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Research Article

# ANTIOXIDANT ACTIVITY OF ILLICIUM GRIFFITHI HOOK. F. & THOMS SEEDS - IN VITRO

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**ABSTRACT**

The antioxidant activity of hexane, ethyl acetate and methanol extracts of *Illicium griffithii* (*I. graffithii*, Family: Schisandraceae) seeds were determined using 1,1-diphenyl-2- picrylhydrazyl (DPPH), phosphomolybdenum, cupric ions ($Cu^{2+}$) reducing antioxidant capacity (CUPRAC), ferric reducing antioxidant power (FRAP), reducing power, lipid peroxidation, hydroxyl and N,N-Dimethyl-p-phenylenediamine (DMPD) methods. Extracts were analyzed for total phenolic content (TPC) and total flavonoids content (TFC) using a spectrophotometric analysis. Total phenolic content 164.91 ± 12.67 GAE mg/g (as gallic acid equivalents) and total flavonoids content 63.94 ± 0.16 CE mg/g (as catechin equivalents) were estimated in the methanol extract of seeds. Among the extracts tested for antioxidant activity, methanol extract showed maximum activity on DPPH (70.96 ± 1.88), CUPRAC (0.988 ± 0.07), reducing power (0.236 ± 0.02), lipid peroxidation (36.95 ± 2.36), hydroxyl (47.52 ± 1.94) and DMPD (64.30 ± 0.31). It also exhibited high activity at 300 µg/ml on total antioxidant activity (0.159 ± 0.04 GAE mg/g) and FRAP (0.297 ± 0.03 mM $Fe^{2+}$/g). The results indicated that the methanol extract of *I. griffithii* seeds is having more of natural antioxidants and it can be considered for further clinical use.

**Keywords:** *Illicium griffithii*; antioxidant activity; DPPH; phosphomolybdenum; CUPRAC; FRAP; reducing power; lipid peroxidation; hydroxyl; DMPD.

## INTRODUCTION

Living cells may generate free radicals and other reactive oxygen species as a result of physiological and biochemical processes. Free radicals can cause oxidative damage to lipids, proteins and DNA, eventually leading to many chronic diseases, such as cancer, diabetes, aging, and other degenerative diseases in humans [1]. In many parts of the world, medicinal plants are used as a source of phytochemicals to cure various illnesses such as urinary infections, cervicitis vaginitis, skin infections, blood infections, and gastrointestinal disorders [2]. The phytochemicals have been found to act as antioxidants by scavenging free radicals, and many have therapeutic potential for free radical associated disorders [3]. Therefore, it is important to assess antioxidant activity of the plants used in the herbal medicine either to elucidate the mechanism of their pharmacological action or to provide information on antioxidant activity of these herbal plants.

*Illicium griffithii* Hook. f. & Thoms is an important medicinal tree species of the temperate broad-leaved forests of Northeast India [4]. Fruits of *I. griffithii* are used in the pharmaceutical and spice industries. In recent years, scientists also found cancer fighting properties especially against lung cancer cells [5]. These reports are sufficient to highlight the use of this species [6]. The main objective of this study was to estimate the total content of polyphenols, flavonoids, and the antioxidant activity of hexane, ethyl acetate and methanol extracts of seeds of *I. griffithii*.

## MATERIALS AND METHODS

### Chemicals

All the chemicals and reagents used in this study were obtained from Himedia, Qualigens and SRL and were of analytical grade. Folin-Ciocalteu reagent, gallic acid, catechin, thiobarbituric acid (TBA), 1,1-Diphenyl-2-picrylhydrazyl (DPPH), sodium hydroxide (NaOH), aluminums chloride ($AlCl_3$), TPTZ (2, 4, 6-tripyridyl-s-triazine), nitroblue tetrazolium (NBT), butylated hydroxytoluene (BHT), copper chloride ($CuCl_2$), neocuproine, deoxyribose, ammonium molybdate, trichloroacetic acid (TCA), deoxyribose, potassium dihydrogen phosphate, phenazine methosulphate (PMS), sodium nitroprusside (SNP), sodium acetate ($CH_3COONa$), acetic acid ($CH_3COOH$), sodium nitrate ($NaNO_2$), sulfanilamide, ammonium acetate, naphthylethylenediamine dihydrochloride (NED), sodium carbonate ($Na_2CO_3$), phosphoric acid ($H_3PO_4$) , mono and di basic sodium phosphate, Ferric chloride ($FeCl_3$), Nicotinamide adenine dinucleotide (NADH), thiobarbituric acid (TBA), dimethyl sulphoxide (DMSO), ethylene diamine tetra acetic acid (EDTA), hydrogen peroxide ($H_2O_2$), ascorbic acid (AA), nitroblue tetrazolium (NBT) and ferrous sulphate ($FeSO_4$), N,N-dimethyl-p-phenylenediamine (DMPD) were used.

