



***In-vitro* antioxidant activity of tender shoot of *Calamus leptospadix* Griff.**

Bitupon Borah^{1*}, Reshma Ahmed², Dibyojyoti Baruah¹, Debojit Kumar Sarmah¹, Sawlang Borsingh Wann² and Brijmohan Singh Bhau¹

¹Plant Genomics Laboratory, Medicinal Aromatic and Economic Plant Division, CSIR-North East Institute of Science & Technology, Jorhat-785006, Assam, India

²Biotechnology Division, CSIR-North East Institute of Science & Technology, Jorhat-785006, Assam, India

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ABSTRACT

Calamus leptospadix Griff. belongs to the family *Arecaceae*, is a very important cluster forming small gregarious cane grown in different parts of the world. Tender shoots of *C. leptospadix* are used in food and have got medicinal value in addition to its predominant use in the furniture industry. Antioxidant properties of the three extract viz. methanolic, ethanolic and aqueous extract of tender shoots of *C. leptospadix* were evaluated in this study. DPPH, H₂O₂ scavenging activity, reducing power assay and total phenolic and flavonoids content analysis were carried out. We found that the in methanolic and ethanolic extract of tender shoots of *C. leptospadix* possess the antioxidant activity in a dose-dependent manner. The total phenolic contents were found to be 79.56 µg GAE in 1mg of methanolic extract and 34.63 µg GAE in 1mg of ethanolic extract. Flavonoids were found to be 162.33 and 150.62 µg quercetin equivalents in 1mg of methanolic extract and ethanolic extract respectively which contributes to its antioxidant properties. Aqueous extract showed less antioxidant properties as compared to methanolic and ethanolic extract.

KEYWORDS: *Calamus leptospadix*, antioxidant, DPPH, H₂O₂, phenolic, flavonoids.



INTRODUCTION

Plant-derived products have potential in fast expanding pharmaceutical and nutraceutical industries, yet these potential are still largely unexplored. Nowadays, there is a growing interest in the exploitation of plant derived products and especially plants which are easily available but not commercially used. However, the fundamental utilization of plant derived products has not yet been fully explored. A number of studies have reported antioxidant activity from various agricultural byproducts. An antioxidant is a chemical that prevents the oxidation of other chemicals. They protect the key cell components by counteracting the damaging effects of free radicals, which are natural by-products of cell metabolism [1-2]. Oxygen is an essential element for the survival in this earth and during the process of oxygen utilization in normal physiological and metabolic processes and approximately 5% of oxygen gets univalently reduced to oxygen derived free radicals [3-4]. This free radicals possess an unpaired electron in the outer shell of the molecule. For this reason, the free radicals are highly reactive

and can react with proteins, lipids, carbohydrates and DNA. Free radicals attack the nearest stable molecules, stealing its electron. When the attacked molecule loses its electron, it becomes a free radical itself, beginning a chain reaction, finally resulting in the description of a living cell [5]. The most common reactive oxygen species (ROS) include superoxide (O²⁻) anion, hydrogen peroxide (H₂O₂), peroxy (ROO[·]) radicals, and reactive hydroxyl (OH[·]) radicals. The nitrogen-derived free radicals are nitric oxide (NO) and peroxynitrite anion (ONOO⁻) [6]. The ROS readily attack and induce oxidative damage to various biomolecules including proteins, lipids, lipoproteins and DNA [7]. This oxidative damage is decisive etiological factor concerned in quite a lot of chronic human diseases such as diabetes mellitus, cancer, atherosclerosis, arthritis, and neurodegenerative diseases and also in the ageing course [8]. Numerous synthetic antioxidants have been registered, but only a few species are permitted as food additives by the law because of toxicity effects and other side effects. Typical antioxidants permitted as the food additives are butylated hydroxy anisole (BHA), butylated hydroxy toluene

*Corresponding Author Address: Bitupon Borah, Medicinal Aromatic and Economic Plant Division, CSIR-North East Institute of Science & Technology, Jorhat-785006, Assam, India; Email: btprbrh3@gmail.com; Mobile: +91-8822077874

(BHT), pueraria glycoside (PG), and tertiary-butylatedhydroquinone (TBHQ) [9].

