INTERNATIONAL JOURNAL OF PHARMACEUTICAL, CHEMICAL AND BIOLOGICAL SCIENCES

Available online at www.ijpcbs.com

Research Article

QUANTIFICATION OF OLEANOLIC ACID AND BETULINIC ACID BY TLC AND BRINE SHRIMP LETHALITY ASSAY OF *NYMPHAEA STELLATA* WILLD. LEAVES

D. Agilandeswari

Department of Pharmaceutics, JSS College of Pharmacy, Ootacamund, Tamilnadu, India.

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 of medicine with a wide range of pharmacological activities. Since the complete phytochemical profile was unavailable, comparative TLC was used to identify and quantify chemical constituents of *Ns* leaves. Brine shrimp lethality assay is a very useful bench-top method for drug discovery process. Hence methanolic extract, 50% methanolic extract, aqueous extract, unsaponified petroleum ether fraction of methanol extract (UPEFME), chloroform fraction of methanol extract (CFME) and the residual fraction of methanol extract (RFME) were screened along with the first timed identified oleanolic acid and betulinic acid. The amount of oleanolic acid and betulinic acid quantified from the leaves were 0.008186 %w/w and 0.078238 %w/w respectively. Oleanolic acid and aqueous extract showed LC_{50} of 120 and 2760 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 compounds.

Key words: HPTLC, brine shrimp lethality assay, betulinic acid, *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 of chemical constituents and 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 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 of medicine. The leaves, roots and flowers have 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 was unavailable, Co-TLC was used to identify and quantify chemical constituents of *Ns* leaves.

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 the 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, fractions and identified chemical constituents.

MATERIALS AND METHODS

Chemicals and reagents

Pure oleanolic acid and betulinic acid were procured from Himedia Laboratories Pvt. Ltd., Mumbai, 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 (20 X 10 cm) were used for the analysis. Chromatography was performed using pre-activated (60 °C for 5 min) silica gel $60F_{254}$ TLC plates (20 X 10 cm; layer thickness 250 µm). 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. Accurately weighed extracts were dissolved in respective solvents to produce a known concentration. The extracts were separated in suitable mobile phase along with standards. The identified chemical constituents were quantified from the calibration curve of peak area versus concentration of the standards. All quantification was performed 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* multiwavelength 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 oleanolic acid

Accurately weighed 10 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 oleanolic acid (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 oleanolic acid as standard. Sample solution was applied in triplicate on the TLC plate and developed with mobile phase toluene: ethyl acetate: glacial acetic acid (7:3:0.1, 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 ^{o}C for 5-10 min[16]. Densitometric scanning was performed in absorptionreflection mode at 540 nm. Peak areas were recorded and the amount of oleanolic acid was calculated using the calibration curve.

Identification and quantification of betunilic acid

Accurately weighed 2.5 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 betunilic acid (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 betunilic acid as standard. Sample solution was applied in triplicate on the TLC plate and developed with mobile phase toluene: ethyl acetate: glacial acetic acid (7:3:0.03, 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 ^{o}C for 5-10 min[16]. Densitometric scanning was performed in absorption-reflection mode at 527 nm. Peak areas were recorded and the amount of betulinic acid was calculated using the calibration curve.

Preparation of extracts and fractions

Coarsely powdered leaves of *Ns* were extracted with methanol, 50% methanol and chloroform water in soxhlet apparatus until exhaustion; the extract was concentrated *in vacuo* by rotary evaporator and dried in desiccator. Further the methanol extract was successively fractioned with petroleum ether and chloroform. The dried petroleum ether fraction of methanol extract was saponified to obtain the unsaponifiable matter[17]. The unsaponified petroleum ether fraction of methanol extract was designated as UPEFME. The chloroform fraction of methanol extract was designated as CFME and the residue left over after chloroform fractionation was designated as RFME.

Methnolic extract (ME), 50% methanolic extract (50% ME), aqueous extract (AE), UPEFME, CFME, RFME, oleanolic acid and betulinic acid were screened for brine shrimp lethality bioassay.

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 4000 μg/ml for extracts/fractions and 2000 μg/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.