### Plant materials

Healthy, disease free seeds of *I. griffithii* were collected from Arunachal Pradesh, India and were identified and authenticated by the taxonomist, Department of Plant Biology and Biotechnology, Loyola College, Chennai, India. All the seeds were separated from fruits and were shade dried at room temperature. The dried seeds were then powdered and stored in airtight containers.

### Preparation of crude extract

Seed Powder (1 kg) was soaked serially in hexane (4 L), ethyl acetate (4 L), and methanol (4 L) for 72 h respectively with intermittent shaking. The solutions were filtered and the filtrates were concentrated under reduced pressure using rotary vacuum evaporator (25 °C–hexane extract; 35 °C–Ethyl acetate extract; 40 °C–Methanol extract). The yield of seed extracts were: hexane extract (IS1, 8.1%, w/w), ethyl acetate extract (IS2, 9.8%, w/w) and methanol extract (IS3, 11.6% w/w). Finally, the crude extracts were obtained and stored at 4°C.

### Phytochemical Analysis

### Determination of Total Phenolic Contents (TPC).

The total phenolic content of the samples was determined using the Folin-Ciocalteu's reagent as described by the method of Slinkard and Singleton [7]. Briefly, a 100 µl aliquot of extract was assayed with 250 µl of Folin reagent and 500 µl of sodium carbonate (20%, w/v). The mixture was vortexed and diluted with water to a final volume of 5 ml. After incubation for 30 min at room temperature, the absorbance was read at 765 nm and total phenols in these extracts were expressed as gallic acid equivalents (GAE), using a calibration curve of a freshly prepared gallic acid solution.

### Estimation of Total Flavonoids Content (TFC).

The total flavonoids content was determined according to the method Kareti et al [8]. One hundred micro litter aliquot of extract was added to a 10 ml volumetric flask containing 4 ml of distilled

water. At zero time, 0.3 ml of 5% $NaNO_2$ was added to the flask. After 5 min, 0.3 ml of 10% $AlCl_3$ was added. At 6 min, 2 ml of 1 M NaOH was added to the mixture. Immediately, the reaction solution was adjusted to 10 ml by adding 2.4 ml of distilled water and thoroughly mixed. Absorbance of the mixture was determined at 510 nm versus blank. The total flavonoids content was expressed in mg catechin equivalents (CE)/g of extract.

**Antioxidant activity**

**DPPH Radical Scavenging Capacity**

DPPH quenching ability of *I. griffithii* hexane, ethyl acetate and methanol extracts were measured by method of Hanato et al [9]. The reaction mixture contained 50 µl of different concentrations (100-1000 µg/ml) of the sample and 2.95 ml of 0.1 mM DPPH in ethanol. After 30min incubation at room temperature, the absorbance was recorded at 517 nm using a spectrophotometer. The experiment was performed in triplicate. The percentage $DPPH^{\bullet}$ scavenging activity at different concentrations was calculated. Ascorbic acid was used as standard. The ability to scavenge the $DPPH^{\bullet}$ radical was calculated using the following equation:

$DPPH^{\bullet}$ scavenging effect (%) = $(A_0 - A_1) / A_0 \times 100$……(1)

Where $A_0$ is the absorbance of the control at 30 min, and $A_1$ is the absorbance of the sample at 30 min. All samples were analyzed in triplicate.

