

**HPTLC STUDY AND *In-Vitro* ANTIOXIDANT ASSAY OF 'NELLIKKAY THENURAL' A SIDDHA DRUG**R. Ganesan<sup>\*1</sup>, Meenakshi<sup>2</sup>, Reena V. L.<sup>1</sup>, R. Shakila<sup>1</sup>, P. Sathyarajeswaran<sup>1</sup> and S. Chitra<sup>3</sup><sup>1</sup>Siddha Central Research Institute, Arumbakkam, Chennai-106.<sup>2</sup>Valliyammal College for women, Annanagar, Chennai-40.<sup>3</sup>Captain Srinivasa Murthy Regional Ayurveda Drug Development Institute, Chennai-106.**\*Corresponding Author: R. Ganesan**

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**ABSTRACT**

To determine the antioxidant activity of the Siddha herbal drug- Nellikay Thenural using different pre-screening method (Poly phenol and Flavonoid), antioxidant assays namely DPPH, Pro-oxidant, Total Antioxidant, Scavenging of Superoxide radical - DMSO method and HPTLC technique. Various biological studies have been done separately in *Phyllanthus emblica* and Honey. The present study (**HPTLC, pre - screening and antioxidant analysis**) is carried out to determine the antioxidant activity of the siddha drug **Nellikay Thenural** in *invitro* condition.

**KEYWORDS:** Antioxidant, DPPH (1,1-diphenyl-2-picryl-hydrazyl), HPTLC (High Performance Thin Layer Chromatography), Superoxide radical scavenging activity.

**INTRODUCTION**

Siddha system of medicine was gifted by 18 siddhars. It said the longevity of the human was up to 100 years,<sup>[1]</sup> but nowadays it was reduced due to lifestyle modification to avoid it, we are in demand to stop oxidation stress. By identifying and using those kayakalpa herbs increase the longevity of the human. One of 18 siddhars called Thirumoolar, says the concept of kayakalpa in his thirumandiram briefly. In siddha, the concept of kayakalpa, antioxidant deals with the prevention of NARAI (Grey hair), THIRAI (Shrinking of Skin), MOOPU (Aging), SAAKADU (Death), kayakalpa herbs were described in Karuvurar vadhakaviyam and Theriary emagavenba.<sup>[2]</sup>

The human system creates reactive oxygen species (ROS), such as superoxide anion radical, hydroxyl radical and hydrogen peroxide by many enzymatic systems through oxygen consumption.<sup>[3]</sup> In small amounts, these ROS can be beneficial as signal transducers and growth regulators.<sup>[4]</sup> However, during oxidative stress, large amounts of these ROS may favor some human disease conditions such as cancer, hepatic diseases, cardiovascular diseases, ageing and neurodegenerative diseases.<sup>[5]</sup> Hence, certain amounts of exogenous antioxidants are constantly required to maintain an adequate level of antioxidants in order to balance the ROS. Recently, many epidemiological studies have suggested that the consumption of natural antioxidants such as polyphenol-rich food, fresh fruits,

vegetables, or tea have protective effects against the aforesaid diseases and this protection has been partly described to the presence of several components, such as vitamins, flavonoids, and other phenolic compounds.<sup>[6]</sup>

*Phyllanthus emblica*, has been used in Siddha and Ayurveda, the ancient Indian system of medicine. It has been used for the treatment of several disorders such as common cold, scurvy, cancer and heart disorders.<sup>[7-10]</sup>

So anti- oxidant present in the siddha medicine can protect against oxidative damage by decreasing the number of free radicals which cause chronic diseases and aging process and many other diseases. Siddhars have formulated different types of drug with antioxidant property one among such formulation is 'Nellikay thenural' made out of amla (Goose berry/ Nellikai /*Phyllanthus emblica*) and Honey.

**MATERIALS AND METHODS****Collection of the Drug**

To prepare the drug, seed have to be removed from Amla fruit and to be soaked equal amount of honey for 45 days. Since the drug is available in market, it was purchased from SKM Pharma Pvt.Ltd.

**Extraction procedure**

75g of the Nelliakkay thenural was soaked in 500 ml of hydroalcohol (1:1) mixture for 48 hours. The extract was filtered and concentrated using rotary evaporation under

reduced pressure (100 mbar) and reduced temperature (55°C). It was transferred to a porcelain dish. Using minimum quantity of ethanol and dried over water bath to free methanol. The obtained extract is used for further antioxidant and HPTLC process.

