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PHYTOCHEMICAL SCREENING, HPTLC ANALYSIS AND *IN VITRO* ANTIOXIDANT POTENTIAL OF ETHANOLIC EXTRACT OF *NELUMBO NUCIFERA* SEEDS.

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ABSTRACT

The main objective of the present work is to investigate the phytochemical screening, HPTLC fingerprint and in vitro antioxidant of ethanolic extract of seeds of Nelumbo nucifera. Various phytochemical constituents are investigated in N.nucifera seeds. HPTLC analysis showed the presence of flavonoids. The experimental conditions as well as general comments on the application of chromatographic fingerprint analysis are discussed. 1-1 Diphenyl-2-picryl-hydroxyl radical scavenging was carried out to evaluate the antioxidant potential of the extract. Free radical scavenging activity of ethanolic extract of seeds of N.nucifera increased in a concentration dependent manner. The ethanolic extracts possess free radical scavenging activity at five different concentrations (50-250µg/ml). These findings suggested that the ethanolic extract of N.nucifera possess in vitro antioxidant activity and acts as an effective free radical scavenger.

KEYWORDS: *Nelumbo nucifera, In vitro* antioxidant, DPPH and HPTLC fingerprint profile.

1. INTRODUCTION

A free radical may defined as a molecule or molecular fragments containing one or more unpaired electrons in its outermost atomic or molecular orbital and are capable of independent existence.[1] Reactive oxygen species (ROS) and reactive nitrogen species (RNS) are describes free radicals and other non-radical reactive derivatives. The reactivity of

radicals is generally stronger than non-radical species though radicals are less stable.[2] Antioxidants are any substance that delay or inhibits oxidative damage to a target molecule. At a time one antioxidant molecule can react with single free radicals and are capable to neutralize free radicals by donating one of their own electrons, ending the carbon-stealing reaction. Antioxidants prevent cell and tissue damage as they act as scavenger. Cell produce defence against excessive free radicals by their preventative mechanisms, repair mechanisms, physical defence and antioxidant defences.[3]

Synthetic antioxidants have serious risks associated with them as these can generate chain reactions leading to synthesis of more free radicals, leading to carcinogenesis. This situation calls for search of alternate sources of antioxidants. Nowadays, researchers have started focusing on search of 'complementary and alternative medicine.[4]

Herbal medicine is the use of plants and plant sources to treat disease, something mankind has always done. Herbal medicine exists in many local varieties depending on the regional flora.[5] Many modern drugs were originally extracted from plant sources, even if they are now made synthetically and many other drugs are descended from plant substances.

N.nucifera comes under the family Nelumbonaceae, which has various local tribal names (Indian lotus, bean of India, Chinese water lily and sacred lotus) and several botanical names (*Nelumbium nelumbo*, *N. speciosa*, *N. speciosum* and *Nymphaea nelumbo*).[6]

The fruit of this plant is an aggregate of indehiscent nutlets. Ripe nutlets are ovoid, roundish or oblongish, up to 1.0 m long and 1.5 cm broad, with a hard, smooth, brownish or greyish black pericarp which is faintly longitudinally striated, pedunculated and single seeded. Seeds fill in the ripe carpel. The seeds are sold as a vegetable in Indian markets, under the name of 'kamal gatta'.[7] Seeds contain β-sitosterol, palmitic acid, glucose, Oxoushine and N-norarmepavine isolated from seeds.[8]

The seeds are used indigenously in Ayurveda, Chinese and folk medicines to treat tissue inflammation, cancer, diuretics, include improving learning and memory, hepatoprotective, anti-obesity, anti-HIV activity, anti-tumour effect, antipyretic activity and antidepressant.

2. MATERIALS AND METHODS

2.1 Collection of Plant materials

Fresh, seeds of *Nelumbo nucifera* were collected from different area of Mannargudi, Thiruvarur District, Tamil Nadu, India.

2.2 Preparation of plant extract

The seeds were first washed well with distilled water and dried at room temperature. The dried seeds were powdered in an electrical grinder and stored at 5°C until further use. One hundred grams of powder were soaked in ethanol for 72hrs. The powder was filtered by means of whatmann filter paper. The extract was kept in a boiling water bath to evaporate ethanol. The ethanol free extract was used for Preliminary Phytochemical Screening, HPTLC analysis, *in vitro* antioxidant activity.

