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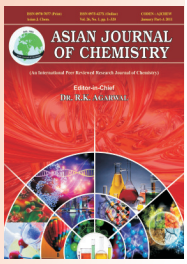

Chemical Standardization and *in vitro* Cytotoxic Studies on *Nellikai lehyam*†

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Plants play a major role in all alternate systems of medicines like Siddha, Ayurveda, Unani and Chinese. In the recent years there has been considerable researches going on traditional siddha medicines focusing towards validation and standardization aspects. In the present work, an immuno-modulatory drug-*Nellikai lehyam* was subjected to physicochemical standardization and *in vitro* cytotoxic studies. A preliminary phytochemical screening was carried out to identify the nature of phytoconstituents present. The yield of tannins, flavonoids, phenols, vitamin C, total fatty matter, were estimated as per standard procedures. Percentage of iron was also estimated and was found to be 77.49 %. Finger printing profile of flavonoids in *Nellikai lehyam* was carried out by HPTLC technique using toluene: ethyl acetate:formic acid (5:4:1 v/v/v) as mobile phase. The bio active constituents in lehyam were also identified through GC-MS and LC-MS analysis. *In vitro* antioxidant activity was carried out using DPPH and reducing assay. This drug possesses strong antioxidant activity which was exposed by its ability to scavenge the stable free radical DPPH. The cytotoxic activity was tested against cells using MTT assay which revealed strong activity. This is the first report on chemical standardization, antioxidant and anticancer activity of this unique siddha formulation *Nellikai lehyam*.

Keywords: HPTLC, GC-MS, LC-MS, DPPH, Cytotoxic activity, *Nellikai lehyam*.

INTRODUCTION

The plants are the major source of naturally occurring medicaments. Indian Systems of Medicine in practice are Siddha, Ayurveda and Unani and the efficacy and popularity of any system of medicine depends on the quality of medicines used for the treatment. Standardization of herbal medicines becomes most important to access the quality of the drug[1,2].

Present paper demonstrates the development of methods for the standardization of *Nellikai lehyam*, a polyherbal siddha formulation constituting sixteen different herbal plants prepared using ghee, tender coconut, ginger juice and sugar. This formulation is used in the treatment of Anemia. *Nellikai lehyam* is an immune modulator as it is rich in iron and vitamin C. *Nellikai lehyam* also proved to be effective in HIV infected persons[3]. As this drug contains high composition of *Phyllanthus emblica,* it may have potential activity against cancer[4].

In the present work, an attempt is made to estimate and to confirm the type of active principles present in the drug by HPTLC, GC-MS and LC-MS techniques. In addition, *in vitro* antioxidant activities and cytotoxic study was also performed.

EXPERIMENTAL

DPPH purchased from Sigma Aldrich. All other reagents are of AR grade.

Physico-chemical standardization: Standardization parameters such as pH, loss on drying, total ash, acid insoluble ash, water and alcohol soluble extractives were analyzed as per standard protocols[5,6].

Phytochemical screening: Phytochemical screening of methanolic extract of *Nellikai lehyam* for Tannins, flavonoids, phenols, alkaloids, proteins, terpenoids and sterols were performed by various tests[7].

Quantitative estimation: Iron, tannins, vitamin C was estimated by titrimetric method[8,9] whereas flavonoids and phenols were estimated spectrometrically by aluminium chloride and Folin Ciocalteu's method, respectively[10].

High performance thin layer chromatography (HPTLC) analysis: Methanolic extract of *Nellikai lehyam* were spotted on a pre-coated silica gel $60F_{254}$ TLC plate (E. Merck) of thickness 0.2 mm, 7 mm wide band using TLC applicator Linomat V using toluene: ethyl acetate: formic acid (5:4:1 v/v/v) as mobile phase. Developed the plate in a twin trough chamber to a height of 8 cm which was previously saturated

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with solvent vapour for 0.5 h. After development, the plate was dried and then scanned densitometrically at 254 nm using TLC scanner 3.

