Original Article

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HPTLC STUDIES AND BRINE SHRIMP LETHALITY ASSAY OF EXTRACTS, FRACTIONS AND IDENTIFIED COMPOUNDS OF NYMPHAEA STELLATA WILLD. LEAVES

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Abstract

TLC due to its simplicity, accuracy, cost effectiveness and rapidity, is often used as an alternative to other chromatographic techniques for quantifying plant products. *Nymphaea stellata* Willd. (*Ns*) of the family Nymphaeceae is an important and well-known medicinal plant in the Ayurvedic and Siddha systems. Complete phytochemical profile of *Ns* leaf is unavailable, hence comparative TLC was used to identify and quantify chemical constituents. The quantity of gallic acid, β-carotene, lupeol and β-sitosterol were found to be 0.094679, 0.01045, 0.013016 and 0.047703 %w/w respectively. Brine shrimp lethality assay is a very useful bench-top method for drug discovery process. Hence methanolic extract, 50% methanolic extract, aqueous extract were screened along with the first timed identified gallic acid, β-carotene, lupeol and β-sitosterol. β-carotene and β-sitosterol showed LC_{50} of 520 and 750 µg/ml respectively in brine shrimp lethality assay. As the polarity of the extracts increased the lethality also increased, suggesting the presence of polar toxic compound/s *Ns* leaf.

Key words: HPTLC, brine shrimp lethality assay, gallic acid, β-sitosterol, lupeol, β-carotene, *Nymphaea stellata*.

Introduction

Thin layer chromatography (TLC) is an important analytical tool in separation, identification and estimation of different classes of natural products. Comparative TLC (co-TLC) with chemical or biological marker compounds can be used for identification and quantification of chemical constituents and also to standardize the herbal raw materials. Moreover, due to its simplicity, accuracy, cost effectiveness and rapidity, TLC is often used as an alternative to other chromatographic techniques. *Nymphaea stellata* Willd. (*Ns*) of the family Nymphaeceae is an important and well-known medicinal plant in the Ayurvedic and Siddha systems of medicine.

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The leaves, roots and flowers has been claimed for a wide range of pharmacological activities and are used for diabetes, eruptive fevers and as cardiotonic, emollient, diuretic, narcotic and aphrodisiac[1,2]. Since complete phytochemical profile of *Ns* leaf is unavailable, co-TLC was used to identify and quantify chemical constituents.

The study of bioactive compounds from plant sources and extracts in the chemical laboratory is often hampered by the lack of a suitable, simple, and rapid screening procedure. But this method, utilizing brine shrimp (*Artemia salina*), is a simple bioassay for natural product research. The procedure determines lethal concentrations of active compounds in brine medium. The activities of a broad range of active compounds are manifested as toxicity to the shrimp. The method is rapid, reliable and has been used for over thirty years in toxicological studies. The commercial availability of inexpensive brine shrimp eggs, the low cost and ease of performing the assay make brine shrimp lethality

assay, a very useful bench-top method[3]. The shrimp lethality assay was proposed by Michael et al.[4], and later developed by Vanhaecke et al.[5], and Sleet and Brendel[6]. It is based on the ability to cause death in the laboratory cultured Artemia nauplii brine shrimp. The assay is considered a useful tool for preliminary assessment of toxicity[7], and it has been successfully used for studying plant extract toxicity[3], teratology screens[8], cytotoxic compounds[9], antimalarial compounds[10], insecticidal compounds[11] and antifeedent compounds[12]. Brine shrimp bioassay has good correlation with the human solid tumour cell lines[13]. Considering brine shrimp lethality as a simple bioassay useful for drug discovery process, the procedure of Meyer et al.[14], was adopted to determine the lethality of *Ns* leaf extract and identified chemical constituents.

Materials and methods

Chemicals and reagents

Pure gallic acid, β-sitosterol and β-carotene were procured from Himedia Laboratories Pvt. Ltd., Mumbai, India and lupeol was procured from Sigma Chemicals, Bangalore, India. Brine shrimp eggs were purchased from Ocean Star International Inc., Snowville, UT, USA. Other solvents and chemicals used were of analytical grade. Silica gel $60F_{254}$ TLC plates were purchased from Merck (Darmstadt, Germany).