2.3 Preliminary Phytochemical Screening

The ethanolic extract of *Nelumbo nucifera* seeds was subjected to preliminary phytochemical screening of various constituents.[9]

2.4 HIGH PERFORMANCE THIN LAYER CHROMATOGRAPHY

HPTLC studies were carried out following the method of Wagner and Bladt, 1996.[10]

2.4.1 Sample preparation

70% ethanolic extracts obtained were evaporated under reduced pressure using rotovac evaporator. Ethanolic extract residue was dissolved in 1ml of chromatographic grade 70% ethanol, which was used for sample application on pre-coated silica gel 60F254 aluminium sheets**.**

2.4.2 Developing solvent system

Developed the plate in the solvent system to a distance of 8cm. HPTLC of the extracts was performed by using the Toluene: Ethyl acetate: Formic acid (5:4:1).

2.4.3 Sample application

Applied 20µl and 30µl of test solution on a precoated silica gel 60F254 TLC Plate (E.Merck) of uniform thickness 0.2mm using Linomat5 sample applicator. Developed the plate in the solvent system to a distance of 8cm. Scanned the plate densitometrically at 254nm using TLC Scanner3. Observed the plate under UV light at 254nm & 366nm using CAMAG Reprostar3.

2.4.4 Development of chromatogram

The extracts were applied point-wise from 1000 µg/ml sample solution; 10µl of the sample was applied on HPTLC aluminium sheets as different tracks in the form of 6 mm wide bands by using a CAMAG semi-automatic Linom at 5 spotters at a distance of 12mm.

2.4.5 Detection of spots

All plates were visualized directly after drying and a fingerprint profile was photo documented using a CAMAG Reproster- 3 under 254 nm and 366 nm in UV and visible light. The digital images of the chromatograms were evaluated with the programme CAMAG Video Scan. The captured images were subjected to a visible on the computer screen. The differences found, are specified by the HPTLC system in which the difference is detected and by the Rf value (and colour) of a compound in the system.

2.5 In vitro Antioxidant activity

2.5.1. DPPH Scavenging Activity

DPPH Scavenging Activity determined by (Blois, 1958).[11] The DPPH is reacted with Methanol or Absolute Ethanol to yield purple colour DPPH radical. The presence of antioxidants which include polyphenolics and flavonoids in the sample will scavenge the formed DPPH radical and thereby a decreased color will be observed which is spectrophotometrically measured at 517 nm. To 0.5 ml of DPPH radical solution, add 2 ml of the extract and the reaction mixture is vortexed for 10 s and allowed to stand at room temperature for 30 minutes. The absorbance was recorded at 517 nm the % of inhibition was calculated. Ascorbic acid was used as standard.

2.5.2. *Calculation*

$$\%\text{ of DPPH scavenging activity} = 1- \frac{\text{Sample Absorbance}}{\text{Blank Sample Absorbance}} \times 100$$

3. RESULTS AND DISCUSSION

In the present study was carried out on the ethanolic seed extract of *Nelumbo nucifera* revealed the presence of medicinally active constituents. The phytochemical constituents of the *Nelumbo nucifera* seeds are investigated and the results are summarized in Table 1.

Phytochemical constituents revealed the presence of Alkaloids, flavonoids, Carbohydrates, steroids, Phenols, protein, Terpinoids and triterpinoids while Tannin, saponin, Glycosides and phytosterol were absent in ethanolic seed extract of *Nelumbo nucifera*.

The preliminary phytochemical screening has revealed the presence of alkaloids and flavonoids in the ethanolic extract. The presence of flavonoids might be responsible for many diseases. Results support the traditional use of the plant in the treatment of various diseases conditions.[12]

Table 1: Phytochemical screening of ethanolic extract of *Nelumbo nucifera* seed.