Recently, peoples are concerned about the problems of human health caused by food additives has once more reminded food scientists to the enthusiasm of seeking natural antioxidants from the various unexplored plant sources. So far, one of the understandings on this issue is that natural antioxidants are primarily plant phenolic compounds occurring in all parts of plant bodies. Common phenolic antioxidants from plant sources include flavonoids, cinnamic acid derivatives, coumarins, tocopherols, carotenoids, and polyfunctional organic acids [10]. In the search of new natural antioxidant rich plant, *C.leptospadix* was selected for this study as the young shoots is being used by the ethnic people of North East India as vegetables and a variety of therapeutic uses in different system of medicine [11]. The people of Nagaland used the fruit and seeds of the plant for treatment of indigestion and stomach disorder [12]. Previous study confirms its anthelmintic property and the presence of alkaloid, saponins, carbohydrates, glycosides, saponins, flavonoids, tannins and phenolic compounds as major phytoconstituents [13]. Considering the above potential beneficial health effects, the present investigation was carried out to study the antioxidant potential of the three different extracts viz. methanol, ethanol and water from tender shoots of *C.leptospadix* by various *in vitro* methods.

MATERIALS AND METHODS

Collection, drying of plant material and Preparation of the extract: The plant *C.leptospadix* was collected from Jorhat (Assam). The tender shoots of *C. leptospadix* were then shade dried in room temperature to attain crunchy enough to be fit for grinding. Plant extracts were prepared by using Soxhlet Apparatus. In the Soxhlet extraction 100 g of coarse powder was extracted with 1000 ml of methanol, ethanol and water separately. The extracts were concentrated by evaporating the solvent using rotary evaporator.

DPPH free radical scavenging assay: Ascorbic acid was taken as standard for this assay. Required quantity of ascorbic acid was dissolved in methanol to give the concentration of 2, 4, 6, 8, and 10 µg/ml. Sample solutions were prepared by dissolving the extracts in methanol to give concentrations of 20, 40, 60, 80, and 100 µg/ml. The DPPH scavenging activity of three extract were determined using the method described by Choi *et al.*, [14] with some minor modifications. The reaction mixture consists of 1 ml of 0.3 mM

DPPH solution and 2 ml of sample solution of different concentrations. Blanks were prepared by adding 1 ml of methanol to 2 ml of sample solution of different concentrations. 1 mL of DPPH solution and to 2 ml of methanol was used as a control. The solutions were allowed to incubate in dark at room temperature for 30 minutes. The absorbance values were measured at 517 nm. Percentage of antioxidant activity was calculated by using the following formula:

$$\text{Scavenging activity (\%)} = [A_0 - A_t/A_0] \times 100$$

Where, A_0 is the absorbance of control, and A_t is the absorbance of the sample or standard solution.

The IC₅₀ values were calculated by linear regression of plots.

Hydrogen peroxide radical scavenging assay:

Ascorbic acid was taken as standard for this assay. Required quantity of ascorbic acid was dissolved in distilled water to make concentrations of 200, 400, 600, 800 and 1000 µg/ml. Sample solutions were prepared by dissolving the extracts in distilled water to final concentrations of 200, 400, 600, 800 and 1000 µg/ml. The ability to scavenge hydrogen peroxide was determined according to the method of Ruch *et al.*, [15]. Test solutions were prepared by adding 0.6 ml of 40 mM H₂O₂ prepared in phosphate buffer saline (pH 7.4) and 1 ml of sample solution of different concentrations. Blanks were prepared without H₂O₂ for each concentration. For Control, 0.6 ml of 40 mM H₂O₂ was added to 1 ml of phosphate buffer saline (pH 7.4). The reaction mixtures were incubated for 10 minutes and the absorbance values were measured at 230 nm. The percentage of scavenging of hydrogen peroxide was calculated using the following equation:

$$\text{Percent scavenged [H}_2\text{O}_2] = [A_0 - A_t/A_0] \times 100$$

Where, A_0 is the absorbance of the control, and A_t is the absorbance of the sample or standard solution.