Collection and authentication of plant materials

Leaves of *Nymphaea stellata* Willd. were collected from Coonoor and Ootacamund, The Nilgiris, India. The plant was identified by Dr. Rajan, Field Botanist, The Survey of Medicinal Plants and Collection Unit, Government Arts College, Ootacamund, India and authenticated by comparing with the voucher specimen.

Thin layer chromatographic study

A Camag TLC system equipped with Camag Linomat V, an automatic TLC sample spotter, Camag glass twin trough chamber (20X10 cm) was used for the analysis. Chromatography was performed using pre-activated (60° C for 5 min) silica gel $60F_{254}$ TLC plates (20X10 cm; layer thickness 250μm). Samples and standards were applied on the plate as 8 mm wide bands with an automatic TLC sampler under a flow of N_2 gas, 10 mm from the bottom and 10 mm from the side and the space between two spots were 15 mm of the plate. The linear ascending development was carried out in a Camag twin trough chamber saturated with 20 ml mobile phase for 20 min at room temperature (25 ± 2°C and 40 % relative humidity). The plates were developed up to 8 cm under chamber saturation conditions. Subsequent to the development, TLC plates were dried in current air with the help of a hair dryer. The post chromatographic derivatization was carried out with specific detecting agents. Evaluations of the plates were performed with Camag scanner 3 (win CATS 4.0 integration software). Densitometric scanning was performed in the absorption-reflection mode, using a slit width of 6 X 0.45 mm, data resolution 100 μm step and scanning speed 20 mm/s with a computerized Camag TLC scanner.

Identification and quantification of chemical constituents

Based on the preliminary qualitative phytochemical screening, co-TLC studies of extracts were performed with known standards. The extracts were separated in suitable mobile phase along with standards. The identified chemical constituents were quantified by external standard method.

Method development and validation

Specificity of the method was determined by analyzing standard and the unknown sample. The spot sample spot was confirmed by comparing the R*f* multi wavelength scanning and spectral overlay of the standard spot. The peak purity was assessed by comparing the spectra at three different levels, i.e., peak start, peak apex and peak end positions of the spot. The method was validated for precision, accuracy and repeatability (ICH, 1996/2005)[15]. Instrumental precision was checked by repeated scanning of the same standard spot at different concentrations and expressed as coefficient of variance (% RSD). Method precision was studied by analyzing standard at lower and higher concentration under the same analytical procedure and laboratory condition on the same day (intra-day precision) and on different day (inter-day precision), the results were expressed as % RSD. Accuracy of the method was tested by performing the recovery studies of pre-analyzed sample with standard at three levels 80, 100 and 120 % and % recovery was calculated.

Identification and quantification of gallic acid

Accurately weighed 7.5 g of coarsely powdered leaves were extracted with methanol (4 X 50 ml) under reflux (30 min each time) on a water bath. The combined extracts were filtered, concentrated and transferred to a 50 ml volumetric flask and the volume was made up with methanol. A stock solution of gallic acid (100μg/ml) was prepared in methanol. Working solutions were prepared by appropriate dilution of the stock solution with the same solvent. Calibration range was 500 to 900 ng. Quantification was performed by external standard method, using pure gallic acid as standard. Sample solution was applied in triplicate on the TLC plate and developed with mobile phase toluene: ethyl acetate: methanol: formic acid (6:3:1:0.5, v/v/v/v). Densitometric scanning was performed in absorption-reflection mode at 282 nm. Peak areas were recorded and the amount of gallic acid was calculated using the calibration curve.

Identification and quantification of β-sitosterol

Accurately weighed 5 g of coarsely powdered leaves were extracted with methanol (4 X 50 ml) under reflux (30 min each time) on a water bath. The combined extracts were filtered, concentrated and transferred to a 50 ml volumetric flask and the volume was made up with methanol. A stock solution of β-sitosterol (100 μg/ml) was prepared in methanol. Working solutions were prepared by appropriate dilution of the stock solution with the same solvent. Calibration range was 100 to 500 ng. Quantification was performed by external standard method, using pure β-sitosterol as standard[16]. 5 μl of the sample solution was applied in triplicate on the TLC plate and developed with mobile phase toluene: chloroform: methanol (4:4:1, v/v/v). The post chromatographic derivatization was carried out with anisaldehyde-sulphuric acid placed in a dipping chamber (CAMAG) followed by heating in an oven at 100 °C for 5-10 min[17]. Densitometric scanning was performed in absorption-reflection mode at 527 nm. Peak areas were recorded and the amount of β-sitosterol was calculated using the calibration curve.