S.No.	Phytochemicals Constituents	Ethanolic extracxt
1.	Alkaloids	+
2.	Carbohydrates	+
3.	Flavonoids	+
4.	Glycosides	-
5.	Phenol	+
6.	Phytosterol	-
7.	Protein	+
8.	Saponin	-
9.	Steroid	+
10.	Tannin	-
11.	Terpenoids	+
12.	Triterpenoids	+

(+) **indicates present**

(-) **indicates absent**

Phytochemicals are biologically active compounds found in small amounts which are not established nutrients but on the other hand contribute significantly to protection against degenerative diseases.[13] These obtained results were corroborative with the reports of that its seeds contain the active principles such as terpenoids, steroids, carbohydrates, flavonoids and phenolic compounds which shows an antioxidant activity.[14]

3.1 HPTLC ANALYSIS

HPTLC analysis of *N. nucifera* seed was conducted in order to determine phytochemical compounds. The plates developed in Mobile phase (Toluene: Ethyl acetate: Formic acid) and visualized under UV 254nm and 366 nm.

The results from HPTLC finger print scanned at wavelength 254 nm for ethanolic extract of *nelumbo nucifera* seeds. There are four polyvalent phytoconstituents and corresponding ascending order of Rf values start from 0.25 to 0.99 in which highest concentration of the phytoconstituents was found to be 30.98% and its corresponding Rf value was found to be 0.91 are shown in (Figure: 3b).

The results from HPTLC finger print scanned at wavelength 366 nm for ethanolic extract of *Nelumbo nucifera* seeds. There are three polyvalent phytoconstituents and corresponding ascending order of Rf values start from 0.22 to 0.89 in which highest concentration of the phytoconstituents was found to be 46.49% and its corresponding Rf value was found to be 0.89 are shown in (Figure: 3c).

The major band was scrubbed from the plate and subjected for phytochemical qualitative analysis. The fraction detected consists of flavonoids (Figure: 2) suggested that flavonoids might have contributed in antioxidant activity.

Analyzing the solvent extract of plant samples by HPLC will help to know its composition and promote biological properties.[15] Various phytoconstituents from plants were reported to be responsible for cardioprotective activity including carotenoids, triterpenes, flavonoids, cardiac glycosides, alkaloids, saponins and terpenoids.[16] In this study, flavonoids compounds were observed in *Nelumbo nucifera* seed extract qualitatively by HPTLC method might have contributed in antioxidant activity, anti-inflammatory activity and also cardioprotective activity. Similarly the identification of natural product, including flavonoids and phenolic compounds which are attributed to antioxidant activity of the soybean by HPTLC method was reported by.[17,18]

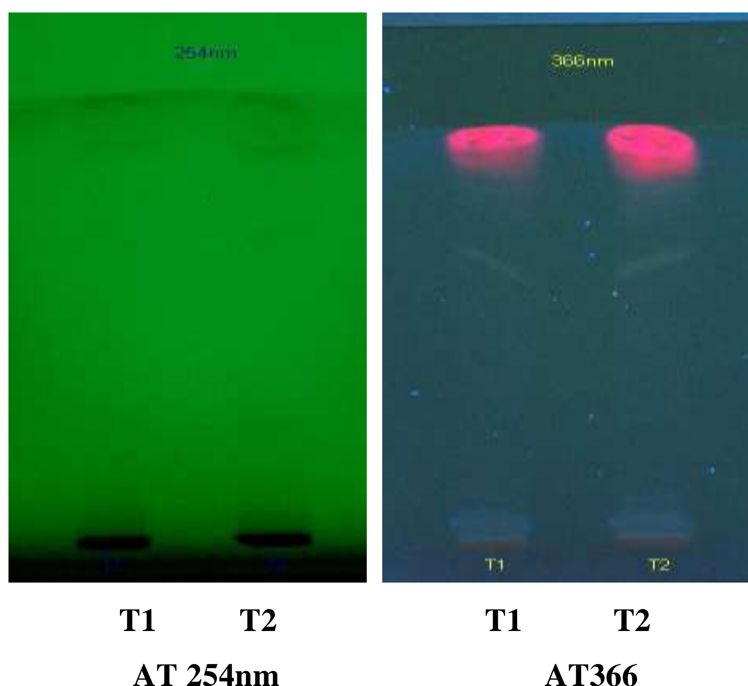

Figure 1: HPTLC Fingerprinting Profile for Hydro-alcoholic extract of *Nelumbo nucifera seed*.