**Cupric-Ion-Reducing Antioxidant Capacity (CUPRAC)**

CUPRAC assay was performed according to the method of Apak et al [10] with some modifications. The test mixture contained 1ml of 10mM of $CuCl_2$, 7.5 mMneocuproine, and 1M ammonium acetate buffer (pH 7.0). Briefly, 1ml of sample in the concentration range of 100 –1000 µg/ml was added to the test mixture to achieve final volume of 4ml. The test mixtures were incubated for 30min at room temperature and then absorbance at 450 nm was recorded against a blank. BHT was used as standard.

**Ferric Reducing Antioxidant Power (FRAP)**

A slightly modified method of Benzie and Strain [11] was adopted for the FRAP assay. A standard or sample extract (300 µg/ml) was mixed with 300 µl of ferric-TPTZ reagent (prepared by mixing 300 mM acetate buffer, pH 3.6, 10 mM TPTZ in 40 mM HCl and 20 mM $FeCl_3.6H_2O$ at a ratio of 10:1:1 (v/v/v)). The mixture was incubated at 37°C and the absorbance readings were taken at 593 nm after 4 min. Results were expressed in mM Fe (II)/g dry mass.

**Determination of Total Antioxidant Capacity (TAC)**

Total antioxidant activity of *I. griffithii* was determined according to the method of Kareti et al [12]. Briefly, an aliquot 300 µg/ml of sample was combined with 3ml molybdenum reagent (50 ml of 0.6 M sulfuric acid, 50 ml of 28 mM sodium phosphate and 50 ml of 4 mM ammonium molybdate). The reaction mixture was incubated in a water bath at 95 ºC for 90 min. After cooling to room temperature, the absorbance was measured at 695 nm. The total antioxidant activity of the sample was expressed as mg gallic acid equivalents (GAE)/g of extracts.

**Reducing Power Ability**

The reducing power of hexane, ethyl acetate and methanol extracts of *I. griffithii* were evaluated according to the method of Oyaizu [13]. Different concentrations of the extracts (100–1000 µg/ ml) were suspended in distilled water and mixed with 2.5 ml of 0.2 M phosphate buffer (pH 6.6), and 2.5 ml of 1% $K_3Fe(CN)_6$. The mixture was incubated at 50 °C for 20 min; 2.5 ml of 10% TCA was added to the mixture and centrifuged at 3000 rpm for10 min. The upper layer of the solution (2.5 ml) was mixed with distilled water (2.5 ml) and $FeCl_3$ (0.5 ml, 0.1%), and the absorbance was measured at 700 nm. Increased in absorbance of the reaction mixture indicates the ability of reducing power. Vitamin C was used as standard.

**Anti-lipid Peroxidation Assay in rat liver homogenate**

The inhibition effect of hexane, ethyl acetate and methanol extracts of *I. griffithii* on lipid peroxidation was determined according to the thiobarbituric acid method [14]. $FeCl_2$–$H_2O_2$ was used to induce liver homogenate peroxidation. In this method, 0.2 ml of different concentrations of extracts (100–1000 µg/ml) were mixed with 1 ml of 1% liver homogenate (each 100 ml homogenate solution contains 1 g rat liver); then 50 µl of $FeCl_2$ (0.5 mM) and $H_2O_2$ (0.5 mM) was added. The mixture was incubated at 37 °C for 60 min; then 1 ml of trichloroacetic acid (15%) with thiobarbituric acid (0.67%) was added and the mixture was heated in boiling water for 15 min. The absorbance was recorded at 532 nm and the percentage of inhibition was calculated using the formula (1). Vitamin C was used as positive control.

**Hydroxyl radical scavenging assay**

The assay was performed as described by Elizabeth and Rao method [15] with minor changes. All solutions were prepared freshly. 1 ml of the reaction mixture contained 100 µl of 28 mM 2-deoxy-2-ribose (dissolved in phosphate buffer, pH 7.4), 500 µl solution of various concentrations of *I. griffithii* hexane, ethyl acetate and methanol extracts (100–1000 µg/ml), 200 µl of 200 µM $FeCl_3$ and 1.04 mM EDTA (1:1 v/v), 100 µl $H_2O_2$ (1 mM) and 100 µl ascorbic acid (1 mM). After an incubation period of 1 h at 37 °C, the extent of deoxyribose degradation was measured by the TBA reaction. The absorbance was read at 532 nm against the blank solution. Vitamin C was used as a positive control. The scavenging activity was calculated using formula (1).