The extract concentration providing 50% inhibition (IC₅₀) was calculated from linear regression of plots.

Reducing power assay: Required quantity of ascorbic acid (standard) was dissolved in methanol to give the concentrations of 200, 400, 600, 800, and 1000 µg/ml. Sample solutions were prepared by dissolving extracts in methanol to give concentrations of 200, 400, 600, 800, and 1000 µg/ml. The reducing power of the tender shoots of *C. leptospadix* was determined according to the method of Oyaizu [16]. 1 ml of different concentrations of the sample solutions were mixed with phosphate buffer (2.5 ml, 0.2 M, pH 6.6) and potassium ferrocyanide (2.5 ml, 1%). The mixture was incubated at 50 °C for 20 min. A portion of

trichloroacetic acid (2.5 ml, 10%) was added to the mixture, which was then centrifuged at 3000 rpm for 10 min. The upper layer of the solution (2.5 ml) was mixed with distilled water (2.5 ml) and FeCl₃ (0.5 ml, 0.1%). The absorbance was measured at 700 nm and compared with standards. Increased absorbance of the reaction mixture indicated increased reducing power.

Total phenolic content determination: 1 mg gallic acid (standard) was dissolved in 1ml of distilled water and serially diluted to concentrations of 20, 40, 60, 80 and 100µg/ml. The total phenolic content was determined using the method of McDonald *et al.*, [17] with minor modifications.

Preparation of the calibration curve was prepared by adding 1 ml of different concentration of gallic acid, 5 ml of 0.2N Folin-ciocalteu and 4 ml of 75 g/l Na₂CO₃. Blank was prepared by using 1 ml of distilled water except gallic acid. Accordingly, 1 ml of sample solution (1000 µg/ml) was also mixed with the reagents above and the absorbance was measured at 765 nm to determine the total phenolic contents. The total content of phenolic compounds in the extract in gallic acid equivalents (GAE) was calculated by the following formula:

$$T = \frac{C.V}{M}$$

Where, T = Total content of phenolic compounds mg/ml plant extract in GAE;

C = The concentration of gallic acid established from the calibration curve in mg/ml;

V = The volume of extract in ml;

M = The weight of plant extract in mg.

Total flavonoids content determination: The total flavonoids content was determined by using the method of Ebrahimzaded *et al.*, [18] with slight modifications. 1 mg of quercetin was dissolved in 1 ml of methanol and diluted to 50, 100, 150, 200 and 250 µg/mL. The reaction mixture contains 1ml of different concentrations of extracts, 2 ml of methanol, 0.2 ml of 10% aluminium chloride, 0.2 ml of 1 M potassium acetate and 5.6 ml of distilled water. For blank, 1 ml of methanol was added except the test solution. 1 ml of sample solution (1mg/ml) was also mixed accordingly. After 30 minutes of incubation the absorbance was measured at 415 nm against the blank. The total flavonoids contents in the extract in quercetin equivalents were calculated by the following formula:

$$T = \frac{C.V}{M}$$

Where, T= Total flavonoids content in mg/ml of plant extract;

C= The concentration of quercetin established from the calibration curve in mg/ml;

V= The volume of extract in ml;

M= The weight of methanolic plant extract in mg.

Statistical Analysis: All the experimental results were mean ± SD of three parallel measurements.