#### High Performance Thin Layer Chromatography

10 µl of Nellikay Thenural extract is applied on TLC plate using Camag's ATS4 applicator and developed by gradient mode the mobile phases, Methanol: 10% formic acid in Chloroform - 90:10 (developed upto 15 mm from the bottom); Methanol: 10% formic acid in Chloroform - 30:70 (developed upto 60 mm from the bottom); 10% formic acid in Ethyl acetate: toluene -70:30 (developed upto 80 mm from the bottom) using the automated multiple developer (AMD2). After development, the plate was dipped in 5% sulphuric acid reagent followed by heating at 105°C till development of coloured spots. The plate was then photo documented in white light and was scanned at 520 nm (Absorption mode and W lamp) and photo documented using Camag's TLC Visualizer for finger print profiles.

#### Estimation of polyphenol content of the drug

The amount of polyphenol in the extract is determined by folin-ciocalteu method. 1mg of the extract was dissolved in 5ml of methanol: water: concentrated-HCl (60:40:0.3). The contents were filtered through Whatman No.1 filter paper. After 2 minutes 100 micro liter of 50% of follins pohenol reagent was added. The tubes were incubated at room temperature 30 min and the absorbance was read at 750 nm by using UV spectrophotometer. Gallic acid was used as standard. Each assay was carried out in triplicate.

#### Determination of flavonoid concentrations in the drug

The content of the flavonoid in the examined drug was determined using spectrophotometric method. 1mg of the extract is dissolved in 1ml of methanol and it was taken in various concentration. To that 1ml of 2% Aluminium chloride solution was dissolved in methanol. The sample were incubated for 30 minutes at room temperature, the absorbance was determined using spectrophotometer 415nm. The samples were prepared in triplicate for each analysis and the mean value of absorbance was obtained. The same procedure for the standard solution of quercitin and the calibration line was constructed. Based on the measured absorbance, the concentration of flavonoids was read and its percentage of inhibition is calculated using the given formulae.

#### Antioxidant assay

##### Determination of DPPH radical scavenging activity

The reagents of DPPH solution (100 mM) was prepared by dissolving 33 mg of DPPH in 100 ml of ethanol. From the stock solution, 10 ml of was taken and made up to 100 ml using ethanol to 100mm DPPH solution and kept in amber coloured bottle to protect from sunlight. Quercitin was used as standard. Drug extract and the

standard was prepared in various concentrations. 0.1ml of various concentration of the extract and 1 ml solution of 100 mM DPPH was incubated at room temperature for 30 minutes and the changes in absorbance of reaction mixture was read at 517 nm, an equal amount of ethanol and DPPH was served as control.

##### Dertermination of pro-oxidant effect of the drug

The chemicals were prepared by dissolving 1g of Potassium ferricyanide in 100 ml of distilled water (1% w/v). phosphate buffer (0.2 M, Ph-6.6) was prepared by dissolving 2.75 g of sodium dihydrogen phosphate and 5.365g of disodium hydrogen phosphate in 100 ml of distilled water. 10 g of Trichloro acetic acid (10%) was dissolved in 100 ml of distilled water and 0.1 g of Ferric chloride in 100 ml of distilled water. Various concentration of the drug, the standard quercitin was used at various concentration, and these were mixed with 2.5 ml of phosphate buffer, potassium ferricyanide. This mixture was kept at 50°C in water bath for 20 minutes. After cooling 2.5 ml of 10% Trichloro acetic acid was added and centrifuged at 3000rpm for 10 min. 2.5ml of supernatent was mixed with equal volume of distilled water and 0.5 of ferric chloride solution was added. The absorbance was measured at 700 nm. Control was prepared in similar manner excluding samples, where the saturation point reaches the concentration was taken as 50% inhibition by the drug.

##### Determination of total antioxidant capacity of the drug

The reagents were prepared to 28mm of sodium phosphate was prepared by dissolving 84 mg of sodium phosphate in 25 ml of distilled water. 4mm of Ammonium molybdate was prepared by dissolving 124 mg of ammonium molybdate in 25 ml of distilled water and 0.6 mm of sulphuric acid was prepared. 100 µg of the extract was dissolved in 0.3 ml of distilled water. To that 3 ml of molybdate reagent containing 0.6 mm sulphuric acid, 28 mm of sodium phosphate and 4 mm of ammonium molybdate was added. The tubes were incubated at 95°C for 90 minutes. Then the mixture was cooled at room temperature and the absorbance was measured at 695 nm. The results were expressed as equivalents of ascorbic acid.