**DMPD Radical scavenging assay**

DMPD radical ($DMPD^{\bullet}$) scavenging ability was performed by the method [16] with slight modification. The coloured radical was formed by adding ferric chloride to the DMPD solution ($Fe^{3+}$: DPMD ratio 1:10) and the absorbance of this solution was measured at 505 nm. 50 µl of the test solution (100-1000 µg/ml) was added to 2.95ml DMPD solution. The absorbance at 505 nm was measured after 10 min at 25°C under continuous stirring. The percentage scavenging activity was calculated.

**Statistical analysis**

The data were analyzed using Microsoft Excel (2007) and expressed as mean ± Standard Deviation.

**RESULTS AND DISCUSSION**

**Phytochemical Analysis**

**Total Phenolic and flavonoids Contents**

Considering the physiological importance of phenolic compounds and their contribution towards total antioxidant capacity, Folin-Ciocalteu method was used to estimate the total phenolic content of extracts. It must be noted that this reagent does not react exclusively with phenolics, but other reducing agents, for example, ascorbic acid as well [17, 18]. Hence, results of this test therefore reflect the total reducing capacity of the extracts and positive controls tested. Total phenolic compounds in extracts varied widely, ranging from 72.75±6.41 and 164.91±12.67 mg/g expressed as gallic acid equivalents (GAE) (Table 1). Methanol extract of seeds (IS3) exhibited the highest total phenolic content. The content of flavonoids expressed as catechin equivalents, varied from 58.83±0.32 to 63.94±0.16 mg catechin equivalent/g extract (Table 1). The IS3 showed the highest amount of flavonoids contents followed by IS2 and IS1.

**Table 1: Total Phenolic and flavonoids Contents of hexane (IS1), ethyl acetate (IS2) and methanol (IS3) extracts of *I. griffithii* seeds. GAE: gallic acid equivalents, CE: catechin equivalents. Data are presented as the mean ± Standard deviation of triplicate measurements.**

| Extracts | TPC mg GAE/g | TFC mg CE/g |
|---|---|---|
| IS1 | 72.75 ± 6.41 | 58.83 ± 0.32 |
| IS2 | 95.02 ± 5.44 | 61.65 ± 0.29 |
| IS3 | 164.91 ± 12.67 | 63.94 ± 0.16 |

**In vitro antioxidant activity**

Several techniques have been used to determine the antioxidant activity in vitro in order to allow rapid screening of substances since substances that have low antioxidant activity in vitro, will probably show little activity in vivo [19]. Free radicals are known to play a definite role in a wide variety of pathological manifestations. Antioxidants fight against free radicals and protect from various diseases. They exert their action either by scavenging the reactive oxygen species or protecting the antioxidant defense mechanisms [20]. Antioxidant capacity of seeds extracts of *I. griffithii* was examined using eight different assays.

**DPPH radical scavenging capacity.**

DPPH$^{\bullet}$ is a stable free radical, due to the delocalization of the spare electron on the whole molecule. Thus, DPPH$^{\bullet}$ does not dimerize, as happens with most free radicals. The delocalization on the DPPH molecule determines the occurrence of a purple colour, with an absorption band with a maximum around 517 nm. When DPPH$^{\bullet}$ reacts with a hydrogen donor, the reduced (molecular DPPH) form is generated, accompanied by the disappearance of the colour. Therefore, the absorbance diminution depends linearly on the antioxidant concentration [21, 22]. A large decrease in the absorbance of the reaction mixture indicates significant free radical scavenging activity of the compound under test [23]. Figure 1 shows the scavenging effects of various extracts of *I. griffithii* seeds on DPPH$^{\bullet}$ in the following order: IS3> IS2>IS1. Among all the extracts tested methanol extract of seeds (IS3), showed highest inhibition percentage and directly correlated with total phenolic content. Results of this study suggest that the extracts contain phytochemical constituents that are capable of donating hydrogen to a free radical to scavenge the potential damage.

