



# Effect of solvent and extraction time on the antioxidant properties of *Citrus macroptera*

Partha Sarathi Gope<sup>1</sup>

Alak Kanti Dutta<sup>2,3</sup>

Sukh Makhnoon<sup>4</sup>

Subrata Banik<sup>2</sup>

Muhammad Ali Siddiquee<sup>2</sup>

Yearul Kabir<sup>3</sup>

<sup>1</sup>Environmental Microbiology Laboratory, Centre for Food and Waterborne Diseases, International Centre for Diarrhoeal Disease Research, Bangladesh (ICDDR, B).

<sup>2</sup>Grain Quality and Nutrition Division, Bangladesh Rice Research Institute (BRRI), Joydebpur, Gazipur, Bangladesh.

<sup>3</sup>Department of Biochemistry & Molecular Biology, University of Dhaka, Dhaka, Bangladesh.

<sup>4</sup>Biochemistry and Biotechnology, Department of Life Sciences, North South University, Dhaka, Bangladesh.

## Corresponding Authors:

Alak Kanti Dutta  
Grain Quality and Nutrition Division,  
Bangladesh Rice Research Institute (BRRI),  
Joydebpur, Gazipur, Bangladesh.  
E-mail: leolak84@yahoo.com,  
leolak@gmail.com

## Introduction

Oxidative stress is a state of physiological condition, induced by overabundance of oxidants, including reactive oxygen species such as free radicals, oxygen ions and peroxide. Oxidative damage by free radicals is associated with many diseases; cancer and heart diseases is the most common (1-3). Antioxidants are micromolecules that act as an armor to inhibit apoptosis, mediated by reactive oxygen species. Antioxidant such as glutathione, ubiquinone, uric acid and the antioxidant enzymes such as glutathione peroxidase, superoxide dismutase

## Abstract:

We aimed to evaluate the effects of solvents and different time intervals on the extractions of phenolic compounds and antioxidant properties of the pulp of the *Citrus macroptera*. The pulp of the fruit was extracted for one and two hours separately with four types of solvent namely- methanol, ethanol, acetone and water.

Total phenolic content was determined by using Folin-Ceocalteu method, while ferric reducing antioxidant power (FRAP), total antioxidant capacity (TAC), 1,1-diphenyl-2-picrylhydrazyl DPPH radical-scavenging activities, alpha amylase inhibition assay, and the inhibition of the lipid per-oxidation were used to test their antioxidant properties.

Both the acetone extractions showed better extraction efficacy in the phenolic content extraction whereas water extractions showed higher antioxidant properties in TAC and DPPH radical scavenging activities.

Our present study indicates that both time and solvent extractions played a vital role in the extraction of phenolic contents and their antioxidant properties of *C. macroptera*. We propose water as more suitable solvent to extract this fruit considering its extraction efficacy as well as its availability and use.

**Keywords:** Phenolic compound, *Citrus macroptera*, Antioxidant properties.

and catalase, can be generated in the body. These are essential to prevent oxidative damage. However, the amount of these antioxidants generated in the body might be inadequate, particularly under conditions of oxidative stress or inflammation when free radicals production increases. Thus, adequate amount of antioxidants is required to prevent building up of free radicals and oxidative damage in our body (4). Fruits, vegetables, herbal and other plant ingredients are considered as alternative source of natural antioxidants, which are complement to antioxidants, produced in human body. These natural food products are enriched with vitamins (A, E and C), phenolic compounds, including

flavonoids, tannins and lignins, all these compounds have antioxidant properties (5-6). Natural antioxidants may act as free-radical scavengers, chain breakers, chelators of pro-oxidant metal ions, and quenchers of singlet oxygen present in the environment (7), thereby reducing oxidative damage to lipids, DNA, and proteins. For these reasons over the past two decades researchers have highlighted on natural antioxidants and still new studies are on-going to investigate phenolic compounds from edible plant products due to their promising source of the natural antioxidant.