GC-MS analysis: GC analysis was carried out on a Clarus 500 gas chromatograph using a non-polar, Elite-5 ms column (30 m × 0.25 mm × 0.25 µm film thickness, coated with 5 % diphenyl-95 % dimethyl polysiloxane) interfaced with mass detector. Helium was used as carrier gas with a flow rate of 1 mL/min. Temperature programme was 50-150 ºC hold for 2 min at the rate of 3 ºC/min and increased to 290 ºC (10 min) at the rate of 8 ºC/min. 1 µL of the methanol extract was injected with split ratio as1:10. Mass spectra were recorded in the EI mode at 70 eV in a scan range of 40-600 amu. Injector and ion source temperature were maintained at 280 and 200 ºC, respectively. The resulted spectra were compared with NIST library database.

LCMS-MS analysis: Liquid chromatography analysis was carried out on a HPLC Dionex C_{18} RP Acclaim 120 interfaced with MSMS Bruker Q-II TOF Mass detector. Liquid chromatography condition was gradient mobile system start with 0.2 min at 1 % ACN and 99 % water (1 % acetic acid) to 75 % ACN at 16th min, this was brought to 100 % ACN at 19th min to 5 % ACN at 21st min and was maintained at same condition till run ends at 23rd min with 0.2 mL/min was set as a flow rate under UV at 325 nm. MS-MS spectra was recorded in the ESI, Negative mode, Nebulizer 30.5 psi with 6.0 L/min N_2 flow, mass scan in the range of 50-1000 *m/z*, Capillary voltage 4500 V, dry heater temperature at 280 ºC.

Antioxidant activity

DPPH assay: The selected concentration of sample and standard gallic acid (7.81-500 µg) was mixed with 2.5 mL of 0.3 mM DPPH in ethanol. The mixture was shaken vigorously and allowed to stand in dark for 0.5 h at room temperature. The intensity of the colour measured at 517 nm[11].

Reducing power capacity assay: The reducing power of the sample was determined using ferricyanide-ferric chloride method[12]. 1 mL of sample (7.81-500 µg/mL) was mixed with 1 mL of 0.2 M phosphate buffer of pH 6.6 and 1 mL of 1 % potassium ferricyanide. Mixture was incubated for 20 min at room temperature. After incubation, 1mL of trichloroacetic acid (10 %) was added and centrifuged at 3000 rpm for 10 min. From this, 1 mL of supernatant was mixed with 1 mL of distilled water and 100 µL of $FeCl_3$ by using ascorbic acid as a reference standard. Absorbance was read at 700 nm.

Cytotoxic studies: In each assay, 0.1×10^6 EAC (*Ehrlich ascites carcinoma*) cells were harvested in culture medium and plated in 96-well flat bottom culture plates and incubated at 37pC for 24 h in humidified 5 % CO_2. After 24 h, 10 µL aliquots of serial dilutions of extract (1000-1.95 µg/mL) in DMSO were added to *Ehrlich ascites carcinoma* cells and incubated for 48 h. Cell viability was assessed through the MTT assay[13]. Briefly, 25 µL of MTT (5 mg/mL) were added and the cells were incubated for an additional 3 h. Thereafter, cells were lysed and the dark blue crystals solubilized with 100 µL of a solution containing 50 % N, N-dimethylformamide, 20 % sodium dodecyl sulfate. The optical density of each well was measured using Epoch micro plate spectrophotometer (BioTek, USA) set at 590 nm filter. Cells viability was calculated and tabulated.

RESULTS AND DISCUSSION

Physico-chemical data: Physico-chemical standard such as pH, loss on drying, total ash, acid insoluble ash and extractive values were evaluated and presented in Table-1. These parameters can be efficiently used for testing identity, purity and strength of this formulation, *Nellikai lehyam*. The ash value of 2.14 % indicates that the siliceous matter is low in the formulation. High percentage of water and alcohol soluble extractive values depicted the strength of bioactive constituents present in the formulation.

TABLE-1
PHYSICO-CHEMICAL CONSTANTS-*Nellikai lehyam*

Parameters	As per analysis
Description	Dark brown coloured semisolid drug with characteristic odour
pH (1 % w/v suspension)	4.87
LOD at 105 ºC	17.0428 %
Total ash	2.1425 %
Acid insoluble ash	0.9299 %
Water soluble extractive	62.0903 %
Alcohol soluble extractive	36.5247 %

Preliminary phytochemical screening: Phytochemical screening of methanolic extract revealed the nature of active constituents present in experimental plant (Table-2).