RESULTS

Direct extraction from the plant parts using various types of solvents is the most common technique employed to obtain extracts with high antioxidant activity. From the table 1 ascorbic acid and three different extract showed DPPH free radical scavenging activity in a concentration dependent manner as the percentage of inhibition increases with the increasing concentrations. Methanolic extract showed inhibition of 29.43 ± 0.46 % at concentration of 50 µg/ml to 95.54±0.48 % at concentration of 250 µg/ml whereas, ascorbic acid showed faster inhibition with 31.34 ± 0.50 % at concentration of 10 µg/ml and achieved 98.56 ± 0.51 % at concentration of 50 µg/ml. Ethanolic extract and aqueous extract showed less percentage of inhibition as compared to methanolic extract and ascorbic acid (Fig 1). From the regression curve analysis IC₅₀ value for the standard ascorbic acid was found to be 16.12 µg/ml whereas IC₅₀ values for methanolic, ethanolic and aqueous extracts were found to be 90.56 µg/ml, 137.94 µg/ml and 171.95 µg/ml respectively in DPPH assay. In H₂O₂ scavenging activity ascorbic acid and all the three extract showed similar concentration dependant activity (Table-2). In comparison to ascorbic acid, which showed inhibition of 97.03±0.46 % at concentration of 1mg/ml with IC₅₀ value 488.65 µg/ml; methanolic, ethanolic and aqueous extracts showed inhibition of only 85.9±0.21%, 60.55±0.33 % and 40.60±0.28% at concentration of 1mg/ml (Fig. 2) and IC₅₀ values were 669.28µg/ml, 868.93µg/ml, and 1256.38µg/ml respectively. In reducing power assay an increase in the absorbance at 700nm indicated the greater reducing power. Methanolic extract showed dose dependent increase in absorbance starting from 0.43±0.12 at concentration of 200 µg/ml to highest absorbance of 0.459±0.06 at concentration of 1mg/ml. Ethanolic and aqueous extracts showed absorbance of 0.396±0.012 and 0.206±0.021 at the highest concentration of 1 mg/ml respectively. These were compared with standard ascorbic acid (Fig.3), which showed absorbance of 0.101±0.01 at concentration of 200 µg/ml and increased up to 0.550 ± 0.045 at concentration of 1mg/ml (Table.3). The total phenolic content was calculated from the calibration graph using gallic acid as standard (Fig.4) and found to be 79.56 µg, 34.63 µg and 30.50 µg GAE in 1mg of methanolic,

ethanolic and aqueous extracts (Table.4). From the calibration graph (Fig.5) total flavonoids contents was calculated as 162.33 μg , 150.62 μg and 70.90 μg quercetin equivalents in 1mg of methanolic, ethanolic and aqueous extracts respectively (Table 5).

DISCUSSION

Scavenging activity for free radicals of 1,1-diphenyl-2-picrylhydrazyl (DPPH) has been widely used to evaluate the antioxidant activity of natural products from plant as it is highly sensitive and rapid assay [19]. When an antioxidant scavenges the free radical by hydrogen donation, the purple color of DPPH in assay solution turns to yellow, which can be monitored spectrophotometrically at 517 nm [20]. In the present study, we investigated the DPPH radical scavenging activity of the three different extracts viz. methanolic, ethanolic and aqueous extracts of *C.leptospadix* tender shoots. It was observed that the scavenging activity was increased with the increasing concentration of the sample and methanolic extract showing highest DPPH radical scavenging activity than ethanolic and aqueous extracts. The results of the present study clearly indicate that methanolic extract has powerful antioxidant activity against DPPH free radical in *in vitro* condition.

Hydrogen peroxide itself is not very reactive, but can sometimes be toxic to cell because it may give rise to hydroxyl radical in the cells [21]. The extracts were capable of scavenging hydrogen peroxide in a concentration-dependent manner. Scavenging of H_2O_2 by extracts is due to their phenolic compounds, which can donate electrons to H_2O_2 , thus neutralizing it to water [22]. In this study, we found that methanolic extracts contains higher phenolic compound as compared to ethanolic and aqueous extract and therefore showed higher H_2O_2 scavenging activity.