Identification and quantification of lupeol

Accurately weighed 7.5g of coarsely powdered leaves were extracted with methanol (4 X 50 ml) under reflux (30 min each time) on a water bath. The combined extracts were filtered, concentrated and transferred to a 50 ml volumetric flask and the volume was made up with methanol. A stock solution of lupeol (100 μg/ml) was prepared in methanol. Working solutions were prepared by appropriate dilution of the stock solution with the same solvent. Calibration range was 100 to 500 ng. Quantification was performed by external standard method, using pure lupeol as standard[18]. 5 μl of the sample solution was applied in triplicate on the TLC plate and developed with the mobile phase toluene: chloroform: ethyl acetate: glacial acetic acid (10:2:1:0.03, v/v/v/v). The post chromatographic derivatization was carried out with freshly prepared antimony trichloride reagent (20% solution of antimony III chloride in chloroform) placed in a dipping chamber (CAMAG) followed by heating in an oven at 110°C for 5-6min[17]. Densitometric scanning was performed in fluorescence mode at 366nm. Peak areas were recorded and the amount of lupeol was calculated using the calibration curve.

Identification and quantification of β-carotene

Accurately weighed 5 g of coarsely powdered leaves were extracted with methanol (4 X 50 ml) under reflux (30 min each time) on a water bath. The combined extracts were filtered, concentrated and transferred to a 50 ml volumetric flask and the volume was made up with methanol. A stock solution of β-carotene (100 μg/ml) was prepared in methanol. Working solutions were prepared by appropriate dilution of the stock solution with the same solvent. Calibration range was 100 to 500 ng. Quantification was performed by external standard method, using pure β-carotene as standard. Sample solution was applied in triplicate on the TLC plate and developed with mobile phase n-hexane: benzene (9:1, v/v). Densitometric scanning was performed in absorption-reflection mode at 445 nm. Peak areas were recorded and the amount of β-carotene was calculated using the calibration curve.

Preparation of extracts

Coarsely powdered leaves of *Ns* were extracted with methanol (ME), 50% methanol (50% ME) and chloroform water (AE) in soxhlet apparatus until exhaustion; the extract was concentrated *in vacuo* by rotary evaporator and dried in desiccator.