*Citrus macroptera* Montr., commonly known as *Shatkara*, is a semi-wild species of citrus, which is naturally available to Malesia and Melanesia. It is also widely available in the Sylhet region of Bangladesh. The tree usually grows up to 5 m and has thorns. Its fruit is about 6–7 cm in diameter, has a fairly smooth, moderately thick peel, and is yellow when ripe. The pulp of the fruit is greenish-yellow and used in cooking due to its sour taste and particularly to impart flavor to savory dishes. In the rural areas people also use this to treat fever and diarrhea. Malik et al, (8) reported *Shatkara* as an endangered wild species, used by locals in Northeast India as medicine for stomach pain and alimentary disorder.

Previous studies on the bark and leaves of the *Shatkara* showed the antioxidant and antimicrobial activities (9), however there is not available information on the other parts of the plant or the effect of the various types of solvent mixtures on the extraction of the antioxidant from this plant. Considering the importance of *Shatkara* in cooking and medicinal uses, we investigated the antioxidant potential of the pulp of *Shatkara* and attempted to optimize the extraction

procedure by using different solvents with one and two hours of extraction.

## Materials and Methods:

### Chemicals and reagents

Sodium carbonate, disodium hydrogen phosphate, potassium dihydrogen phosphate, Folin–Ciocalteu’s phenol reagent, sodium salicylate, trichloroacetic acid, methanol, ethanol and iron (II) sulfate-7-hydrate were purchased from Merck Chemicals. DPPH was purchased from Sigma Aldrich. All the reagents were analytical and HPLC grade.

### Extraction and preparation of sample

The fruit was collected from the Sylhet region of Bangladesh, as it is the native of Sylhet. After collection the specimen was identified by the plant taxonomist of the National Herbarium of Bangladesh, Mirpur, Dhaka, Bangladesh. The fruit was cleaned in the running water and subsequently peeled off to take the pulp. The pulp was then extracted with the 50% fraction of the acetone, methanol, ethanol and water, separately for one hour and two hour. The extracts were filtered through Whatman No. 1 filter paper and used directly for the estimation of total phenolic compound and assessment of antioxidant properties.

### Total phenolic content

The total phenolic content (TPC) of the extracts was determined according to the Folin–Ciocalteu method (10). The total phenolic content was expressed as gallic acid equivalents in mg/100g of sample dried weight.

### Ferric reducing antioxidant power (FRAP)

The ferric reducing antioxidant power (FRAP) assay reported by Chu et al.(11) was adopted.

FRAP of extracts was expressed mg ascorbic acid equivalent/100g of sample dried weight.

#### Total antioxidant capacity

The total antioxidant capacity (TAC) of extracts was evaluated by the method of Prieto et al. (12). TAC of extracts was expressed mg ascorbic acid equivalent/100g of sample dried weight.

#### DPPH radical scavenging activity

The 1,1-diphenyl-2-picrylhydrazyl (DPPH) radical scavenging activity reported by Ranilla et al.(13) was adopted. Radical-scavenging ability was calculated as IC50.

#### Alpha amylase inhibition assay

The  $\alpha$ -amylase inhibitory activity was determined according to the method by Ranilla et al.(13). It was expressed as the mg maltose/g.

#### Lipid peroxidation assay

A modified thiobarbituric acid-reactive species (TBARS) assay (14) was used to measure the lipid peroxide formed, using egg yolk homogenates as lipid rich media (15).

### Statistical analysis

Three replicates of each sample were used for statistical analysis and the values were reported as mean  $\pm$  SD. Data were also subjected to the analysis of variance and mean values were compared by Duncan's post-hoc multiple comparison tests. Differences at  $P < 0.05$  were considered to be significant.

## Results

#### Total phenolic content

Phenolic compounds contribute to overall antioxidant activities of *Shatkara* extracts. Total phenolic contents (TPC) of the antioxidant extracts prepared from *Shatkara* samples were

examined and presented in Table 1. *Shatkara* extracts differed significantly ( $P < 0.05$ ) in their total phenolic contents among the different extractions in two different time extraction periods. Acetone-2H extracts contained the highest level of total phenolics ( $91.30 \pm 4.07$  mg GAE/100g), suggesting that 50% acetone is a better choice for extracting phenolics from *Shatkara*. Besides, ethanol 2H ( $85.09 \pm 1.93$  mg GAE/100g) and acetone-1H ( $86.33 \pm 0.54$  mg GAE/100g) also showed very high level of phenolic content.