The reducing capacity of a compound may serve as a significant indicator of its potential antioxidant activity [23]. The presence of reductants such as antioxidant substances causes the reduction of Fe^{3+} ferricyanide complex to Fe^{2+} ferrous form. Therefore, the reducing power of the sample could be monitored by measuring at 700 nm [24]. The reducing power of the extracts may be due to the

biologically active compounds in the extract which possess potent donating abilities. In this study, all the three extract showed reducing power activity with the highest in methanolic extract due to its high phenolic and flavonoids contents though it was found to be less in comparison to that of a standard drug ascorbic acid.

Phenolics are an important class of secondary plant metabolites possessing an impressive array of pharmacological activity with excellent radical scavenging ability. The antioxidant activities of phenolics are due to their redox properties. The phenol moiety (hydroxyl group on aromatic ring) helps them to work as reducing agents, hydrogen donors and singlet oxygen quenchers [25] and the flavonoids are one of the most diverse and widespread group of natural compounds, are probably the most important natural phenolics. These compounds possess a broad spectrum of chemical and biological activities including radical scavenging properties [26]. The result showed that all the three extract contains phenolic compounds and flavonoids with the highest found in methanolic extract that contains phenolic compounds and flavonoids in significant amount, which attributes to its rationality of possessing antioxidant activity by neutralizing the free radicals.

CONCLUSION

It was demonstrated that the use of several test methods for radical scavenging and antioxidant activity provides valuable data for better understanding of purposeful application of the plant extract. The *in vitro* antioxidant study suggested that the *Calamus leptospadix* Griff. plant extract possess antioxidant activity, which might be helpful in preventing or slowing the progress of various oxidative stress-related diseases. Further, investigation on the isolation and identification of antioxidant component(s) in the plant may lead to chemical entities with potential benefits for clinical use.

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Table-1. DPPH scavenging activity by ascorbic acid (standard), methanolic extract, ethanolic extract and aqueous extract of *Calamus leptospadix*

Concentration of Ascorbic acid (µg/ml)	% of inhibition Ascorbic acid	Concentrations of extracts (µg/ml)	% of inhibition MECL	% of inhibition EECL	% of inhibition AECL
10	31.34±0.50	50	29.43±0.46	15.33±0.23	5.11±0.27
20	55.89±0.35	100	54.56±0.51	38.8±0.33	25.50±0.31
30	89.83±0.87	150	78.25±0.25	59.77±0.55	40.94±0.83
40	94.50±0.50	200	94.57±0.51	75.63±0.42	60.56±0.53
50	98.56±0.51	250	95.54±0.48	80.70±0.36	78.13±0.33
IC 50(µg/ml) →	16.12 (y=1.7305x+22.109 R ² =0.8751)		90.56 (y=0.3445x+18.801 R ² =0.9249)	137.94 (y=0.3351x+3.775 R ² =0.9549)	171.95 (y=0.3662x+12.282 R ² =0.9987)

Table-2. H₂O₂ scavenging activity by ascorbic acid (standard), methanolic extract, ethanolic extract and aqueous extract of *Calamus leptospadix*

Concentration of (µg/ml)	% of inhibition Ascorbic acid	% of inhibition MECL	% of inhibition EECL	% of inhibition AECL
200	2.34±0.57	4.22±0.53	3.54±0.43	2.1±0.33
400	42.18±0.28	17.2±0.37	15.47±0.50	9.3±0.23
600	81.33±0.28	40.1±0.55	27.80±0.26	18.5±0.33
800	94.46±0.50	65.8±0.72	45.57±0.51	25.89±0.55
1000	97.03±0.46	85.9±0.21	60.55±0.33	40.6±0.28
IC 50(µg/ml) →	488.65 y=0.120x-9.03 R ² =0.8854	669.28 y=0.106x-20.944 R ² =0.9901	868.93 y=0.0721x-12.65 R ² =0.9938	1256.38 y=0.0468x-8.799 R ² =0.9802