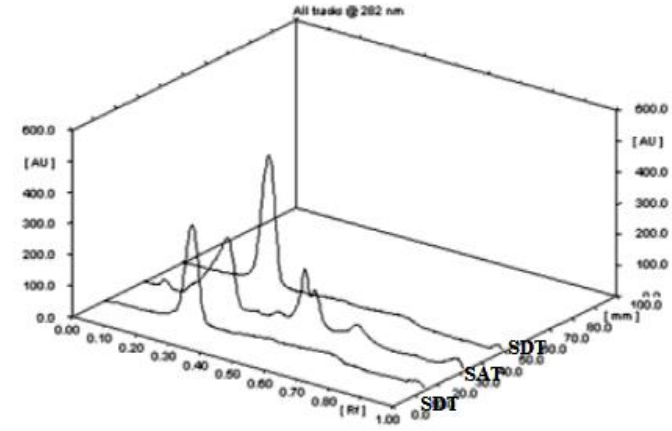
Brine shrimp lethality bioassay

Brine shrimp lethality bioassay was performed as per the method of Meyer et al.[14]. Brine shrimps (*Artemia salina*) were hatched using brine shrimp eggs in a conical shaped vessel (1L), filled with sterile artificial seawater (prepared using sea salt 38 g per liter and adjusted to pH 8.5 using 1N NaOH) under constant aeration for 36 h. After hatching, active nauplii free from egg shells were collected from brighter portion of the hatching chamber and used for the assay. Ten nauplii were drawn through a glass capillary and placed in each vial containing 4.5 ml of brine solution (24 % of sodium chloride in water). In each experiment, 0.5 ml of the extracts/fractions/identified compounds was added to 4.5 ml of brine solution and maintained at room temperature for 24h under the light and surviving larvae were counted. Experiments were conducted at different concentrations (up to 4000µg/ml for extracts/fractions and 2000µg/ml for identified compounds) of the test substances in a set of six tubes per dose. Extracts/fractions/identified compounds were dissolved in minimum volume of DMSO and made up with water. The concentration of DMSO used was also studied as vehicle control. The percentage lethality was determined by comparing the mean surviving larvae of the test and control tubes. LC_{50} values were obtained from the best-fit line plotted concentration verses percentage lethality.

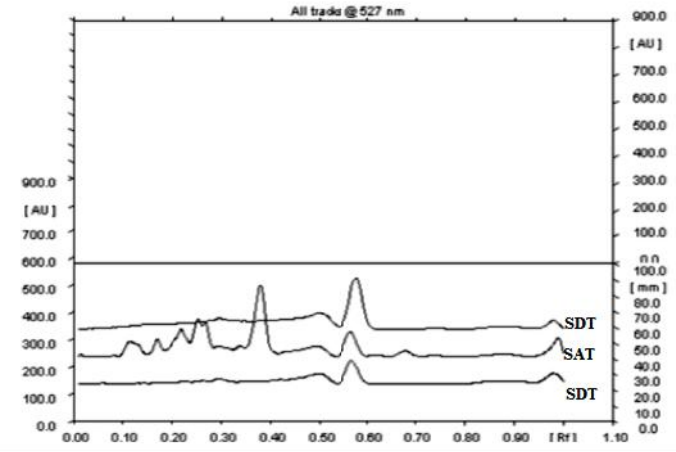
$$\% \text{ lethality} = \frac{\text{NSN Control} - \text{NSN Test}}{\text{NSN Control}} \times 100$$

Where,

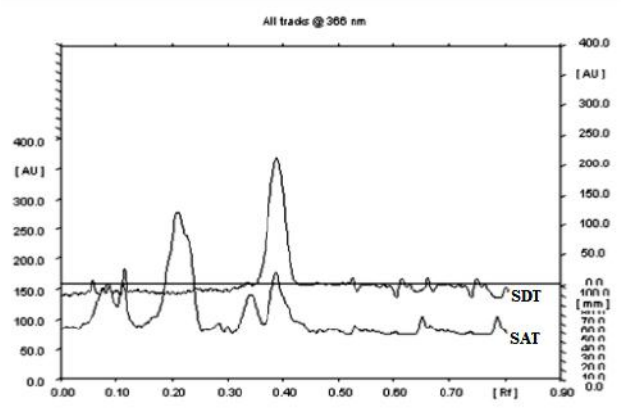
NSN Control - Number of surviving nauplii in control;
NSN Test - Number of surviving nauplii in test.



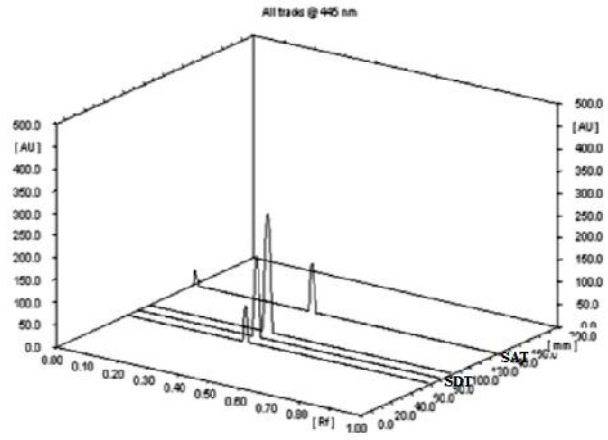
***Ns* leaf extract with gallic acid**



***Ns* leaf extract with β-sitosterol**



***Ns* leaf extract with lupeol**



***Ns* leaf extract with β-carotene**

Figure 01: Densitogram of *Ns* leaf extract showing identical peak with standards (SAT-Sample tract; SAD-Standard track)

Table 01: Linearity regression data for quantification of gallic acid, β-sitosterol, lupeol and β-carotene