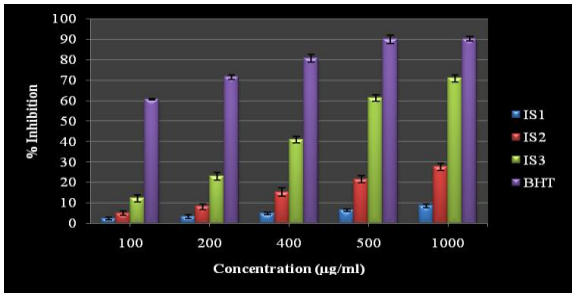


**Figure 1:Antioxidant capacity of hexane (IS1), ethyl acetate (IS2) and methanol (IS3) extracts of *I. griffithii* seeds by DPPH method. BHT-butylated hydroxytoluene. Each value represents the mean ± standard deviation of triplicate experiments.**

**Cupric-ion-reducing antioxidant capacity.**

The CUPRAC assay utilizes copper(II)-neocuproine (Cu(II)-Nc) reagent as the chromogenic oxidizing agent. It is based on the measurement of absorbance at 450 nm by the formation of stable complex between neocuproine and copper (I) [24]. The cupric ion ($Cu^{2+}$) reducing ability of various extracts of *I. griffithii* seeds is shown in Figure 2. $Cu^{2+}$ reducing capability measured by this method was found to be concentration-dependent. $Cu^{2+}$ ions reducing power of extracts and standard compounds were in the following order: BHT> IS3>IS2>IS1.

**Ferric reducing antioxidant power.**

When a $Fe^{3+}$-TPTZ complex is reduced to the $Fe^{2+}$ form by an antioxidant under acidic conditions, an intense blue color develops with an absorption maximum at 593 nm. The antioxidant effect (reducing ability) can be evaluated by monitoring the formation of a $Fe^{2+}$-TPTZ complex with a spectrophotometer [25]. The antioxidant potential of *I. griffithii* extracts was estimated from their ability to reduce TPRZ-Fe (III) complex to TPTZ-Fe (II). Increasing absorbance indicates an increase in reductive ability. The FRAP values of the studied extracts were calculated and the results are presented in Table 2. Among all the extracts, the methanol extract of seeds (IS3) showed the highest FRAP value (2.146 ± 0.23) at 300 µg/ml.

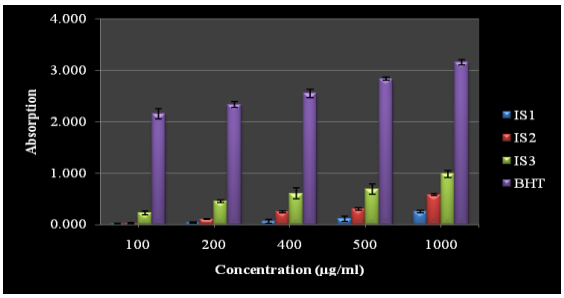


**Figure 2: Antioxidant capacity of hexane (IS1), ethyl acetate (IS2) and methanol (IS3) extracts of *I. griffithii* seeds by CUPRAC method. Each value represents the mean ± standard deviation of triplicate experiments.**

**Table 2: Antioxidant capacity of hexane (IS1), ethyl acetate (IS2) and methanol (IS3) extracts of *I. griffithii* seeds by FRAP and TAC method. GAE: gallic acid equivalents. BHT-butylated hydroxytoluene. AA-ascorbic acid. Data are presented as the mean ± Standard deviation of triplicate measurements. --- : not done.**

| Sample 300 µg/ml | FRAP mM Fe(II) / g | TAC mg GAE/g |
|---|---|---|
| IS1 | 0.108 ± 0.03 | 0.009 ± 0.01 |
| IS2 | 0.142 ± 0.02 | 0.028 ± 0.02 |
| IS3 | 0.297 ± 0.03 | 0.159 ± 0.04 |
| BHT | 2.312 ± 0.12 | --- |
| AA | 2.270 ± 0.05 | 0.043 ± 0.01 |

**Determination of total antioxidant capacity.**

The total antioxidant capacity of the extracts was measured spectrophotometrically through phosphomolybdenum method, based on the reduction of Mo (VI) to Mo (V) by the test sample and the subsequent formation of green phosphate-Mo(V) compounds. The antioxidant capacity of various solvent extracts of seeds of *I. griffithii* was found to decrease in this order: IS3 > IS2 > IS1 (Table 2). Recent studies have shown that many flavonoids and related polyphenols contribute significantly to the phosphomolybdate scavenging activity of medicinal plants [26].