#### FRAP

Table 1 shows the antioxidant capacity of the *Shatkara* using different types of solvent, which ranged between  $46.75 \pm 0.45$  mg AAE/100g (methanol-1H) to  $55.65 \pm 0.16$  mg AAE/100g (ethanol-1H) and  $55.25 \pm 1.52$  mg AAE/100g (methanol-2H). Significant variation ( $P < 0.05$ ) was found among the extraction methods. With the exception of the acetone extraction, the influence of time was observed between the two different time intervals in the same solvent.

#### Total antioxidant capacity

The total antioxidant capacity was determined by the phosphomolybdenum method and the results are shown in Table 1. Water-1H extraction displayed the highest antioxidant capacity with the value of  $206.21 \pm 2.02$  mg AAE/100g, followed by water-2H extraction ( $201.52 \pm 13.30$  AAE/100 g), ethanol-2H ( $182.77 \pm 3.51$  AAE/100g), methanol-1H ( $166.37 \pm 4.05$  AAE/100g), acetone-1H ( $160.51 \pm 5.36$  AAE/100g), methanol-2H ( $158.17 \pm 3.51$  AAE/100g), acetone-2H ( $158.17 \pm 3.51$  mg AAE/100g) and finally ethanol-1H ( $157.00 \pm 2.02$  mg AAE/100g). The antioxidant capacity can be divided into three major groups based on the level of significance. Both the water extractions are in the highest group. The medium group comprised only ethanol-2H and the remaining five

extractions methanol-1H, methanol-2H, ethanol-1H, acetone-1H, acetone-2H were in the lowest group. Only ethanol extractions showed significant difference ( $P < 0.05$ ) between the two different time intervals.

#### DPPH radical scavenging activity

We investigated the influence of solvent and time on DPPH scavenging activities of *Shatkara* samples. The ethanol-2H extract exhibited the highest DPPH radical scavenging activity ( $0.28 \pm 0.02$  mg/mL), and followed by acetone-2H ( $0.34 \pm 0.02$  mg/mL), water-2H ( $0.39 \pm 0.02$  mg/mL), water-1H ( $0.41 \pm 0.00$  mg/mL), ethanol-1H ( $0.45 \pm 0.02$  mg/mL), methanol-2H ( $0.52 \pm 0.01$  mg/mL), acetone-1H ( $0.57 \pm 0.00$  mg/mL) and methanol-1H ( $0.61 \pm 0.00$  mg/mL) under the experimental conditions. Significant difference ( $P < 0.05$ ) was found among the solvents as well as within the same solvent between the two time periods.

#### Alpha amylase inhibition activity

Alpha amylase inhibition activities were calculated in mg maltose/g. The more maltose liberated, the less the amylase inhibition. Both the water-1H and the methanol-1H showed the highest inhibition ( $31.20 + 1.24$  mg maltose/g and  $31.21 + 2.17$  mg maltose/mg respectively) and the acetone-2H displayed the lowest ( $50.56 + 1.43$  mg maltose/g). Besides, the methanol-2H ( $36.84 + 1.67$  mg maltose/g) and the ethanol-1H ( $38.96 + 5.69$  mg maltose/g) also displayed the considerable alpha amylase inhibition activity. From the graph, it is evident that the inhibition activities decreased in the second hour extraction. Significant reduction ( $P < 0.05$ ) of inhibition activity was observed in each solvent group with different extraction times.

#### Lipid peroxidation inhibition activity

Lipid peroxidation inhibition activity was measured by using the TBARS method, where the egg yolk

was used as the lipid rich media. The findings of this method were quite similar to the results of the alpha amylase inhibition assay. In case of methanol and the ethanol extractions, second hour extractions showed less activity compare to the first hour, whereas the other two solvents showed the increased activity in the second hour. Overall the activity ranged between the 20.50% (water-1H) to 60.10% (ethanol-1H) (Fig-2). Besides the water-2H, both the methanol extractions, and the water-2H fraction showed the potential inhibition activities. Based on the inhibition activity of lipid, which was: water > acetone > methanol > ethanol, the choice of solvents can be preferred.

