In-vitro Antioxidant activities of hydro alcoholic extracts of *Trigonella foenum-graecum* seeds, *Cinnamomum verum* bark and *Carica papaya leaves* and seeds

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ABSTRACT

Background: Oxidative stress and free radical damage have been implicated in the pathogenesis of various diseases like cancer. Apart from the human body's antioxidant mechanisms several dietary constituents could aid in mitigating oxidative stress.

Aim and objectives: To assess the in-vitro antioxidant activity of hydro alcoholic extracts of *Trigonella foenum graecum* seeds, *Cinnamomum verum* bark and CO.2 strain *Carica papaya* male leaves, female leaves and seed extracts.

Materials and methods: Hydroalcoholic extracts of *Trigonella foenum graecum* seeds, *Cinnamomum verum* bark, CO.2 strain *Carica papaya* male leaves, female leaves and seeds were prepared and in-vitro antioxidant activities were assessed by Total antioxidant activity assay, Reducing power assay, Nitric oxide scavenging activity assay, DPPH radical scavenging assay and Superoxide radical scavenging activity assay. Ascorbic acid and quercetin were used as standards. The experiments were done in triplicates and the results were subjected to statistical analysis.

Results: The results obtained indicated that all the herbal extracts studied, showed significant antioxidant activity.

Summary and Conclusion: The results of our study demonstrated that all the herbs studied exerted antioxidant effects through different mechanisms. Hence a polyherbal formulation comprising of all the above herbal extracts would be a good adjuvant in prevention and management of various diseases including cancer.

Key words: *Trigonella foenum-graecum*, *Cinnamomum verum*, *Carica papaya,* phytochemicals, oxidative stress, antioxidants.

INTODUCTION

Cells in tissues and organs are continuously exposed to free radicals generated exogenously from ionizing radiation, pollution, and smoking and endogenously by inflammatory reactions and mitochondria. Free radicals are unstable molecules that have an unpaired electron in their outermost shell. These molecules can accept electrons from adjacent atoms or molecules to attain stability, thereby causing damage to cellular structures. Cells withstand and counteract this condition by enzymatic and non-enzymatic antioxidant mechanisms. The imbalance between free radicals and antioxidant systems is termed as oxidative stress. This process is implicated in the pathogenesis of diseases like cancer (M. Valko, 2006). Several hundred years ago Charaka and Susrutha used medicinal herbs for management of various diseases. An attempt to re-explore the medicinal properties of herbs needs to be done for management of diseases such as oral cancer. In this regard, dietary phytochemicals like polyphenols, flavonoids, vitamins and various inorganic micronutrients with potent antioxidant properties have been the subject of extensive research for their potential benefits to reduce the risk of several types of cancer and as therapeutic adjuncts for cancer management as indicated by various studies in-vitro and in-vivo. These compounds are generally regarded safe as they are diet derived, and possess a long history of use in diet **(**T. Anbazhagi, 2009).

Fenugreek also called *Trigonella foenum graecum* is an annual plant belonging to the family Fabaceae. The plant is cultivated worldwide as a semi arid crop. *Trigonella foenum graecum* is one of the oldest medicinal herbs that originated from the Mediterranean region and Asia. It was a part of Indian diet even before 3000 years. (V. A. Parthasarathy et al., 2008). Fenugreek is known to stimulate digestive and metabolic process. It

is used for the management of diabetes and hypertension. (E. Edwin Jarald, 2006)

Cinnamomum verum also known as true cinnamon or Ceylon cinnamon is an evergreen tree whose barks and leaves are strongly aromatic. Cinnamon is indigenous to Sri Lanka. It has been cultivated in India, Seychelles, Madagascar, Brazil, South East Asia and other tropical countries since early times. Cinnamon is used as an analgesic, antiseptic, anti-rheumatic, antispasmodic, demulcent, digestive, expectorant, stomachic, diaphoretic, antibacterial and antifungal. It has been known to be used for the management of vomiting, diaorrhea, flatulence, bronchitis, common cold, palpitations, nausea, congestion and liver problems. (WHO Monographs on Selected Medicinal Plants)