**Reducing Power Ability**

In reducing power assay, the yellow colour of the test solution changes to various shades of green and blue, depending on the reducing power of the sample. The presence of reducing agents causes the conversion of $Fe^{3+}$-ferricyanide complex to the ferrous form that may be followed at 700 nm due to the formation of Perl's Prussian blue $Fe_4[Fe(CN)_6]_3$. Increasing absorbance at 700 nm indicates an increase in reducing ability [27]. The antioxidants present in the extracts of seeds of *I. griffithii* caused their reduction of $Fe^{3+}$-ferricyanide complex to the ferrous form, and thus proved the reducing power. Figure 3 shows the reducing powers of various extracts of *I. griffithii*. It was found that the reducing power increased with concentration of the sample. The ranking order for reducing power was IS3 > IS2 > IS1. Significantly higher reducing power (0.236 ±0.02 at 1000 µg/ml) was evident in IS3.

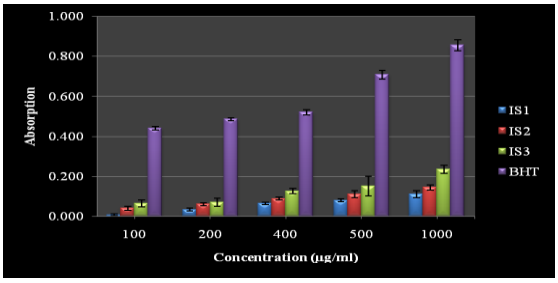


**Figure 3: Antioxidant capacity of hexane (IS1), ethyl acetate (IS2) and methanol (IS3) extracts of *I. griffithii* seeds by reducing power method. BHT - butylated hydroxytoluene. Each**

**value represents the mean ± standard deviation of triplicate experiments.**

**Anti-lipid peroxidation assay in rat liver homogenate.**

Lipid peroxidation induced by free radical species results in breakdown of membrane integrity, affecting its fluidity and permeability [28]. The initial step, that is, peroxidation of polyunsaturated fatty acid components in low-density lipoproteins of membrane produces several byproducts which can damage biomolecules. Transition ions may either generate hydroxyl radicals to initiate the lipid peroxidation process or propagate the chain process via decomposition of lipid hyperoxides [29].The lipid peroxidation induced by $Fe^{2+}$ was estimated by the presence of thiobarbituric acid reactive substances (TBARS). The ability of the extracts to inhibit peroxidation of phospholipids in rat liver is shown in Figure 4. The percentage inhibition of lipid peroxidation by various solvent extracts of seeds of *I. griffithii* was found to decrease in the following order: IS2>IS3>IS1.

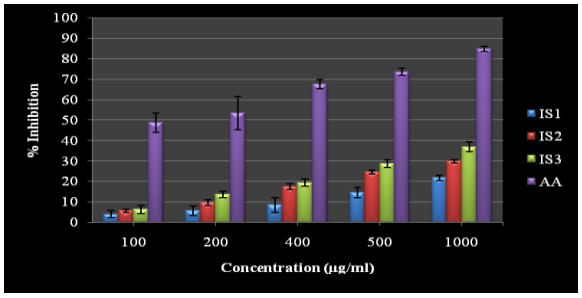


**Figure 4: Antioxidant capacity of hexane (IS1), ethyl acetate (IS2) and methanol (IS3) extracts of *I.griffithii* seeds by Lipid peroxidation method. AA - Ascorbic acid. Each value represents the mean ± standard deviation of triplicate experiments.**