RESULTS AND DISCUSSION

Identification and quantification of oleanolic acid and betulinic acid

The leaf extract of *Ns* when subjected to TLC showed the presence of oleanolic acid and betulinic acid peak (Figure 1, Figure 3). A comparison of the spectral characteristics of the peak for standard compound and that of the sample further confirmed the identity of oleanolic acid and betulinic acid present in the sample. The peak area versus concentration plot was found to be linear in the range of 100-500 ng spot^{-1} for oleanolic acid and betulinic acid (Figure 2, Figure 4). The regression equation and correlation coefficient for oleanolic acid and betulnic acid indicated good linearity (Table 1). The LOD and LOQ of oleanolic acid and betulinic acid were found to be 5.31, 16.08 and 6.16, 18.66 respectively (Table 1). The oleanolic acid and betulinic acid content of the leaves calculated from the area calibration curve by this method was found to be 0.008186 %w/w and 0.078238 %w/w respectively (plant dry weight basis). 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). 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/fractions/identified compounds have been presented in Table 4. The tested compounds followed the order oleanolic acid>betulinic acid in lethality to brine shrimps. UPEFME, CFME, RFME and methanolic extract showed no lethality till 4000 µg/ml. Inspite of oleanolic acid and betulinic acid being present in UPEFME, it showed no lethality, may be due to respective elimination or neutralization of toxic effects of oleanolic and betulinic acid by other unidentified constituents in the fraction. Aqueous extract showed higher lethality when compared to 50% methanolic extract. Although LC_{50} values < 1000 µg/ml are considered significant for crude extracts[18], the lethality of the extracts/fractions of *Ns* leaves increased with polarity.

CONCLUSION

Oleanolic acid with an LC_{50} of 120 µg/ml can be a potent candidate for anticancer, antimalarial, insecticidal and antifeedent studies. As the polarity of the extracts/fraction increased the lethality also increased, suggesting the presence of polar toxic compound/s in *N.stellata* leaves.

Table 1: Linearity regression data for quantification of oleanolic acid and betulinic acid

Parameter	Oleanolic acid	Betulinic acid
R_f	0.53	0.56
Dynamic range (ng spot^{-1})	100-500	100-500
Equation	y=3397.724+7.504x	y=510.618+7.004x
Slope	7.504	7.004
Intercept	3397.724	510.618
Limit of detection (LOD)	5.31 ng	6.16 ng
Limit of quantification (LOQ)	16.08 ng	18.66 ng
Linearity (Correlation coefficient)	0.99613	0.99670
Amount of compound quantified[a]	0.008186 %w/w	0.078238 %w/w

[a] plant dry weight basis

Table 2: Precision and recovery studies data for quantification of oleanolic acid betulinic acid

Precision studies				
TLC Method	Concentration (ng spot^{-1})	Instrumental precision (%RSD)	Method precision (%RSD) Intra-day	Method precision (%RSD) Inter-day
Oleanolic acid	100	0.32	0.51	1.23
	500	0.42	0.49	0.66
Betulinic acid	100	0.47	0.74	0.94
	500	0.57	0.58	0.75
Recovery studies				
	Amount in the sample (µg)	Amount added (µg)	Amount found (µg)	Recovery (%)
Oleanolic acid	8.2	6.5	14.5	98.64
	8.2	8.2	15.9	96.95
	8.2	9.8	17.8	98.89
Betulinic acid	78..2	62.5	139.4	99.07
	78.2	78.2	154.5	98.79
	78.2	93.84	168.1	97.71

Table 3: LC_{50} values of extracts/fractions/ identified compounds of *N. stellata*

Extracts/fractions/identified compounds	LC_{50} values (μg/ml)
ME	> 4000
50 % ME	3690
AE	2760
UPEFME	> 4000
CFME	> 4000
RFME	> 4000
Oleanolic acid	120
Betulinic acid	940