Table-3. Absorbance of ascorbic acid (standard), methanolic extract, ethanolic extract and aqueous extract of *Calamus leptospadix* at 700 nm in reducing power assay

Conc. (µg/mL)	200	400	600	800	1000
Standard (Ascorbic acid)	0.101±0.01	0.209±0.007	0.312±0.009	0.456±0.041	0.550±0.045
MECL	0.043±0.12	0.119±0.03	0.210±0.03	0.350±0.11	0.459±0.06
EECL	0.025±0.013	0.086±0.033	0.172±0.023	0.301±0.002	0.396±0.012
AECL	0.011±0.02	0.056±0.011	0.110±0.013	0.159±0.009	0.206±0.021

Table-4. Total phenolic contents (µg/ml)

	Equation	Conc. of gallic acid(µg/mL)	Equation	Total phenol content (µg/mg)
MECL	Y=0.009x-0.061 r ² =0.999	79.56	$T = \frac{C.V}{M}$	79.56
EECL		34.63		34.63
AECL		30.50		30.50

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 Table-5. Total flavonoids content ($\mu\text{g/ml}$)

	Equation	Conc. of gallic acid($\mu\text{g/mL}$)	Equation	Total phenol content ($\mu\text{g/mg}$)
MECL	$Y=0.0039x+0.0289$	162.33	$T = \frac{C.V}{M}$	162.33
EECL	$r^2=0.9997$	150.62		150.62
AECL		70.90		70.90

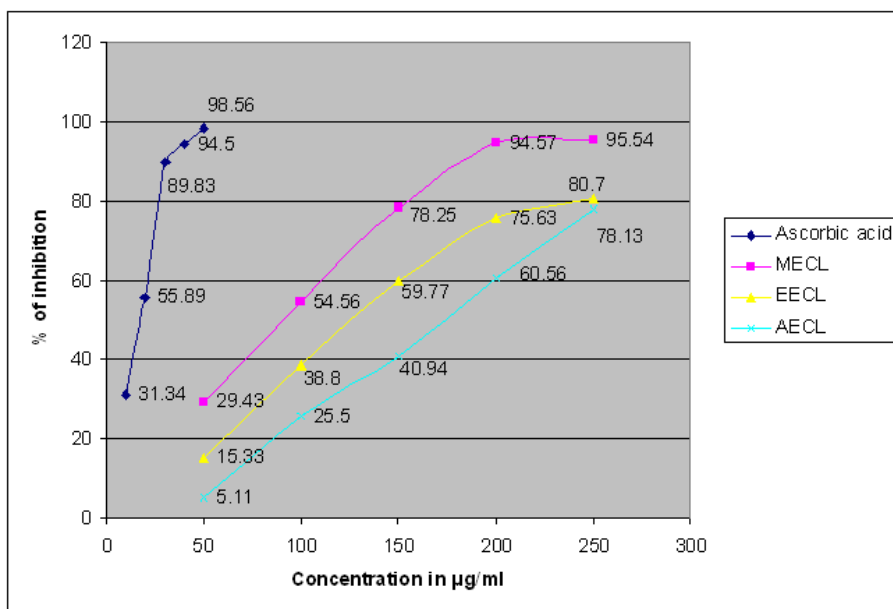


Fig-1. DPPH free radical scavenging activity of ascorbic acid, methanolic extract, ethanolic extract and aqueous extract