##### Scavenging of superoxide radical by alkaline DMSO method

The superoxide scavenging effect of the drug was studied using the alkaline DMSO method. The Reagents were prepared to 0.1 ml of NBT was prepared by dissolving 1 mg of NBT (Nitro blue Tetra Zolium) in 1 ml of DMSO (Dimethyl Sulfoxide). 1 ml of alkaline DMSO was prepared by dissolving 5 mm of sodium hydroxide in 1 ml of DMSO. The extract was dissolved in DMSO was taken in various concentration to that 0.1 ml of NBT (1 mg / ml of DMSO) and 1 ml of alkaline DMSO (5 mm of sodium hydroxide / ml of DMSO) was added. Then the absorbance was measured at 560 nm using spectrophotometer.

## RESULT AND DISCUSSION

### High Performance Thin Layer Chromatography (HPTLC)

HPTLC is a standardization technique used to separate the various compounds present in the herbal drug (Nellikay Thenural). The HPTLC procedure is done by taking Silica Gel 60 F<sub>254</sub> as stationary phase and Methanol : 10% formic acid in Chloroform (90:10 and 30:70) and 10% formic acid in Ethyl acetate: toluene (60:40) as mobile phase. HPTLC process is done to indicate the tendency of the sample (hydroalcoholic

extract of Nellikay Thenural) tends to develop various bands in the selected mobile phase. The developed HPTLC plate was derivitized and then visualized using TLC visualizer at 520 nm. Various bands were developed having different R<sub>f</sub> values ranged from 0.08 to 0.90, and colour of the formed band are gray, black and yellow and indicate the concentration of various compounds in the herbal siddha drug in which the highest concentration of compounds present in the Nellikay Thenural was found to be 44.66% and its corresponding R<sub>f</sub> value is 0.24 respectively.

R <sub>f</sub> value(s)	Color
0.08	Grey
0.09	Grey
0.13	Grey
0.26	Black
0.39	Black
0.59	Yellow
0.66	Yellow
0.74	Yellow
0.90	Yellow

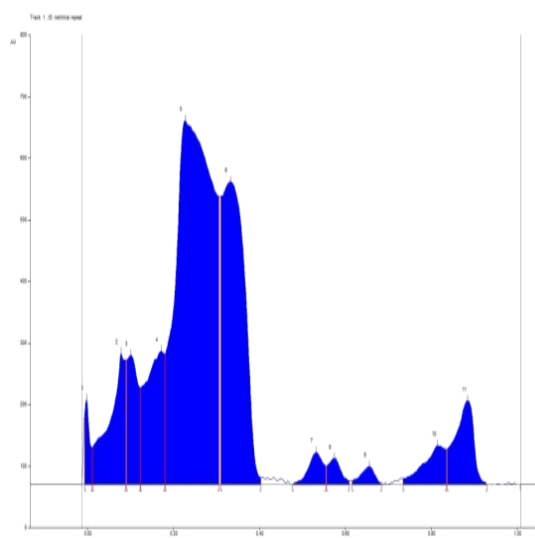
HPTLC Chromatogram @ 520nm:



$\lambda = 520 \text{ nm}$  (Derivatized)

Peak	Start Position	Start Height	Max Position	Max Height	Max %	End Position	End Height	Area	Area %
1	-0.01 Rf	113.3 AU	-0.00 Rf	137.7 AU	6.31 %	0.01 Rf	60.0 AU	1383.5 AU	1.38 %
2	0.01 Rf	60.7 AU	0.08 Rf	212.7 AU	9.75 %	0.10 Rf	02.3 AU	7012.1 AU	6.98 %
3	0.10 Rf	202.4 AU	0.11 Rf	209.9 AU	9.62 %	0.13 Rf	57.1 AU	4775.2 AU	4.76 %
4	0.13 Rf	157.2 AU	0.18 Rf	215.9 AU	9.90 %	0.19 Rf	11.8 AU	8133.8 AU	8.10 %
5	0.19 Rf	212.2 AU	0.24 Rf	590.7 AU	27.08 %	0.33 Rf	67.1 AU	44838.7 AU	44.66 %
6	0.33 Rf	467.8 AU	0.36 Rf	491.8 AU	22.54 %	0.43 Rf	11.0 AU	22953.8 AU	22.86 %
7	0.51 Rf	0.2 AU	0.57 Rf	51.9 AU	2.38 %	0.60 Rf	29.6 AU	1541.9 AU	1.54 %
8	0.60 Rf	29.6 AU	0.62 Rf	42.7 AU	1.96 %	0.65 Rf	5.5 AU	1088.8 AU	1.08 %
9	0.66 Rf	5.8 AU	0.70 Rf	29.1 AU	1.33 %	0.73 Rf	1.8 AU	820.3 AU	0.82 %
10	0.79 Rf	9.6 AU	0.87 Rf	62.9 AU	2.88 %	0.90 Rf	56.3 AU	2801.3 AU	2.79 %
11	0.90 Rf	56.9 AU	0.95 Rf	136.1 AU	6.24 %	1.00 Rf	0.3 AU	5058.5 AU	5.04 %

R<sub>f</sub> values and their percentage of HPTLC finger printing profile of Nellikay Thenural.