Carica papaya commonly known as papaya is a tree cultivated throughout India for its fleshy fruits. Papaya originated in Southern Mexico and Costa Rica. It then was introduced as a plantation crop in Australia, Hawaii, Sri Lanka, South Africa and India. Young papaya leaves are used for the management of jaundice, urinary complaints, colic, fever, beriberi and asthma. In traditional medicine practice, leaves of papaya are dipped in hot water or warmed over fire and applied to painful parts in nervous pain. The seeds of papaya possess anti-fertility activity. They are also known to exhibit anti-helmintic action against Ascaris lumbricoides. Papaya seeds have been used as a carminative, emmenagogue, counter irritant and as a paste formulation in the management of ring worm and psoriasis and anti-fertility problems in males (K.L. Krishna et al., 2008).

With this available background literature, we conducted this study to estimate the in-vitro antioxidant activity of hydro alcoholic extracts of *Trigonella foenum graecum* seeds, *Cinnamomum verum* bark, CO.2 strain *Carica papaya* male and female leaves and CO.2 strain Carica papaya seeds.

MATERIALS AND METHODS

Plant Material: The study material comprised of *Trigonella foenum graecum* seeds which were procured from Chennai, *Cinnamomum verum* bark which was procured from Coimbatore and CO.2 strain *Carica papaya* male leaves, female leaves and seeds which were procured from Tamil Nadu Agricultural University, Coimbatore. The collected plant materials were authenticated at Plant Anatomy Research Center, Tambaram, Chennai, Tamil Nadu.

Preparation of Extracts: The plant materials were washed to remove impurities, shade dried and coarsely powdered. Hydroalcoholic extracts with an ethanol water ratio of 60:40 were prepared using *Trigonella foenum graceum* seeds, *Carica papaya* leaves and seeds. Similarly a hydroalcoholic extract with an ethanol water ratio of 70:30 was prepared using *Cinnamomum verum* bark by maceration for 72, 48 and 24 hours. The pooled extracts obtained were collected and concentrated using rotary flash and further concentration was done in a water bath and vaccum dessicator. Physical characteristics of the extracts were also observed. The extracts were stored at 2-8 degree Celsius until use.

Preliminary Phytochemical Analysis: Tests for phytochemical constituents were performed according to Harborne

Test for Terpenoid (Noller's Test): The substance was warmed with tin and thionyl chloride. Pink coloration indicated the presence of triterpenoids. If the terpenoid was in a bound form Libermann Burchard test was performed.

Test for Flavonoids: To the substance in alcohol, 10% Sodium hydroxide solution or ammonia was added. Dark yellow color indicated the presence of flavonoids.

Test for Steroids and Bound Terpenoids (Libermann Burchard Test): The test sample was dissolved in a few drops of 3ml of acetic anhydride to which addition of 3ml glacial acetic acid was done. Following this the reaction mixture was warmed and cooled under running tap water. To this cold concentrated sulfuric acid was added along the sides of the test tube drop by drop. Formation of bluish green color indicated the presence of steroids and brown layer indicated the presence of bound terpenoids.

Test for Glycosides: The substance was mixed with a little amount of anthrone on a watch glass. One drop of concentrated sulphuric acid was added, made into a paste and warmed gently on a water bath. Dark green coloration indicated the presence of glycosides.

Test for Carbohydrates: The substance was warmed with Fehling solution I and II. Appearance of blue, green or red coloration indicated the presence of carbohydrates.

Test for Polyphenols: To the test substance few drops of alcohol and ferric chloride solution were added. Bluish green or red color indicated the presence of phenols.

Test for Tannins: The test substance was mixed with basic lead acetate solution. Formation of a white precipitate indicated the presence of tannins.

Test for Saponins: The test substance was shaken with water. Copious lather formation indicated the presence of saponins.

Test for Amino acids: The test substance was warmed with ninhydrin reagent. Formation of a purple color indicated the presence of free amino acids.