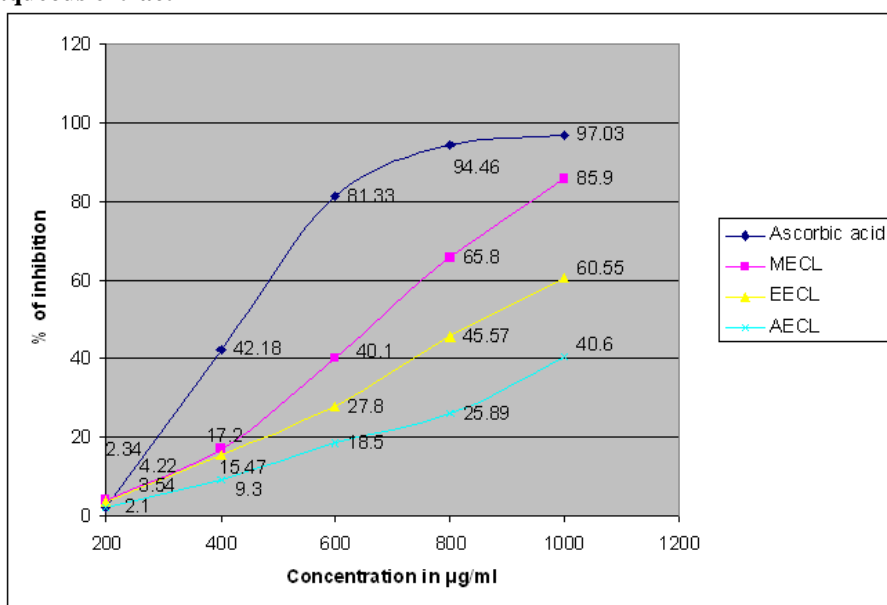


Fig-2. H₂O₂ radical scavenging activity by ascorbic acid, methanolic extract, ethanolic extract and aqueous extract

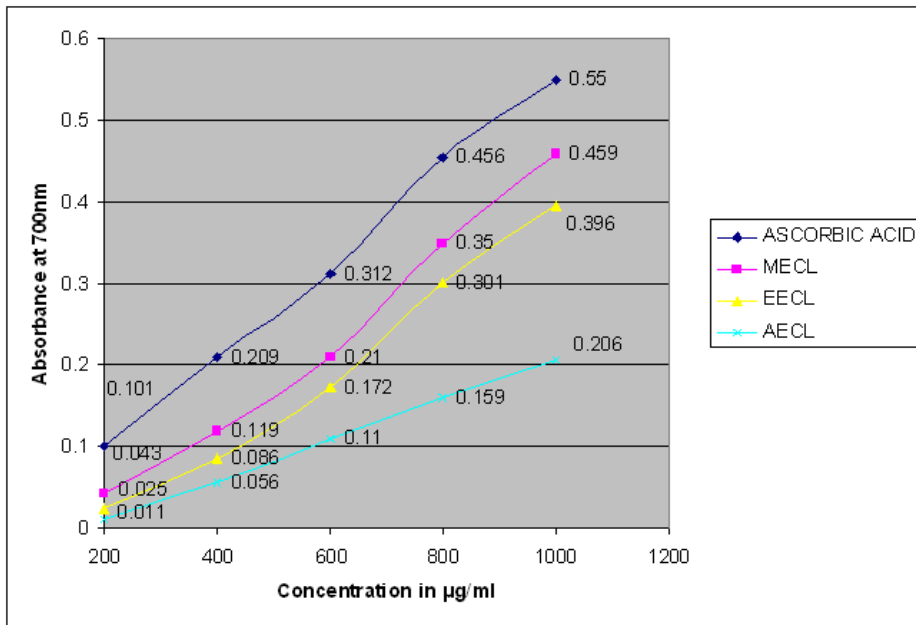


Fig-3. Comparison of reducing power activity of ascorbic acid, methanolic extract, ethanolic extract and aqueous extract