TABLE-2
PRELIMINARY PHYTOCHEMICAL SCREENING OF METHANOLIC EXTRACT OF *Nellikai lehyam*

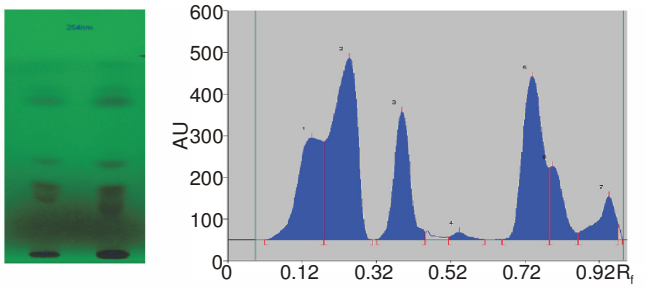
Tests	Methanol extract
Flavonoids	+
Tannins	+
Carbohydrates	+
Sterols	+
Alkaloids	+
Terpenoids	+
Proteins	+

Estimation of major phytochemical constituents: Table-3 shows the quantitative estimation of iron, tannins,vitamin C, flavonoids, phenols, total fatty matter and was found to be 77.49, 39.8199, 0.9898, 3.6109, 2.5728 and 2.0783 %, respectively. Tannin was found to be higher as compared to other ingredients. Tannins possess excellent anticancer activity[14]. Vitamin C, phenols and flavonoids possess significant antioxidant activities[15,16]. Different types of fatty acids identified through GC-MS analysis also provided supportive evidence to cytotoxic activity[17]. The percentage of iron was found to be 77.49 %. Deficiency of iron affects the immune system leading to cancer. The maximum amount of Iron in this drug plays a vital role in immuno-modulatory system[18].

TABLE-3
QUANTITATIVE ESTIMATION OF ORGANIC, INORGANIC AND VITAMIN-C CONTENT

Parameters	Result (%)
Iron	77.49
Vitamin C	0.9898
Tannins	39.8199
Flavonoids	3.6109
Phenols	2.5728
Total fatty matter	2.0783

HPTLC finger printing: HPTLC finger printing profile of flavonoids was identified in the methanolic extract of *Nellikai lehyam* and presented in Fig. 1. The number of bands and the maximum R_f value indicates the presence of flavonoids. The maximum R_f values were observed at 0.15, 0.25, 0.39, 0.55, 0.75, 0.80 and 0.95.



Peak	Start Rf	Start Height	Max Rf	Max Height	Height %	End Rf	End Height	Area	Area %
1	0.02	0.1	0.15	244.9	14.57	0.18	235.9	12202.1	17.78
2	0.18	236.1	0.25	436.8	25.98	0.32	0.2	21879.2	31.87
3	0.33	1.8	0.39	308.2	18.33	0.46	19.0	9687.0	14.11
4	0.52	7.2	0.55	18.2	1.08	0.62	0.8	572.1	0.83
5	0.66	1.6	0.75	391.4	23.28	0.79	173.5	15844.2	23.08
6	0.79	173.9	0.80	176.6	10.51	0.87	17.1	4766.3	6.94
7	0.87	17.3	0.95	105.0	6.25	0.98	39.6	3693.8	5.38

Fig. 1. HPTLC finger printing profile of flavonoids identified in the methanolic extract of *Nellikai lehyam*

GCMS analysis: GC-MS chromatogram of the methanolic extract was presented in Fig. 2. GCMS data presented in Table-4 shows the presence of twenty three fragmented compounds of which 5-HMF, phenols, flavonoid and sugar moieties were found to be higher. 5-Hydroxy methyl furfural (HMF) with area of 51.75 %, is proved to possess antioxidant activity[19]. 4*H*-Pyran-4-one, 2,3-dihydro-3,5-dihydroxy-6-methyl-which is a fragmentation of flavonoid and other phenolic compounds like benzene carboxylic acid and Benzoic acid, 3-hydroxy, methyl ester which also has strong antioxidant activities as discussed above. Sugar based compounds like D-allose and

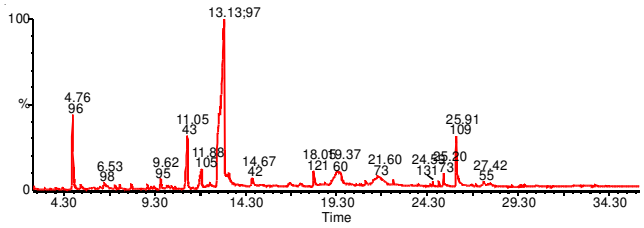


Fig. 2. GC-MS chromatogram of methanolic extract of *Nellikai lehyam*

1,6-anhydro-α-D-glucofuranose with area of 7.39 and 4.96 % are known to possess antioxidant and anticancer activities[20].