Antioxidant activity: Antioxidant activity of various concentrations of extracts of *Trigonella foenum graecum seeds*, *Cinnamomum verum* bark, CO. 2 Strain *Carica papaya* male, female leaves and seeds from 1000µg/ml to 10µg/ml were assessed by determining Total Antioxidant Activity, Reducing Power, Nitric oxide scavenging activity assay, 1,1 Di phenyl 2 picrylhydrazide (DPPH) scavenging assay and Superoxide radical scavenging activity assay. The stock solution of extracts was prepared by dissolving 10mg of the extract in 1 ml of Dimethylsulfoxide and 9ml of distilled water. Serial dilutions were prepared by dissolving the stock solution in distilled water to obtain concentrations of 1000µg/ml, 800µg/ml 400µg/ml, 200µg/ml, 100µg/ml, 50µg/ml, 10 µg/ml.

Total antioxidant activity (Prieto et al., 1999): 2ml of the reagent mixture (0.6M of sulfuric acid, 28mM sodium phosphate, 4mM Ammonium molybdate in 1000ml of water) was added to 0.2ml of the test substance. Incubation was done for 90 minutes at 95ºC using quercetin as standard and distilled water as blank. The reading was taken at 695nm using a UV spectrophotometer.

Reducing power (Oysizu M et al., 1986): 2.5ml Phosphate buffer (0.2M, pH 6.6) and 2.5ml of 1% Potassium ferricyanide were added to 1 ml of various concentrations of the extracts ranging from 1000µg/ml to 10 µg/ml. Following incubation at 50ºC for 20 minutes, 2.5ml of 10% Tricarboxylic acid was added and the mixture was centrifuged at 3000rpm for 10 minutes. To 2.5ml of the supernatant , 2.5ml of distilled water and 0.1% Ferric Chloride were added and the reading was taken at 700nm using a UV spectrophotometer with distilled water as blank and quercetin as standard.

DPPH radical scavenging assay (Yohozowa et al., 1998): To 0.1 ml of the test substance, 200µM of DPPH dissolved in 1.9ml ethanol was added and incubation was done in the dark for 20 minutes following which reading was taken at 517nm using UV spectrophotometer using ethanol as blank. Dimethylsulfoxide and distilled water in a ratio of 1:9 and 200µM of DPPH dissolved in 1.9ml ethanol were used as control and ascorbic acid was used as standard. The percentage inhibition was calculated using the formula $\{1 – (AB_{sample}/ ABS_{control})\}$ X 100

Nitric oxide scavenging activity (Alderson et al., 2006):10mM of Sodium Nitro prusside in 2ml of Phosphate Buffered Saline was added to 1 ml of the test substance and mixed well using vortex and incubated for 4hours at 37ºC. To this 0.5ml of Griess Reagent was added and the absorbance was measured at 546nm using a UV spectrophotometer. Ascorbic acid was used as standard and Dimethylsulfoxide in distilled water (1:9) was used as control. Percentage inhibition was calculated using the formula:$\{1 – (AB_{sample}/ ABS_{control})\}$ X 100

Superoxide radical scavenging activity assay (Robak and Gryglewski et al., 1998): 0.5ml of 16mM TRIS HCl buffer (pH 8), 0.5ml of 0.3mM Nitrobluetetrazolium, 0.5ml of 0.936mM Nicotinamide Adenine Dinucleotide Reduced and 0.5ml of 0.12mM PhenazineMethosulphate were mixed well with 1ml of the test substance in a vortex and incubated in dark for 5 minutes and absorbance at 560 nm was measured using a UV spectrophotometer with distilled water as blank. Quercetin was used as standard and Dimethylsulfoxide and distilled water in a ratio of 1:9 was used as control. Percentage inhibition was calculated using the formula:

$\{1 – (AB_{sample}/ ABS_{control})\}$ X 100

Statistical analysis: All the experiments were done in triplicates and calculations of mean and standard deviation was done. The results of each antioxidant assay were subjected to Kruskall Wallis test for analyzing variations in antioxidant activities between the different herbal extracts and Mann Whitney test to analyze the differences in activities of each extract at different concentrations using SPSS software version 16.