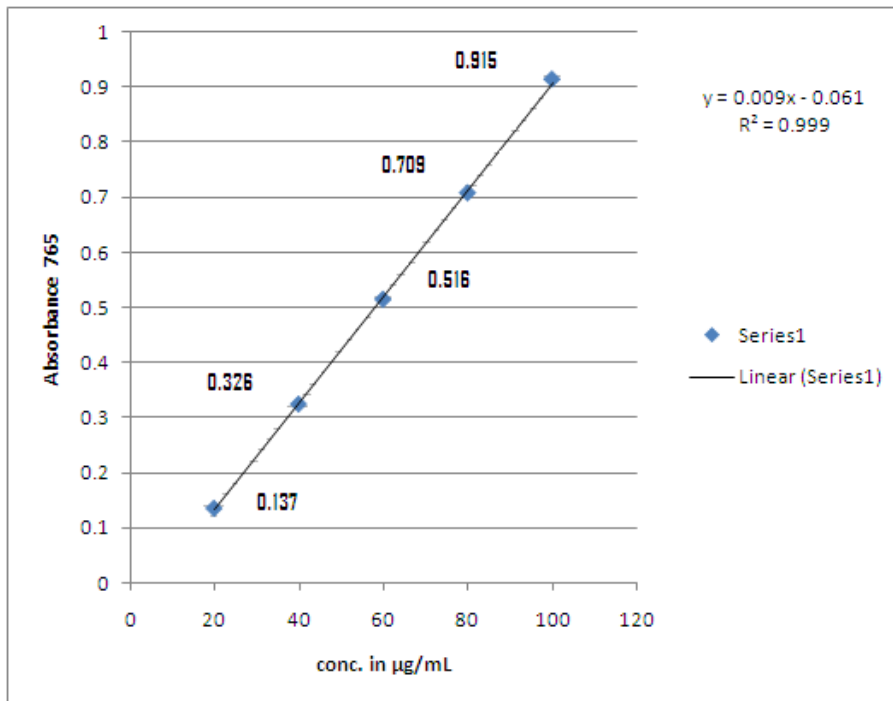


Fig-4. Calibration curve for determination of total phenolic contents.

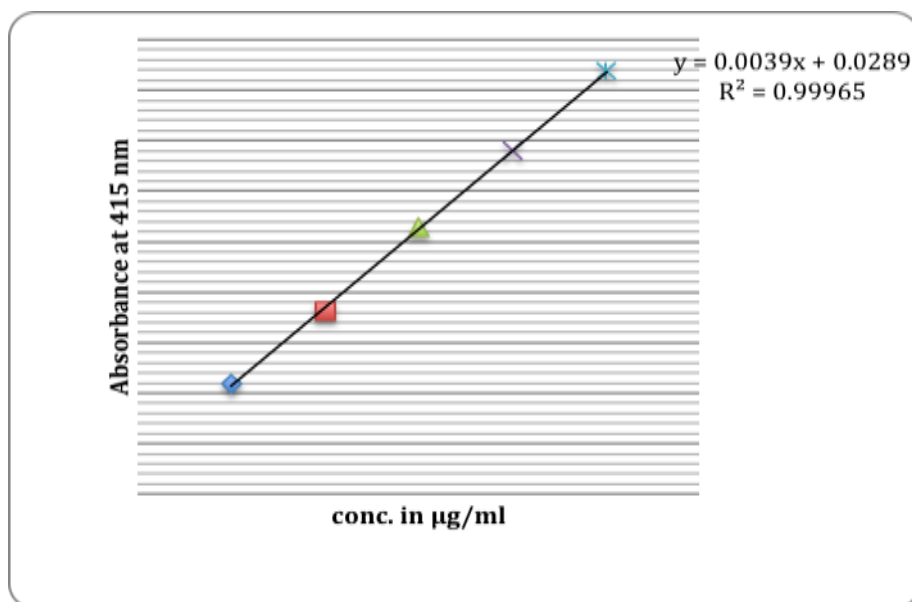


Fig-5. Calibration curve for determination of total flavonoids.

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