LC-MSMS analysis: Figs. 3 and 4 shows the LC-MS chromatogram and MS-MS spectrum of the sample. MS-MS spectrum confirms the presence of flavonoids like dihydro-quercetin, hesperetin, baicalin, liquiritin and 7-O-glycosyl luteolin. These flavonoids are proved to have strong antioxidant and anticancer activities.

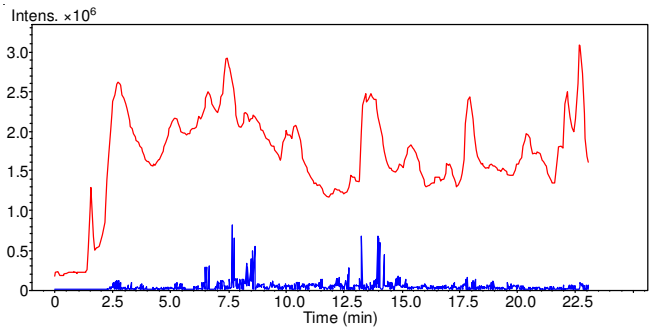


Fig. 3. LCMS chromatogram of *Nellikai lehyam*

***in vitro* antioxidant studies:** Fig. 5 shows the graphical representation of antioxidant activity revealed in DPPH and reducing assay. DPPH free radical scavenging activity showed the potent inhibitory effect when compared with gallic acid as standard. The percentage inhibition of free radical increased

TABLE-4
TABLE SHOWING BIOACTIVE MOLECULES OF *Nellikai lehyam*

Peak name	m.f.	Retention time	Peak area (%)
Furfural	$C_5H_4O_2$	4.76	5.6046
1,6;3,4-Dianhydro-2-O-acetyl-α-d-allopyranose	$C_8H_{10}O_5$	6.53	1.1230
Benzoic acid, 2-hydroxy-6-methyl-, methyl ester	$C_9H_{10}O_3$	8.57	0.0300
Imidazole, 5-hydrazinocarbonyl-	$C_4H_6N_4O$	9.62	0.9844
Levoglucosenone	$C_6H_6O_3$	10.24	0.1738
4*H*-Pyran-4-one, 2,3-dihydro-3,5-dihydroxy-6-methyl-	$C_6H_8O_4$	11.05	6.2900
Benzene carboxylic acid	$C_7H_6O_2$	11.88	3.2308
5-Acetoxymethyl-2-furaldehyde	$C_8H_8O_4$	12.32	0.8660
2-Furancarboxaldehyde, 5-(hydroxymethyl)-(5-HMF)	$C_6H_6O_3$	13.13	51.7496
1,6; 3,4-Dianhydro-2-O-acetyl-α-d-allopyranose	$C_8H_{10}O_5$	14.67	1.7258
Isopentyl 3-hydroxy-2-methylenebutanoate	$C_{10}H_{18}O_3$	16.72	1.1609
Benzoic acid, 3-hydroxy-, methyl ester	$C_8H_8O_3$	18.05	2.1884
D-Allose	$C_6H_{12}O_6$	19.37	7.3922
1,6-Anhydro-α-D-glucofuranose	$C_6H_{10}O_5$	21.60	4.9604
Tetradecanoic acid	$C_{14}H_{28}O_2$	22.43	0.2807
Hexadecanoic acid, methyl ester	$C_{17}H_{34}O_2$	24.47	0.1068
2-Propenoic acid, 3-phenyl-, 2-methylpropyl ester	$C_{13}H_{16}O_2$	24.59	0.2392
Benzaldehyde, 2,4-dihydroxy-6-methyl-	$C_8H_8O_3$	24.96	0.3106
n-Hexadecanoic acid	$C_{16}H_{32}O_2$	25.20	0.7036
p-Mesyloxyphenol	$C_7H_8O_4S$	25.91	6.7418
9-Octadecenoic acid, methyl ester, (E)-	$C_{19}H_{36}O_2$	26.71	0.1011
Oleic acid	$C_{18}H_{34}O_2$	27.42	0.9785
Gingerol	$C_{17}H_{26}O_4$	29.36	0.0526