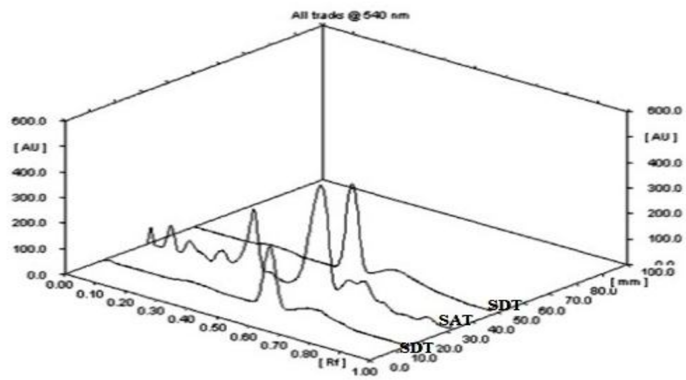

Fig. 1: Ns leaf extract showing identical peak with standard oleanolic acid SAT-Sample tract; SAD-Standard track

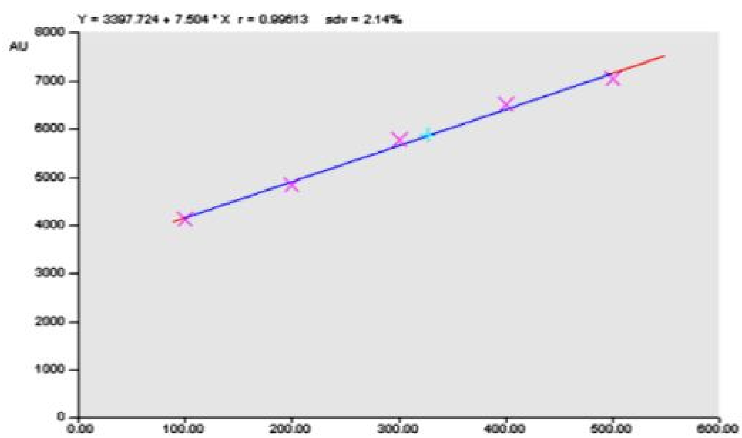

Fig. 2: Calibration curve of peak area versus concentration for oleanolic acid

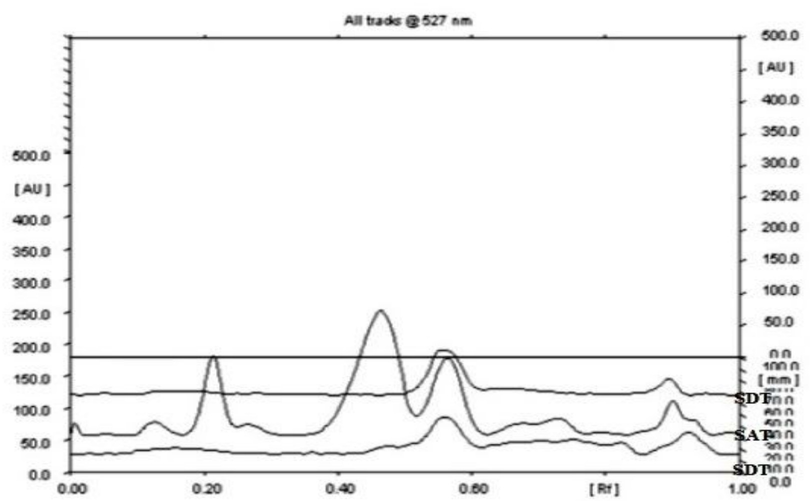

Fig. 3: *Ns* leaf extract showing identical peak with standard betulinic acid SAT-Sample tract; SAD-Standard track