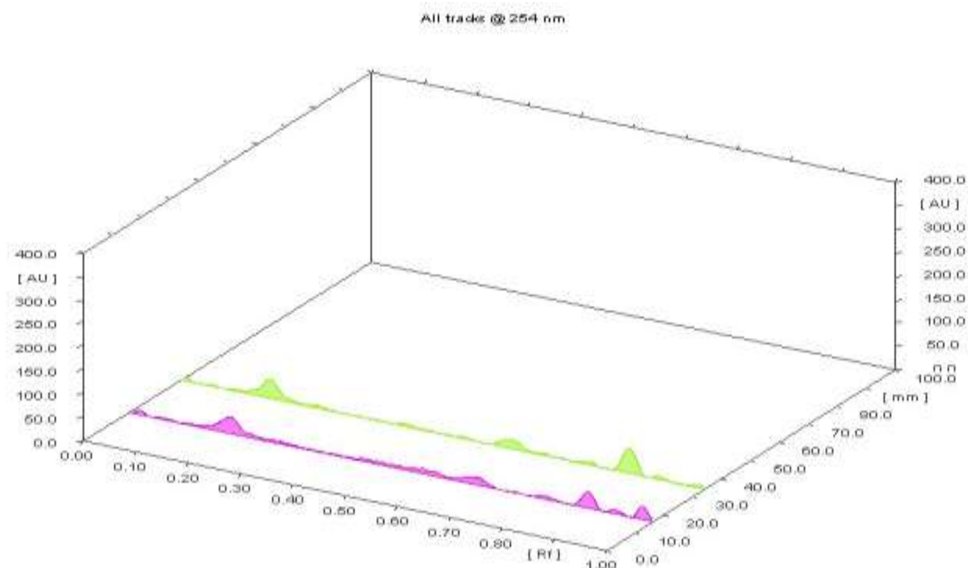

Figure 2(a): Three dimensional representation of HPTLC Chromatogram of *Nelumbo nucifera* ethanolic seed extract measured at 254 and 366nm.

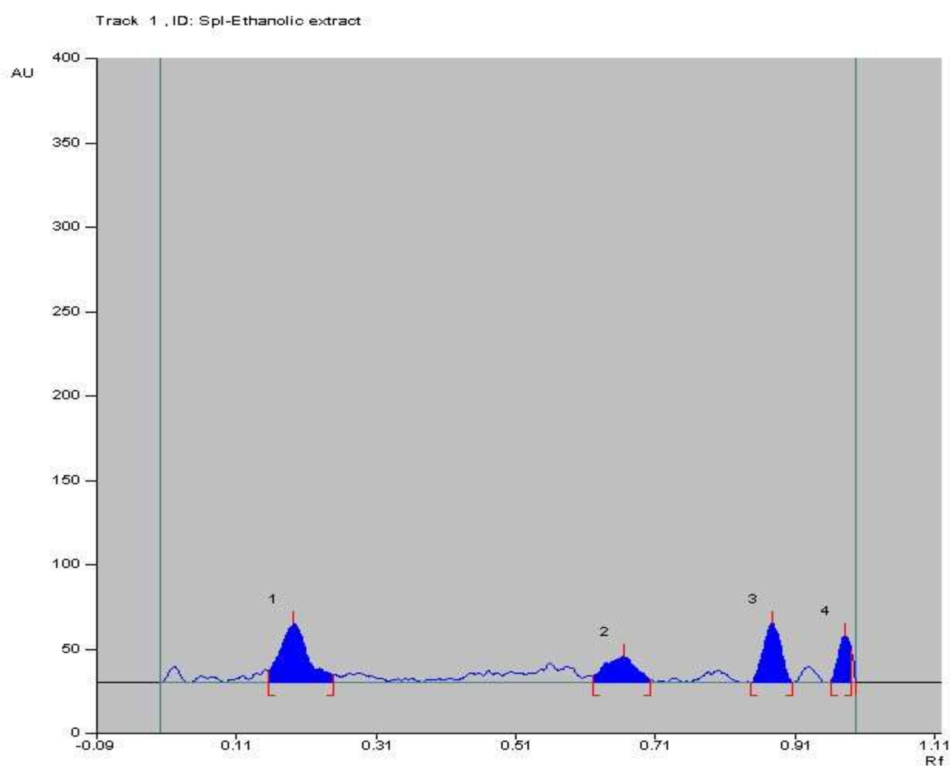

Figure 2(b): HPTLC Chromatogram of *Nelumbo nucifera* seeds (20µl of Sample).

