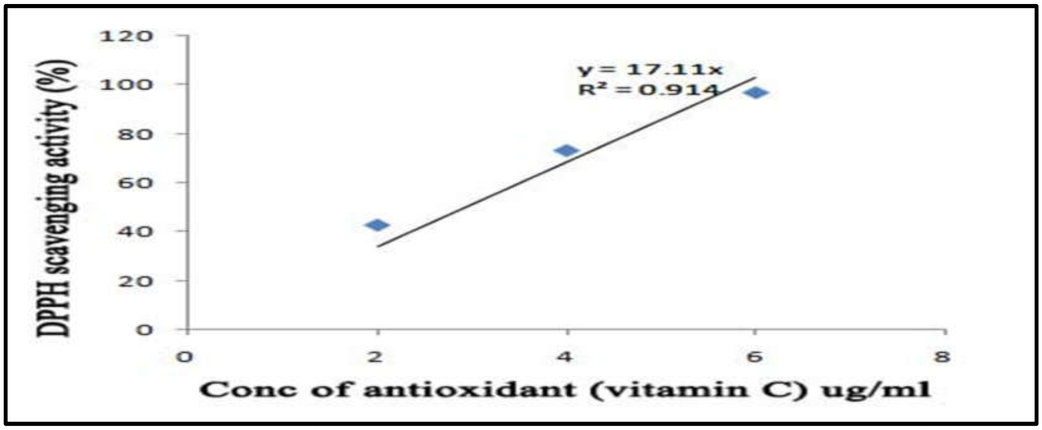


Figure No.4: Concentrations of antioxidants (Vitamin C) µg/ml or standard and DPPH scavenging activity in percentage

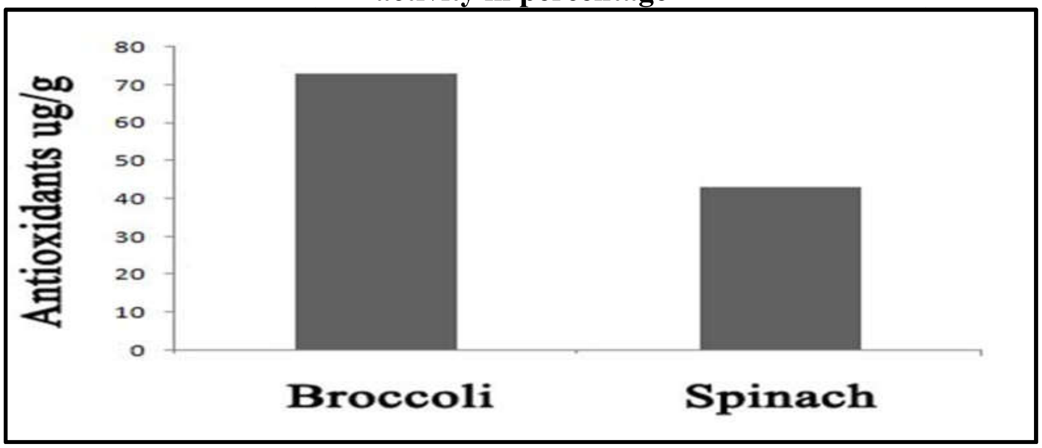


Figure No.5: Concentration of antioxidants in broccoli and Spinach

CONCLUSION

Broccoli (*Brassica oleracea*) contains more antioxidant concentrations comparing to Spinach and our study discussed about, evaluating the cardio protective efficiency of Broccoli in ISO induced myocardial infarction in Swiss Albino mice. There is a correlation between the concentration of antioxidants and the expression of cardiac markers. The high concentrations of antioxidants decrease the expression of various cardiac markers. Comparing to them the Broccoli has low cardiac markers and increasing concentration of antioxidants. The increasing concentration of antioxidants present in leafy vegetables should be included in our daily diet; there is a decrease level in cardiovascular diseases.

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CONFLICT OF INTEREST

The authors declare no conflicts of interest.

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