Peak Table @ 520 nm:

**Estimation of polyphenol in Nellikay Thenural**

The Polyphenol content of the Nellikay thenural extract were detected and compared with Gallic acid. The percentage of inhibition at various concentration of drug- Nellikay Thenural as well as standard Gallic acid (10-50µg/ml) were calculated and plotted in graph. The test

drug - Nellikay Thenural shows higher inhibition of about 45% at 50 µg concentration in 750 nm and similarly the standard Gallic acid exhibits 12.9% of inhibition at 50 µg. For the above drug, the control value is obtained as 0.340. The absorbance were measured at 750 nm using UV- spectrophotometer.

**Estimation of polyphenol in quercetin and Nellikay Thenural at various concentration****Table 1.**

Concentration(µg)	Standard OD	% of Inhibition	Sample OD	% of inhibition
10	0.296	0.8	0.266	21.7
20	0.302	2.9	0.264	22.3
30	0.305	10.2	0.257	24.4
40	0.330	11.1	0.239	29.7
50	0.337	12.9	0.187	45

**Estimation of flavonoid content in Nellikay Thenural**

The Flavonoid content of the extract Nellikay thenural were estimated and compared with Quercetin. The percentage of inhibition at various concentration of the drug Nellikay Thenural as well as standard Quercetin (1-5µg/ml) were calculated and plotted in the graph The drug- Nellikay Thenural shows higher inhibition 34.8% at 1µg/ml and lower inhibition of about 20.9 at 1µg/ml

sample concentration at 415 nm. The standard Quercetin exhibits a higher inhibition of about 44.2% of inhibition at 5 µg/ml and similarly lower inhibition of about 20.9 at 1µg/ml. The test drug - Nellikay Thenural shows a higher percentage of inhibition of about 34.8% in lower concentration 5 µg/ml. For the above drug, the control value is obtained as 0.339. The absorbance was measured at 415 nm using UV-spectrophotometer.

**Estimation of flavonoid and nellikkay tehural at various concentration****Table 2.**

Concentration (µg/)	Standard OD	% of Inhibition	Sample OD	% of Inhibition
1	0.268	20.9	0.268	20.9
2	0.261	23	0.259	23.5
3	0.220	35.1	0.255	24.7
4	0.219	35.3	0.252	25.6
5	0.189	44.2	0.221	34.8

**Antioxidant assay****Determination of DPPH radical scavenging activity**

The DPPH radical scavenging activity of the Nellikay Thenural extract were detected and compared with Quercetin. The percentage of inhibition at various concentration of the drug Nellikay Thenural as well as standard Quercetin (5-25 µg) were calculated and plotted

in graph. The test drug shows higher inhibition of about 46.2% at 25 µg sample concentration in 517 nm. The standard Quercetin exhibits 91.1% of inhibition at 25µg/ml. In lower concentration 5 µg/ml, the test drug shows 29.1% of inhibition. For the above drug, the control value is obtained as 0.158. The absorbance was measured at 517nm using uv-spectrophotometer.

**Determination of DPPH in Quercetin and Nellikay Thenural at various concentration****Table 3.**

Concentration (µg)	Standard OD at 517nm	% of Inhibition	Sample OD at 517nm	% of Inhibition
5	0.022	86.7	0.112	29.1
10	0.019	88.5	0.109	31
15	0.018	89.1	0.096	39.2
20	0.014	91.5	0.087	44.9
25	0.014	91.5	0.085	46.2

**Determination of pro-oxidant effect of the drug**

The prooxidant effect of the extract of Nellikay Thenural were detected and compared with Quercetin. In this method. The percentage of inhibition at various concentration (5-25 µg) of drug as well as standard

Quercetin (5-25µg/ml) were calculated and plotted in graph. The test drug shows higher inhibition of about 98 % at 25 µg sample concentration at 700nm. The standard Quercetin exhibits 97.4 % of inhibition at 25 µg concentration. For the above drug, the control value is

obtained as 1.506. The absorbance was measured at 700 nm using uv-spectrophotometer.