RESULTS

Trigonella foenum graecum seeds, *Cinnamomum verum* bark, CO.2 strain *Carica papaya* leaves (male and female), and seed extracts showed presence of polyphenols and flavonoids and several other phytochemical constituents as shown in Table 1. *Carica papaya* male and female leaves exhibited differences in phytochemical constituents (Table 1). All the extracts showed potent antioxidant activity in Total antioxidant activity assay (Figure 1), Reducing power assay (Figure 2), Nitric oxide scavenging activity assay (Figure 3), Diphenyl picryl hydrazyl scavenging activity assay (Figure 4), Superoxide radical scavenging activity assay (Figure 5) in a dose dependent manner. *Carica papaya* female leaf extracts exhibited maximum Total antioxidant activity and Superoxide radical scavenging activity at a concentration of 1000µg/ml in comparison with other extracts. *Trigonella foenum graecum* seed extracts exhibited maximum Reducing power and Nitric

oxide scavenging activity at 1000 µg/ml in comparison with other extracts. *Carica papaya* seeds showed highest Diphenyl picryl hydrazyl radical scavenging activity at 1000µg/ml in comparison with other extracts.

Table.1.Phytochemical constituents of *Trigonella foenum graecum* seeds, *Cinnamomum verum* bark, *Carica papaya* male and female leaves and seed extract

Name of the compound	*Trigonella foenum graecum* seeds	*Cinnamomum verum* bark	*Carica papaya* male leaf	*Carica papaya* female leaf	*Carica papaya* seed
Triterpenoids	+	+	-	-	+
Flavonoids	+	+	+	+	+
Steroids	+	-	-	-	+
Glycosides	+	+	+	+	+
Carbohydrates	+	+	+	+	-
Polyphenols	+	+	+	+	+
Tannins	-	+	-	-	-
Saponins	+	+	+	-	+
Amino acids	+	-	-	-	+

In the above table, + indicates the presence of the active constituent, - indicates absence of the active constituent

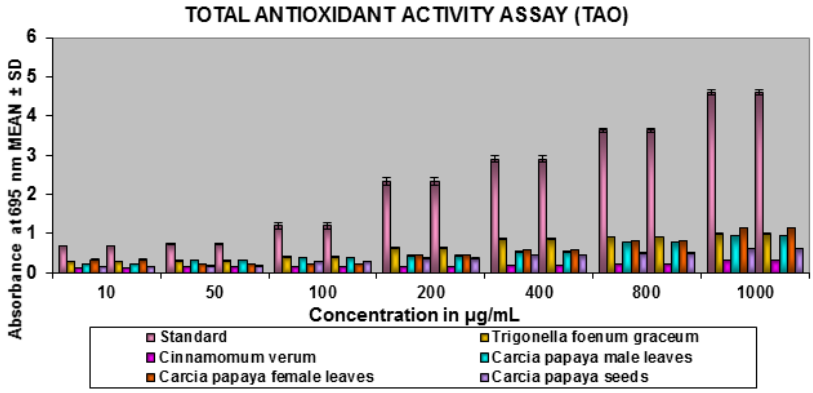

Figure.1. Total antioxidant activity assay

Bar Diagram representing Total Antioxidant Activity Assay (TAO) of Standard (Quercetin), *Trigonella foenumgraecum* seeds (F), *Cinnamomum verum* bark (C), *Carica papaya* male (PML) and female (PFL) leaves and seed (PS) extracts. (Values are represented as mean± Standard Deviation of triplicates) All the extracts exhibited significant antioxidant activity in a dose dependent manner with p<0.01.