Table 2: HPTLC Fingerprint profile Peak list and Rf value of the chromatogram of ethanolic extract of *Nelumbo nucifera* seeds.

Peak	Start Rf	Start Height	Max Rf	Max Height	Height %	End Rf	End Height	Area	Area %
1	0.15	6.5	0.19	34.6	30.63	0.25	4.5	1052.4	40.37
2	0.62	4.1	0.66	15.6	13.81	0.70	1.9	524.0	20.10
3	0.85	0.0	0.88	35.0	30.98	0.91	0.2	664.2	25.48
4	0.96	0.1	0.98	27.8	24.59	0.99	18.2	366.3	14.05

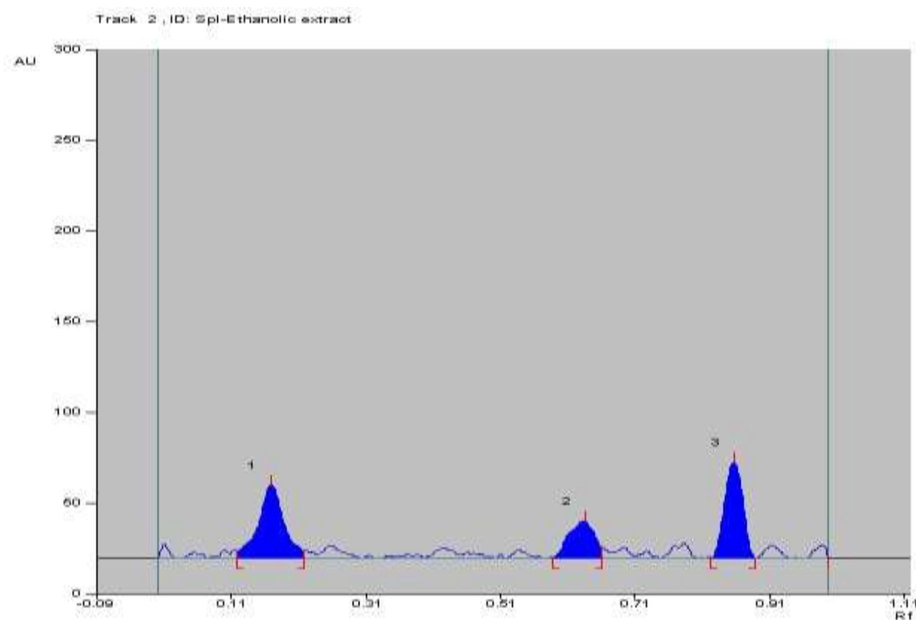

Figure 3: HPTLC Chromatogram of *Nelumbo nucifera* seeds (30µl of Sample).

Table 3: HPTLC FINGERPRINT profile Peak list and Rf value of the chromatogram of ethanolic extract of *Nelumbo nucifera* seeds.

Peak	Start Rf	Start Height	Max Rf	Max Height	Height %	End Rf	End Height	Area	Area %
1	0.12	4.0	0.17	40.3	35.46	0.22	4.0	1164.3	41.08
2	0.59	0.5	0.63	20.5	18.05	0.66	6.2	589.8	20.81
3	0.82	0.1	0.86	52.9	46.49	0.89	0.3	1080.0	38.11

3.2 IN VITRO ANTIOXIDANT ACTIVITY

3.2.1 DPPH radical scavenging activity

Excessive generation of ROS, induced by various stimuli and which exceed the antioxidant capacity of the organism, leads to a variety of patho-physiological processes such as inflammation, diabetes, genotoxicity and cancer.[19] The relatively stable organic radical DPPH is widely used in modeling systems to investigate the scavenging activities of ethnolic extract of *Nelumbo nucifera* seeds. The DPPH radical is scavenged by antioxidants through

the donation of electrons forming the reduced DPPH. The colour changes from purple to yellow after reduction and the accompanying decrease in absorbance can be quantified at wavelength 517 nm. The free radical scavenging activity of ethnolic extract of *Nelumbo nucifera* seeds are shown in Table 4.