**Determination of pro-oxidant in standard and nellikkay thenural at various concentration**  
Table 4.

Concentration ( $\mu\text{g}$ )	Standard OD at 700nm	% of Inhibition	Sample OD at 700nm	% of Inhibition
5	0.100	93.3	0.049	96.7
10	0.082	94.5	0.042	97.2
15	0.051	96.6	0.039	97.4
20	0.041	97.2	0.032	97.8
25	0.039	97.4	0.029	98

**Determination of total antioxidant capacity of the drug**

The Total antioxidant capacity of the extract of Nellikkay Thenural were detected and compared with Ascorbic acid. In this method, Ascorbic acid was used as standard. The percentage of inhibition at various concentration (0.5-2.5  $\mu\text{g}$ ) of drug as well as standard Ascorbic acid (0.5-2.5 $\mu\text{g}$ ) were calculated and plotted in

graph. The test drug shows higher inhibition of about 22.4% at 2.5  $\mu\text{g}$  concentration in 695 nm. The standard Ascorbic acid exhibits 16.55% of inhibition at 2.5  $\mu\text{g}$ . In lower concentration 0.5 $\mu\text{g}$ , the test drug shows 9.6 % of inhibition. For the above drug, the control value is obtained as 0.290. The absorbance was measured at 695 nm using uv-spectrophotometer.

**Determination of total antioxidant capacity in standard and Nellikkay Thenural at various concentration**  
Table 5.

Concentration ( $\mu\text{g}$ )	Standard OD	% of Inhibition	Sample OD	% of Inhibition
0.5	0.287	1.03	0.262	9.6
1.0	0.282	2.7	0.250	13.7
1.5	0.272	6.2	0.243	16.2
2.0	0.262	9.6	0.238	17.7
2.5	0.242	16.55	0.225	22.4

**Scavenging of superoxide radical by alkaline DMSO method**

The superoxide radical scavenging of the extract of Nellikkay Thenural were detected and compared with Ascorbic acid. The percentage of inhibition at various concentration (0.5-2.5  $\mu\text{g}$ ) of drug as well as standard Ascorbic acid (0.5-2.5  $\mu\text{g}$ ) were calculated and plotted in

graph. The test drug shows higher inhibition of about 61.1 % at 2.5  $\mu\text{g}/\text{ml}$  sample concentration in 560 nm. The standard Ascorbic acid exhibits 52.2 % of inhibition at 2.5  $\mu\text{g}/\text{ml}$ . In lower concentration 0.5  $\mu\text{g}$ , the test drug shows 31.7 % of inhibition. For the above drug, the control value is obtained as 0.485. The absorbance was measured at 560 nm using uv-spectrophotometer.

**Determination of superoxide radical in standard and Nellikkay Thenural at various concentration**  
Table 6.

Concentration ( $\mu\text{g}$ )	Standard OD	% of inhibition	Sample OD	% of Inhibition
0.5	0.285	41.2	0.377	31.7
1.0	0.274	43.5	0.365	33.8
1.5	0.255	48.2	0.326	40.9
2.0	0.251	49.8	0.300	45.6
2.5	0.243	52.5	0.220	60.1

**SUMMARY AND CONCLUSION**

The current study helps in the determination of biological activity (antioxidant property) of Nellikkay Thenural. HPTLC has become the most potent tool for qualitative analysis of compounds because of its simplicity and reliability. It serves as the basis for the identification of various compounds present in the herbal drug (Nellikkay Thenural), and the analysis can be used as a diagnostic tool for the correct identification of

photochemical constituents present in the Siddha herbal drug (Nelliakkay Thenural).

HPTLC procedure is used as standardization technique to determine the various chemical compounds present in the herbal drug (Nellikkay thenural), during the process various bands were developed in the selected mobile phase and the 3-Dimensional chromatogram and the graph shows the level (concentration) of various compounds present in the Nellikkay Thenural extract. To determine the antioxidant compounds, present in the

hydroalcoholic extract of the herbal drug, two pre-screening technique such as estimation of **Polyphenol** and **Flavonoids** were done, and the analysis shows a positive result.

The antioxidant activity of (Nellikay thenural) is analyzed and estimated using four different methods such as scavenging of DPPH radical and superoxide dismutase, determination of total antioxidant capacity and effect of pro-oxidant were done. The result showed a good antioxidant activity of Nellikay thenural. Since Nellikay Thenural showed a great antioxidant activity it may have the tendency to cure Hyperthermia and may prevent many chronic diseases caused due to Hyperthermia.

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