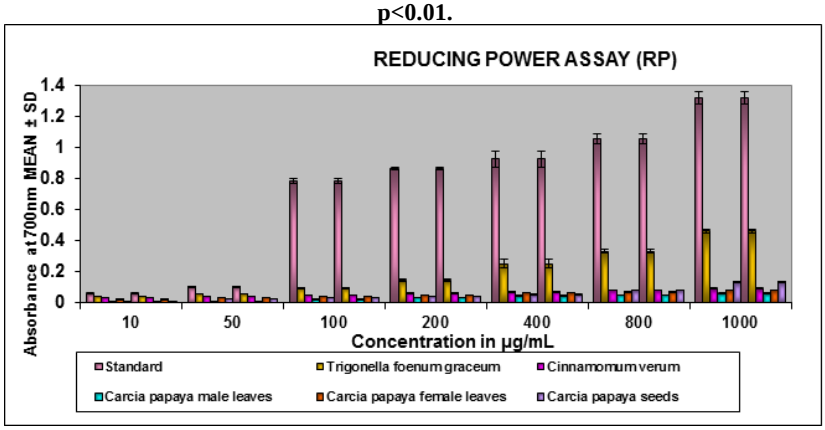

Figure.2. Reducing power assay

Bar Diagram representing Reducing power activity of Standard (Quercetin), *Trigonella foenum graecum* seeds (F), *Cinnamomum verum* bark (C), Carica papaya male (PML) and female (PFL) leaves and seed (PS) extracts(Values are represented as mean± Standard Deviation of triplicates) All the extracts exhibited significant antioxidant activity in a dose dependent manner with p<0.01

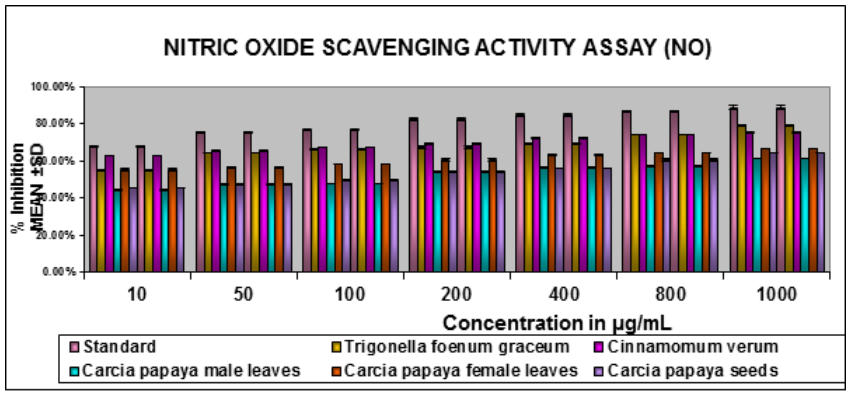

Figure 3:Nitirc oxide radical scavenging activity assay

Bar Diagram representing Nitric oxide scavenging activity of Standard (Ascorbic acid), *Trigonella foenum graecum* seeds (F), *Cinnamomum verum* bark (C), *Carica papaya* male (PML) and female (PFL) leaves and seed (PS) extracts. (Values are represented as mean± Standard Deviation of triplicates) All the extracts exhibited significant antioxidant activity in a dose dependent manner with p<0.01