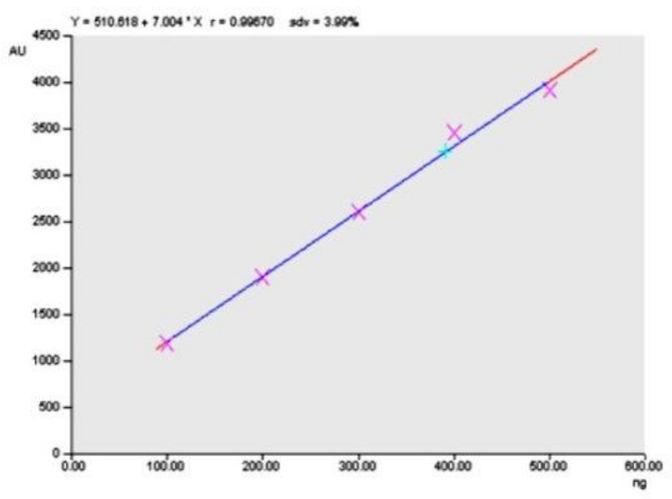

Fig. 4: Calibration curve of peak area versus concentration for betulinic acid

REFERENCES

1. Nadkarni KM. Indian materia medica, vol. I. Popular Prakashan Pvt. Ltd.: Mumbai. 1982: 859.
2. Kirtikar KR and Basu BD. Indian medicinal plants, vol. I. International Book Distributors: Dehradun. 1999: 114.
3. McLaughlin JL, Chang CJ and Smith DL. Bench-top bioassays for the discovery of bioactive natural products: an update. In Studies in Natural Products Chemistry. Edited by Rhaman AU. Elsevier, Amsterdam. 1991.
4. Michael AS, Thompson CG and Abramovitz M. *Artemia salina* as a test organism for a bioassay. Science 1956; 123: 464.
5. Vanhaecke P, Persoone G, Claus C and Sorgeloos P. Proposal for a short-term toxicity test with Artemia nauplii. Ecotoxicol Env Safety 1981; 5: 382-387.
6. Sleet RB and Brendel K. Improved methods for harvesting and counting synchronous populations

of Artemia nauplii for use in developmental toxicology. Ecotoxicol Env Safety 1983; 7: 435-446.

7. Solis PN, Wright CW, Anderson MM, Gupta MP and Phillipson JD. A microwell cytotoxicity assay using *Artemia salina*. Plant Med 1993; 59: 250-252.
8. Carballo JH, Hernandez-Inda ZL, Perez P and Garcia-Gravalos MD. A comparison between two brine shrimp assays to detect in vitro cytotoxicity in marine natural products. BMC Biotechnol 2002; 2: 17.
9. Siqueira MJ. Bomm DM, Pereira NFG, Gareez WS and Boaventura MAD. Estudo fitoquimico de Unonopsis lindmanii- Annonaceae, biomonitorado peloensaio de toxicidade sobre *Artemia salina* LEACH. Quimica Nova 1998; 21: 557-559.
10. Perez H, Diaz F and Medina JD. Chemical investigation and *in vitro* antimalarial activity of *Tabebuia ochracea* ssp. neochrysantha. International Journal of Pharmacog 1997; 35: 227-231.
11. Oberlies NH, Rogers LL, Martin JM and McLaughlin JL. Cytotoxic and insecticidal constituents of the unripe fruit of Persea. American Journal of Natural Products 1998; 61: 781-785.
12. Labbe C, Castillo M and Connoly JD. Mono and sesquiterpenoids from *Satureja gilliesii*. Phytochemistry 1993; 34: 441-444.
13. Anderson JE, Goetz CM, McLaughlin JL and Suffness M. A blind comparison of simple bench-top bioassays and human tumour cell cytotoxicities as antitumor prescreens. Phytochem Anal 1991; 2: 107-11.
14. Meyer BN, Ferrigni NR, Putnam JE, Jacobsen LB, Nichols DE and McLaughlin JL. Brine shrimp: a convenient general bioassay for active plant constituents. Planta Med 1982; 45: 31-34.
15. ICH Guideline Q2R1. 1996/2005. Validation of analytical procedures: Text and methodology. Geneva, Switzerland.
16. Wagner H and Bladt S. Plant Drug Analysis – A Thin Layer Chromatography Atlas. Springer India Pvt Ltd, New Delhi. 1996.
17. Khandelwal KR. Practical Pharmacognosy. Nirali Prakashan, Pune. 2002.
18. Garza BAA, Gonzalez-Gonzalez GM, Salazar-Aranda R, Waksman de Torres N and Rivas-Galindo VM. Screening of antifungal activity of plants from the northeast of Mexico. J Ethnopharmacol 2007; 114: 468-471.