Different concentrations namely 50, 100, 150, 200, 250µg/mL of ethanolic extracts were used with ascorbic acid as standard. Which is based on the ability of DPPH, a stable free radical, to decolorize in the presence of antioxidants is a direct and reliable method for determining radical scavenging action. The ethanolic extract showed strong antioxidant (72.8%) activity. The maximum scavenging activity on hydroxyl radicals (72.8%) could be achieved when seed extract was more than 250µg/ml. It was noticed that ethanolic extract of *N. nucifera* showed strong hydrogen donating abilities to act as an effective antioxidant. The scavenging effect increased with increasing concentration of the extract.[20]

Table 4: DPPH scavenging activity ethanolic extract of *Nelumbo nucifera* seed at different concentration.

S.No	Concentration (µg/ml)	DPPH radical scavenging activity (%)	
		Standard (µg/ml)	Ethanolic extract
1.	50	20.6±0.8	45.7±1.6
2.	100	28.9±o.3	52.9±1.8
3.	150	32.7±1.2	62.8±1.03
4.	200	40±2.3	67.8±0.6
5.	250	46±0.1	72.8±0.12

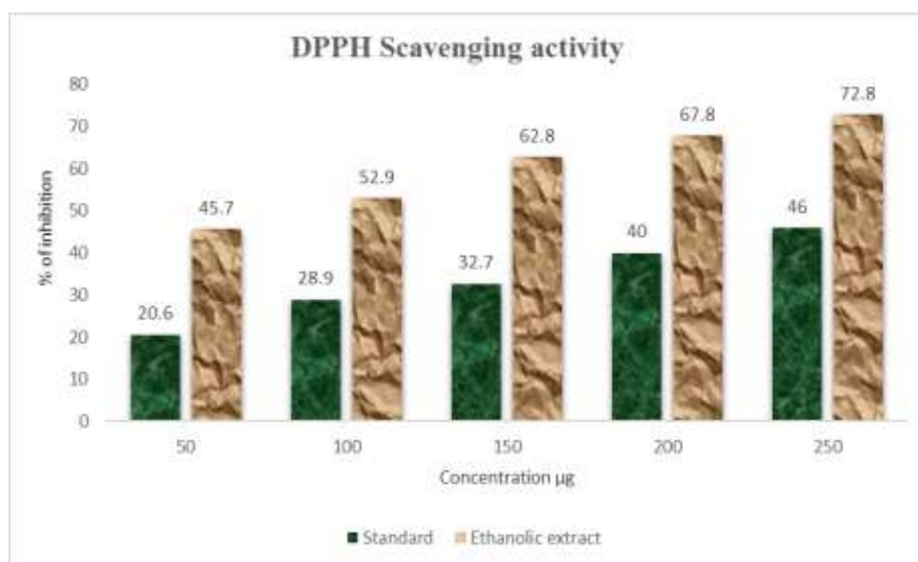

Figure 4: DPPH Scavenging activity recorded for the ethanolic extract of *Nelumbo nucifera* seeds.

CONCLUSION

Based on above results it is concluded that ethanolic extract of *Nelumbo nucifera* seeds showed strong *in vitro* free radical scavenging effect in free system. Phytochemical studies showed that the seed extract contain free radical scavenging molecules, such as proteins, terpenoids, flavonoids, alkaloids, carbohydrates and other metabolites, which are rich in antioxidant activity.

HPTLC study as recommended in this study provides a chromatographic fingerprint of phytochemicals and is suitable for confirming the identity and purity of medicinal plant raw material. HPTLC pre-coated plates with the mobile phase Toluene: Ethyl acetate: Formic acid developed chromatograms which showed distinct phytochemical variations in hydro alcoholic extracts. It can be conclude that HPTLC finger print analysis of *Nelumbo nucifera* seeds extract can be used as a diagnostic tool for the correct identification of the plant and it is useful as a phytochemical marker and also good estimator of genetic variability in plant population.

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