## Discussion

#### Total phenolic content

Phenolic compounds are secondary plant metabolites with beneficial biological effects, e.g. as anti-bacterial, anti-inflammatory and anti-allergic agents (16). In this study, all of the extractions showed potential content of the phenolic compound. Here in this study, the *Shatkara* content differed significantly ( $P < 0.05$ ) in most of the solvents indicating the possible time influence among them. These results were in agreement with previous studies which showed that solvent nature exert a great power in phenolic extraction capacities in many species (17,18). Besides, the study by Michiels et al., (19) showed that the extraction conditions can greatly influence antioxidant capacity assays in plant food matrices.

#### Ferric reducing antioxidant power (FRAP)

FRAP assay provides a simple and effective method for measuring the ability of antioxidants in plant samples to act as reducing agents. From the Table-1, it was evident that all the solvent

extractions showed significant difference among them as well as the different time intervals, although the range is not as high as compared to the TAC or TPC. In most instances, the methanol extracts of the different plant parts contained substantial ferric reducing activities compared to the other extracts (4).

#### **Total antioxidant capacity**

The phosphomolybdenum method is a quantitative assay for the analysis of the total antioxidant capacity and it is expressed as the number of equivalents of ascorbic acid. Phosphomolybdenum method measures the antioxidant activity of the flavonoids, ascorbic acid and citric acid. As it is evident from the results displayed in the Table-1, only the ethanol extractions showed the influence of time in the antioxidant activity. Besides, the time influence was not found in other extraction methods.

#### **DPPH radical-scavenging activity**

DPPH is a nitrogen containing free radical, scavenged by an antioxidant that can act as a proton radical scavenger or nitrogen donors. In this study, all the extractions showed potential antioxidant power with the significant difference ( $P < 0.05$ ) among the different extractions as well as within the same extraction solvent between the two different time periods. A dose dependent relationship, ranging between 0.1-2.5 mg was observed. Previous study by Chowdhury et al., (8) reported DPPH radical scavenging activity of the bark of *C. macroptera* was 178.96 $\mu$ g/mL. However, the comparison with the previous study was not possible as different parts of the plant were analyzed.

#### **Alpha amylase inhibition activity**

Alpha amylase hydrolyzes the starch, which results in the release of glucose and the rapid increase of blood glucose level, which is characterized by the

type-2 diabetes. This may be controlled in the inhibition of enzymes involved in the digestion of carbohydrates. The consumption of inhibitors from the naturally occurring diets could be an effective way for managing post-prandial hyperglycemia with minimal side effects in contrast to the traditional treatments with drugs like acarbose (20). Unlike the previous antioxidant methods, water extractions did not show as much antioxidant power as it did in the previous antioxidant methods. Each solvent showed better efficacy in the second hour of extraction indicating that the longer extraction time is better for the alpha amylase inhibition. A previous study by Ranilla et al., (13) on some medicinal plants, herbs and spices found diminished or no activity which is not concordant with our study.

#### **Lipid peroxidation inhibition activity**

Lipid peroxidation inhibition activity is an important parameter for the food industry as it contributes to the flavor of the food and subsequently deteriorates the food quality. Some of them are aldehydes and cause serious health concerns. Therefore, the some synthetic antioxidants are added in the food to prevent oxidation. Here we found the effect of both extraction time and solvents affected the inhibition of the peroxidation, thus contributing to the food from the deterioration.

### **Conclusion**

The recovery of phenolic compound and its corresponding antioxidant properties are largely dependent on the choice of solvent as well as the extraction time, which is evident from this study. No single extraction method showed the best extraction and the antioxidant properties, i.e., acetone displayed the highest yield for the

phenolic content and the alpha amylase inhibition properties whereas water displayed the better total antioxidant capacity, DPPH and the anti-lipid peroxidation activity. Based on our

findings, we would like to suggest the use of the water for the extraction of phenolic compound and determination of antioxidant properties, as it is non-toxic, cheap and easily available.