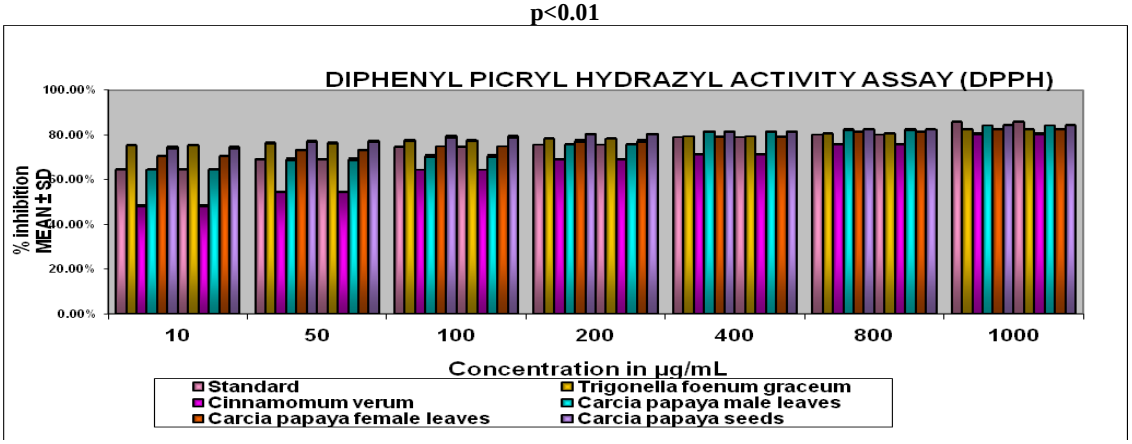

Figure 4:Diphenyl PicrylHydrazyl (DPPH) scavenging activity assay

Bar Diagram representing Diphenylpicrylhydrazyl (DPPH) scavenging activity of Standard (Ascorbic acid), *Trigonella foenum graecum* seeds (F), *Cinnamomum verum* bark (C), *Carica papaya* male (PML) and female (PFL) leaves and seed (PS) extracts. All the extracts exhibited significant antioxidant activity in a dose dependent manner with p<0.01

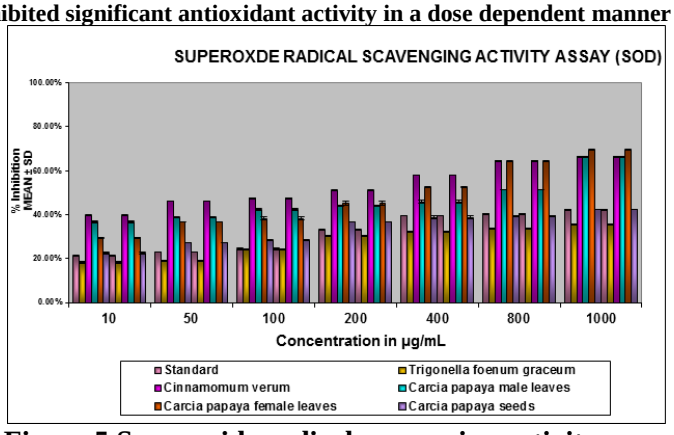

Figure.5.Superoxide radical scavenging activity assay

Bar Diagram representing Superoxide oxide scavenging activity of Standard (Quercetin), *Trigonella foenum graecum* seeds (F), *Cinnamomum verum* bark (C), *Carica papaya* male (PML) and female (PFL) leaves and seed (PS) extracts. All the extracts exhibited significant antioxidant activity in a dose dependent manner with p<0.01

DISCUSSION

It is well known that oxidative stress or free radical damage has been implicated in the pathogenesis of various diseases like cancer. The human body has evolved several potent antioxidant systems to tackle and mitigate the deleterious effects of reactive oxygen species and free radicals. A diseased state often is characterized by overzealous free radical damage or a weak antioxidant mechanism. Other than the human body's natural antioxidant defense system; diet is a source of several important antioxidants. Herbs, fruits, vegetables and other dietary components have natural antioxidant property, thereby conferring medicinal properties to diet. Polyphenol compounds like flavonoids present in plants exert antioxidant activity by scavenging free radicals. (R.Q. Wu et al., 2011).

Plant derived antioxidants are less likely to produce the side effects as they are diet derived. With the available background information, we made an attempt to study the in-vitro antioxidant activity of *Trigonella foenum graecum* seeds, *Cinnamomum verum* bark, and *Carica papaya* leaf and seed extracts. The choice of herbs used for our study was based on the fact that fenugreek, cinnamon and papaya are cost effective, easily grown and obtainable herbs. Moreover India is one of the important producers of all the three herbs. The results of our study showed that fenugreek seeds, cinnamon bark, papaya leaves (male and female), papaya seed extracts tested positive for all assays and demonstrated varying antioxidant properties at varying concentrations.

The antioxidant property of the hydro alcoholic extracts could be attributed to the presence of polyphenols. Phenolic compounds are diverse plant secondary metabolites comprised of aromatic rings, bearing one or more hydroxyl substituents and range from simple phenolic molecules like phenol to highly polymerized compounds often referred to as polyphenols. Plant polyphenols exert antioxidant activity by various mechanisms like reduction, hydrogen donation and quenching singlet oxygen species. Of the various polyphenols, flavonoids form the largest and most important group. Flavonoids and derivatives of flavonoids protect human body against reactive oxygen species with their antioxidant property (H.O. T. Iyawel et al., 2011).