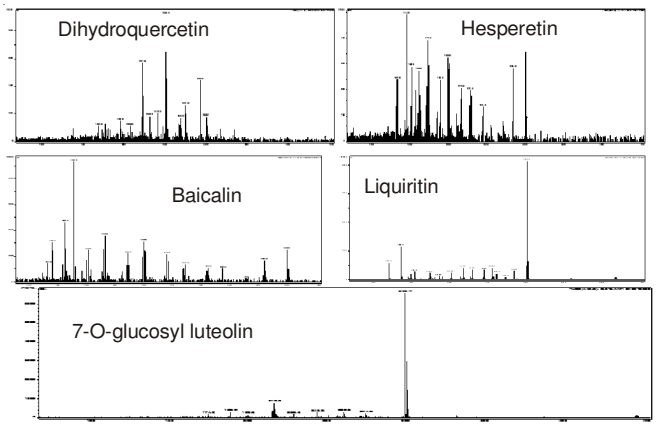


Fig. 4. MS-MS spectrum of flavonoids

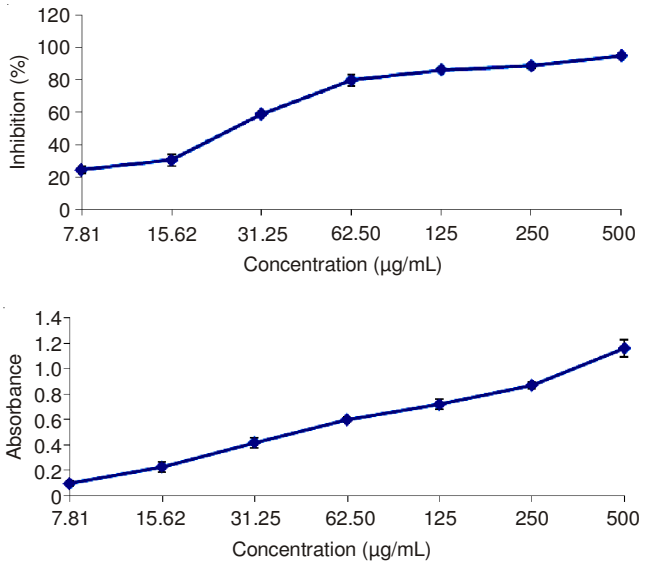


Fig. 5. Graph showing DPPH and reducing capacity efficacy of *Nellikai lehyam*

with increase in concentration of substrates. The IC_{50} value of *Nellikai lehyam* extract was found to be 31.25 µg/mL. Fig. 5 also shows the reducing ability of *Nellikai lehyam* compared with ascorbic acid (vitamin C) as standard. The reducing power of lehyam was found to be effective and increased with increase in concentration.

***In vitro* cytotoxic studies (MTT assay):** Cytotoxicity was evaluated using MTT test. This test is based on the reduction of 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide (MTT) in living cells which is measured colorimetrically. IC_{50} value of the aqueous extract was found to be 13.25 µg/mL. IC_{50} is defined as the concentration (µg/mL) of the substrate that causes 50 % death of cells. Previous studies have demonstrated that flavonoids are found to possess potent cytotoxic activity[21]. This formulated drug contains tannins, flavonoids, phenols, percentage of these constituents were estimated and also identified through chromatographic techniques. The data revealed from cytotoxic assay proves that lehyam shows better anticancer activity due to the rich content of tannins, flavonoids and phenolic compounds.

Conclusion

To conclude, this formulation consists of natural ingredients which were proven to be effectively bio-active and had fewer side effects. Presence of bio-molecules like tannins, flavonoids, phenols, vitamin C and iron contributes to the anticancer and antioxidant potentials of *Nellikai lehyam*. Chemical constituents identified in the present work will provide supporting evidences for the therapeutic activity of the selected Siddha formulation and standardization parameters determined will help in deciding the identity, purity and strength of this siddha formulation "*Nellikai lehyam*".

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