**Table 1:** Total phenolic content, ferric reducing antioxidant power and total antioxidant capacity of the *Shatkara*

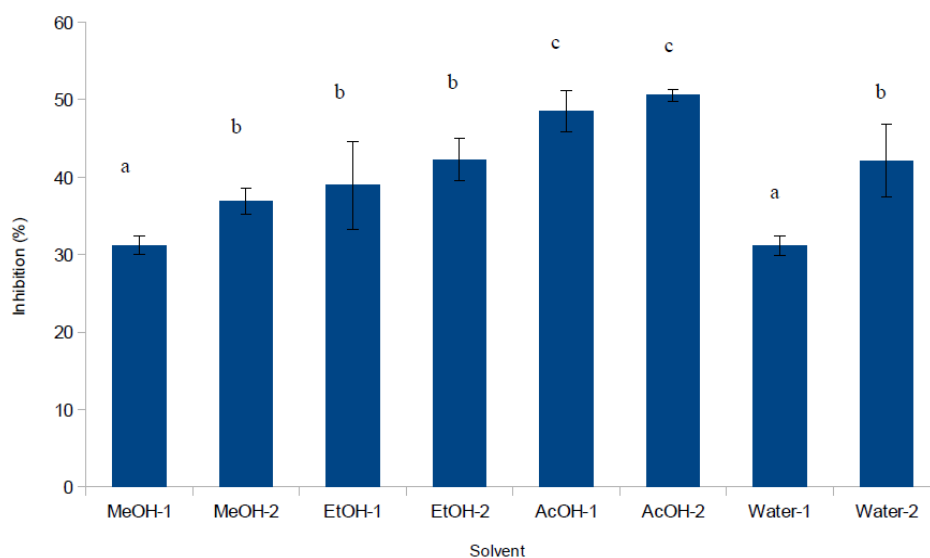
Solvent	Time (Hour)	TPC (mg GAE/100g)	FRAP (mg AAE/100g)	TAC (mg AAE/100g)
Methanol	1	33.85 ± 3.42 <sup>a</sup>	46.75 ± 0.45 <sup>a</sup>	166.37 ± 4.05 <sup>a</sup>
	2	77.84 ± 4.17 <sup>b</sup>	55.25 ± 1.52 <sup>bc</sup>	158.17 ± 3.51 <sup>a</sup>
Ethanol	1	62.73 ± 3.49 <sup>c</sup>	55.65 ± 0.16 <sup>b</sup>	157.00 ± 2.02 <sup>a</sup>
	2	85.09 ± 1.93 <sup>bd</sup>	50.91 ± 1.11 <sup>d</sup>	182.77 ± 3.51 <sup>b</sup>
Acetone	1	86.33 ± 0.54 <sup>bd</sup>	53.48 ± 0.74 <sup>c</sup>	160.51 ± 5.36 <sup>a</sup>
	2	91.30 ± 4.07 <sup>d</sup>	53.67 ± 0.51 <sup>c</sup>	158.17 ± 3.51 <sup>a</sup>
Water	1	51.14 ± 11.12 <sup>e</sup>	47.15 ± 1.85 <sup>a</sup>	206.21 ± 2.02 <sup>c</sup>
	2	82.19 ± 2.81 <sup>bd</sup>	50.02 ± 0.74 <sup>d</sup>	201.52 ± 13.30 <sup>c</sup>