The antioxidant property of *Trigonella foenum graceum* seeds can be attributed to the presence of polyphenolic compounds such as narigenin, quercetin (Kaviarasan et al., 2007) and coumarin (Syeda Birjees Bukhari et al., *2008*) which possess good hydrogen donating ability thereby contributing to the free radical scavenging activity of the extract. Flavonoids like vixetin, tricin, naringenin, quercetin and tricin 70 beta glucopuranoside (Shang M et al., *1998*) present in *Trigonella foenum graceum* seeds are good metal chelators and possess hydroxyl groups which are the ideal structural components for scavenging free radicals by hydroxyl ion donation, thereby contributing to the reducing power ability and radical scavenging activity exhibited in our study (A. Doss et al., 2010**,** Pittella et al., 2009)**.** Flavonoids also exert antioxidant property by inhibition of lipid peroxidation, platelet adhesion and aggregation (Thirunavukkarasu et al., 2003). Saponins present in our extract could possibly contribute to the antioxidant property by inhibition of lipid peroxidation.

The free radical scavenging activity of *Cinnamomum verum* extracts could be attributed to polyphenols like catechin, epicatechin, caffeic acid and procyanidin B2 (Peng X et al., *2008*). These compunds exhibit free radical scavenging activity by hydrogen donation. The reducing power exhibited by *Cinnamomum verum* extract might be due to the presence of di and mono-hydroxyl substitution in the aromatic ring of flavoring compounds in the extract which possess hydrogen donating abilities (Mathew. S. et al., 2006). Eugenol, the major aromatic and flavoring compound could be responsible for Diphenylpicrylhydrazyl (DPPH) radical scavenging activity by hydrogen donation (Chia-Wen Lin et al., 2009). Cinnamaldehyde, the aromatic and volatile compound in *Cinnamomum verum* could contribute to the antioxidant property of cinnamon by activating Nrf2 dependent antioxidant response (Georg T et al., 2010). The antioxidant property of *Cinnamomun verum* extract could also be due to the presence of phenolic compounds such as cinnamaldehyde, cinnamic acid, cinnamyl alcohol, coumarin, caffeic acid, ferulic acid, coumaric acid, protocatechuic acid and vanillic acid (Pramote Khuwijitjaru et al., *2012*) all of which exert antioxidant activity by hydrogen donation and inhibiting the chain reaction of lipid peroxidation.

Antioxidant property of *Carica papay*a leaves could be attributed to the presence of compounds like papain, chymopapain, cystatin, tocopherol, ascorbic acid, flavonoids, cyanogenic glucosides and glucosinolates that can increase the total antioxidant power in blood and reduce lipid peroxidation level (Otsukia et al., *2010*). To the best of our knowledge, this is the first study to have

investigated the variations in antioxidant activities in male and female leaves. The difference in antioxidant activities of male and female leaves observed in our study may be attributed to the differences in phytochemical constituents; however further studies are required to substantiate our results.

The antioxidant property exhibited by *Carica papaya* seed extracts could be attributed to the presence of polyphenolic compounds like vanillic acid, parahydroxybenzoic (Zhou et al., *2011*) which are known to exert redox property that plays a pivotal role in neutralizing free radicals, singlet and triplet oxygen molecules.

CONCLUSION

Thus all the herbs exerted antioxidant effects through different mechanisms and were potent at different concentrations. To the best of our knowledge this is the first study to have compared the antioxidant activity of hydroalcoholic extracts of *Trigonella foenum graceum* seeds, *Cinnamomum verum* bark, *Carcia papaya* leaves and seeds by various model systems and has compared the phytochemical constituents and antioxidant properties of *Carica papaya* male and female leaves. Hence a combination of these would possibly have synergistic effect in mitigating oxidative stress. Future research can be done to develop a polyherbal formulation comprising all the above herbal extracts to combat oxidative stress thereby aiding in the prevention and management of diseases like cancer.

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