**Hydroxyl radical scavenging assay.**

Hydroxyl radicals ($OH^{\bullet}$**)** are short-lived species possessing high affinity toward other molecules. $OH^{\bullet}$ is a powerful oxidizing agent that can react at a high rate with most organic and inorganic molecules in the cell, including DNA, proteins, lipids, amino acids, sugars, and metals. $OH^{\bullet}$ is considered the most reactive radical in biological systems; due to its high reactivity, it interacts at the site of its production with the molecules closely surrounding it. It reacts with polyunsaturated fatty acid moieties of cell membrane phospholipids and causes damage to cell [30]. There are several ways to ascertain the ability to form hydroxyl radicals. One of them is the deoxyribose method. In this method the $OH^{\bullet}$ generated

through Fenton reaction degrades deoxyribose into several fragments using $Fe^{2+}$ as an important catalytic component. Some of these fragments are capable of reacting with TBA after heating and in an acidic pH, originating a pink pigment that can be quantified by spectrophotometry [31]. The $OH^{\bullet}$ scavenging activity of various solvent extracts of seeds of *I. griffithii* can be ranked as IS3 > IS2 > IS1 (Figure 5). The markedly strong antioxidant activity of IS3 and IS2 in comparison with ascorbic acid might be helpful in characterizing the significant sources of natural antioxidants.

**DMPD Radical scavenging assay.**

In the presence of an oxidant solution ($FeCl_3$) at acidic pH, DMPD is converted to stable and colored DMPD radical cation ($DMPD^{\bullet+}$, absorption maxima 505 nm). The antioxidant compounds, which were present in the sample were able to transfer a hydrogen atom to $DMPD^{\bullet+}$ and caused discoloration, which was proportional to their concentration [32]. The $DMPD^{\bullet}$ scavenging activity of various solvent extracts of *I. griffithii* seeds, were found to in the order IS3>IS2 > IS1 (Figure 6).

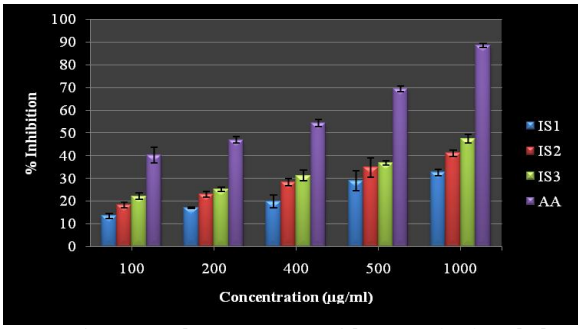


**Figure 5: Antioxidant capacity of hexane (IS1), ethyl acetate (IS2) and methanol (IS3) extracts of *I. griffithii* seeds by Hydroxyl radical method. AA - Ascorbic acid. Each value represents the mean ± standard deviation of triplicate experiments.**

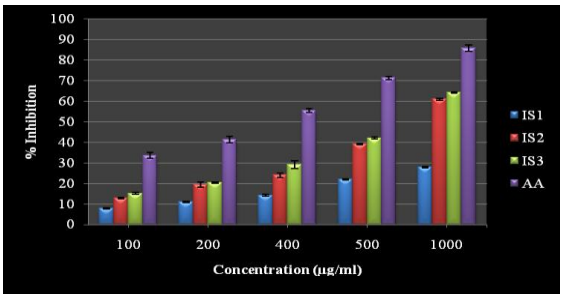


**Figure 6: Antioxidant capacity of hexane (IS1), ethyl acetate (IS2) and methanol (IS3) extracts of *I. griffithii* seeds by DMPD method. AA - Ascorbic acid. Each value represents the mean ± standard deviation of triplicate experiments.**

**Conclusions**

The results of the study have shown that the methanol extract of seeds of *I. griffithii* is potentially a good source of free radical scavenging compounds. It contains high phenolic and flavonoids and hence exhibits high antioxidant activities. However, the components responsible for the antioxidant activity are currently unclear. Therefore, further investigation is needed to isolate and identify the antioxidant compounds present in the methanol extract. Furthermore, the *in vivo* antioxidant activity of this extract needs to be assessed prior to clinical use.

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