n=3, Duncan p <0.05

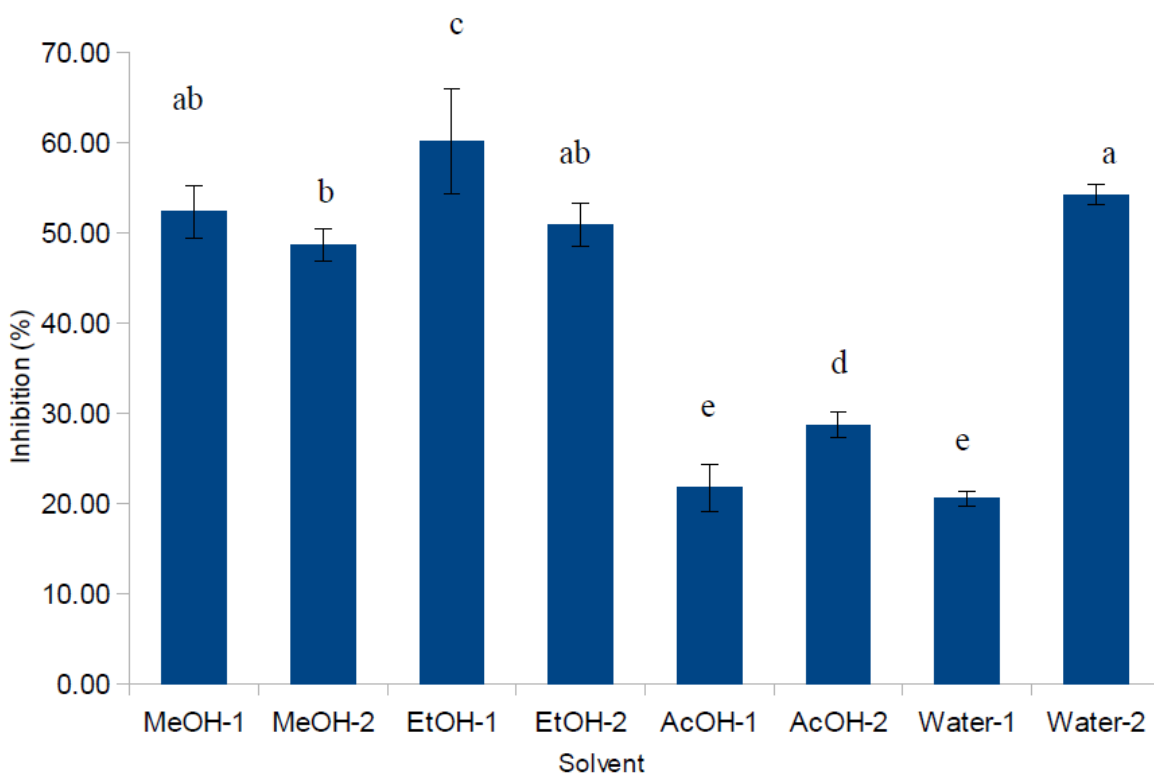
**Table 2:** IC50 values of *Shatkara*

Solvent	Time(Hour)	IC50(mg/mL)
Methanol	1	0.61 ± 00 <sup>a</sup>
	2	0.52 ± 0.01 <sup>b</sup>
Ethanol	1	0.45 ± 0.02 <sup>c</sup>
	2	0.28 ± 0.02 <sup>d</sup>
Acetone	1	0.57 ± 00 <sup>a</sup>
	2	0.34 ± 0.02 <sup>e</sup>
Water	1	0.41 ± 00 <sup>cf</sup>
	2	0.39 ± 0.02 <sup>f</sup>

n=3, Duncan p<0.05



**Fig 1:** a-amylase inhibition activity of the *C. macroptera* in different solvents in one and two hour extractions. MeOH, EtOH and AcOH represent methanol, ethanol and acetone respectively. Number 1 and 2 represent one hour and two hour extractions respectively. Vertical bars represent the standard deviation of each data point. Values marked by the same letter are not significantly different (P < 0.05).



**Fig 2:** Inhibition of lipid peroxidation activity of the *C. macroptera* in different solvents in one and two hour extractions. MeOH, EtOH and AcOH represent methanol, ethanol and acetone respectively. Number 1 and 2 represent one hour and two hour extractions respectively. Vertical bars represent the standard deviation of each data point. Values marked by the same letter are not significantly different ( $P < 0.05$ ).