Parameter	Gallic acid	β-sitosterol	Lupeol	β-carotene
R_f	0.26	0.57	0.40	0.39
Dynamic range (ng spot^{-1})	500-900	100-500	100-500	100-500
Equation	y=2732.345+6.654x	y=280.581+8.778x	y=553.192+10.463x	y=169.086+4.235x
Slope	6.654	8.778	10.463	4.235
Intercept	2732.345	280.581	553.192	169.086
Linearity (Correlation coefficient)	0.99941	0.98515	0.99646	0.99808
Amount of compound quantified[a]	0.094679%w/w	0.047703%w/w	0.013016%w/w	0.01045%w/w

[a] plant dry weight basis

Table 02: Precision data for quantification of gallic acid, β-sitosterol, lupeol and β-carotene

TLC Method	Concentration (ng spot^{-1})	Instrumental precision (%RSD)	Method precision (%RSD)	
			Intra-day	Inter-day
Gallic acid	500	0.94	0.96	1.28
	900	0.80	0.78	0.87
β-sitosterol	100	0.58	0.79	1.31
	500	0.17	0.22	0.37
lupeol	100	0.51	0.34	1.31
	500	0.57	0.42	0.71
β-carotene	100	0.74	0.65	0.47
	500	0.94	0.74	0.41

Table 03: Recovery studies data for quantification of gallic acid, β-sitosterol, lupeol and β-carotene

TLC Method	Amount in the sample (µg)	Amount added (µg)	Amount found (µg)	Recovery (%)
Gallic acid	94.7	75.8	168.2	98.65
	94.7	94.7	185.3	97.83
	94.7	113.6	204.3	98.07
β-sitosterol	47.7	38.2	83.7	97.43
	47.7	47.7	93.5	98.01
	47.7	57.2	102.4	97.61
lupeol	13.1	10.5	23.2	98.31
	13.1	13.1	25.4	96.95
	13.1	15.7	27.9	96.88
β-carotene	10.5	8.4	17.5	92.59
	10.5	10.5	19.0	90.48
	10.5	12.6	21.1	91.34

Table 04: LC_{50} values of extracts and identified compounds of *N. stellata* leaves

Extracts/fractions/identified compounds	LC_{50} values (µg/ml)
ME	> 4000
50 % ME	3690
AE	2760
Gallic acid	>2000
β-sitosterol	750
Lupeol	>2000
β-carotene	520

Results and Discussion

HPTLC studies

The leaf extract of *Ns* when subjected to TLC showed the presence of gallic acid, β-sitosterol, lupeol and β-carotene (Figure 1). A comparison of the spectral characteristics of the peak for standard compound and that of the sample further confirmed the identity of gallic acid, β-sitosterol, lupeol and β-carotene present in the sample. The peak area versus concentration plot was found to be linear. The regression equation and correlation coefficient indicated good linearity (Table 1). The quantity of gallic acid, β-sitosterol, lupeol and β-carotene are shown in Table 1. Instrumental precision was checked by repeated scanning of the same spots of standards three times and % RSD values were calculated (Table 2). To determine the precision of the methods, standards were analyzed three times inter-day and intra-day (Table 2). Recovery studies on all four compounds were found to be within limits (Table 3). This TLC procedure may be used effectively for identity, quality evaluation as well as quantitative determination for this plant or its derived products.

Brine shrimp lethality bioassay

The LC_{50} values of the brine shrimp lethality bioassay obtained for extracts/identified compounds have been presented in Table 4. The tested compounds followed the order gallic acid, lupeol>β-sitosterol>β-carotene in lethality to brine shrimps. β-carotene and β-sitosterol with an low LC_{50} can be a potent candidate for anticancer, antimalarial, insecticidal and antifeedent studies. Methanolic extract showed no lethality till 4000 µg/ml, inspite of the presence of β-sitosterol and β-carotene. The reason may be due to respective elimination or neutralization of toxic effects of β-sitosterol and β-carotene by other unidentified constituents in the extract. Aqueous extract showed higher lethality when compared to 50% methanolic extract. Although LC_{50} values < 1000 µg/ml are considered significant for crude extracts, the lethality of the extracts of *Ns* leaves increased with polarity, suggesting the presence of polar toxic compound/s in *N.stellata* leaves.

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