## References

- 1) Azad N, Rojanasakul Y, Vallyathan V. (2008). Inflammation and lung cancer: Roles of reactive oxygen/nitrogen species. *J Toxicol Environ Health Part B: Critical Reviews*, 11, 1–15.
- 2) Heitzer T, Schlinzig T, Krohn K, Meinertz T, Münzel T. Endothelial dysfunction, oxidative stress, and risk of cardiovascular events in patients with coronary artery disease. *Circulation*. 2001; 104, 2673–2678.
- 3) Madamanchi NR, Vendrov A, Runge MS. Oxidative stress and vascular disease. *Arterios Thromb Vascular Biol*. 2005; 25 (1): 29–38.
- 4) Razali N, Mat-Junit S, Abdul-Muthalib AF, Subramaniam S, Abdul-Aziz A. Effects of various solvents on the extraction of antioxidant phenolics from the leaves, seeds, veins and skins of *Tamarindus indica*L. *Food Chem*. 2012; 131 (2): 441-448.
- 5) Boots AW, Haenen GRMM, Bast A. Health effects of quercetin: From antioxidant to nutraceutical. *Eur J Pharmacol*. 2008; 585 (2-3): 325–337.
- 6) Valko M, Rhodes CJ, Moncol J, Izakovic M, Mazur M. Free radicals, metals and antioxidants in oxidative stress-induced cancer. *Chemico-Biol Interac*. 2006; 160 (1): 1–40.
- 7) Amarowicz R, Pegg R, Rahimi-Moghaddam P, Barl B, Weil J. Free-radical scavenging capacity antioxidant activity of selected plant species from the Canadian prairies. *Food Chem*. 2004; 84 (4): 551-562.
- 8) Malik SK, Chodhury R. The cryopreservation of embryonic axes of two wild and endangered *Citrus* species. *Plant Gen Res: Charac Utilization*. 2006; 4 (3): 204-209.
- 9) Chowdhury SA, Sohrab MH, Datta BK, Hasan CM (2008). Chemical and Antioxidant Studies of *Citrus macroptera*. *Bang J Sci Indus Res*.
- 10) Marwah RG, Fatope MO, Al Mahrooqi R, Varma GB, Al Abadi H, Al-Burtamani SKS. Antioxidant

- capacity of some edible and wound healing plants in Oman. *Food Chem.* 2007; 101(2): 465–470.
- 11) Chu YH, Chang CL, Hsu HF. Flavonoid content of several vegetables and their antioxidant activity. *J Sci Food Agric.* 2000; 80(5): 561–566.
  - 12) Prieto P, Pineda M, Aguilar M. Spectrophotometric quantitation of antioxidant capacity through the formation of a phosphomolybdenum complex: specific application to the determination of vitamin E. *Anal Biochem.* 1999; 269(2): 337–341.
  - 13) Ranilla LG, Kwon YI, Apostolid E, Shetty K. Phenolic compounds, antioxidant activity and in vitro inhibitory potential against key enzymes relevant for hyperglycemia and hypertension of commonly used medicinal plants, herbs and spices in Latin America. *Biores Technol.* 2010; 101(12): 4676–4689.
  - 14) Ohkawa H, Ohisi N, Yagi K. Assay for lipid peroxides in animal tissue by thiobarbituric acid reaction. *Anal. Biochem.* 1979; 95 (2): 351–358.
  - 15) Ruberto G, Baratta MT, Deans SG, Dorman HJD. Antioxidant and antimicrobial activity of *Foeniculum vulgare* and *Crithmum maritimum* essential oils. *Planta Med.* 2000; 66 (4): 687–693.
  - 16) Park J, Lee J, Jung E, Park Y, Kim K, Park B, Jung K, Park E, Kim J, Park D. In vitro antibacterial and anti-inflammatory effects of honokiol and magnolol against *Propionibacterium sp.* *Eur J Pharmacol.* 2004; 496 (1-2): 189 – 195.
  - 17) Akowuah GA, Ismail Z, Norhayati I, Sadikum A. The effect of different extraction solvents of varying polarities on polyphenols of *Orthosiphon stamineus* and evaluation of the free radical-scavenging activity. *Food Chem.* 2005; 93 (2): 311–317.
  - 18) Turkmen N, Sari F, Velioglu YS. Effects of extraction solvents on concentration and antioxidant activity of black and black mate tea polyphenols determined by ferrous tartrate and Folin–Ciocalteu methods. *Food Chem.* 2006; 99 (4): 835–841.
  - 19) Michiels JA, Kevers C, Pincemail J, Defraigne JO, Dommes J. Extraction conditions can greatly influence antioxidant capacity assays in plant food matrices. *Food Chem.* 2012; 130 (4): 986–993.
  - 20) Kwon YI, Vatter DA, Shetty K. Evaluation of clonal herbs of *Lamiaceae* species for management of diabetes and hypertension. *Asia Pac J Clin Nutr.* 2006; 15(1):107-118.

**Article History:** -----

Date of Submission: 12-01-2014

Date of Acceptance: 29-01-2014

Conflict of Interest: NIL

Source of Support: NONE

