Innovare Academic Sciences

International Journal of Pharmacy and Pharmaceutical Sciences

ISSN- 0975-1491 Vol 6, Issue 5, 2014

Original Article

OPTIMISATION AND VALIDATION OF HPLC-DAD METHOD FOR ANALYSIS OF NEGUNDOSIDE IN THE EXTRACTS AND FORMULATIONS CONTAINING *VITEX NEGUNDO* LINN

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Received: 20 Nov 2014 Revised and Accepted: 28 Jan 2014

ABSTRACT

Objective: The present work describes development and validation of new, simple, sensitive and precise HPLC-DAD method for quantification of biomarker negundoside in leaves extracts of *Vitex negundo* Linn.

Methods: The analysis was performed on Eclipse XBD C18 column (150 mm X 4.6 mm I.D., 5 µm) from Agilent Technologies with gradient elution using acetonitrile and 0.1% trifluoroacetic acid (TFA) at a flow rate of 1 ml/min. The column temperature was set at 35 ^{0}C. The detection was carried out at 254 nm. The validation data shows that the method is specific, sensitive, accurate, precise and robust.

Results: The method was found to be linear, over a range of 1.0-100 µg/ml with detection limit and quantitation limit of 0.33 µg/ml and 1.0 µg/ml, respectively. The evaluation of the accuracy was done using an addition/recovery assay. The recoveries of negundoside were between 95.85 % - 98.54 %. The low values of intra and inter-day RSD (1.98% and 1.47 %, respectively) indicated good precision of the developed method.

Conclusion: The developed method was applied for evaluation of three methanol extracts and marketed Ayurvedic formulations such as tablets, capsules, gel, *Vati* and *Taila* for the content of negundoside. The developed and validated method can be very well used to determine batch to batch variations and routine analysis of formulations containing *Vitex negundo* by herbal manufacturers.

Keywords: Negundoside, Irridoid glycosides, Nirgundi, *Vitex negundo*.

INTRODUCTION

Vitex negundo Linn belonging to family Verbenaceae, is a large aromatic shrub distributed throughout India [1]. The plant has been used in various traditional systems of medicine and reported to have variety of biological activities. The plant has got potent anti-noceceptive, anti-inflammatory and antipyretic activities [2-5]. Negundoside and agnuside are the two main irridoid glycosides which have been identified from this plant. Negundoside also known as 2'-p-hydroxy benzoylmussaenosidic acid is chemically (1S,4aS,7S,7aS)-1-[(2R,3S,4R,5R,6S)-4,5-dihydroxy-3-(4-hydroxy benzoyl)oxy-6-hydroxy methyl)oxan-2-yl]oxy-7-hydroxy-7-methyl-4a,5,6,7a-tetrahydro-1H cyclopenta[c]pyran-4-carboxylic acid (Fig.1).

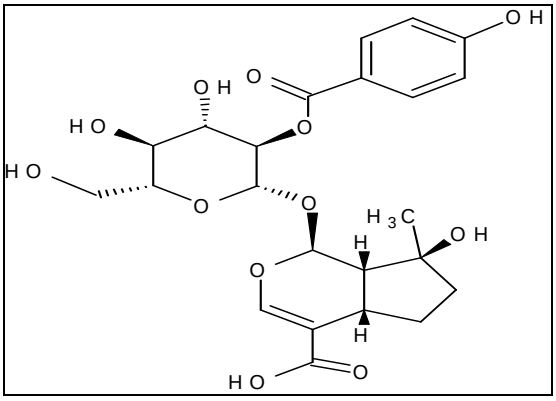

Fig. 1: Structure of negundoside

Negundoside is a bioactive compound having hepatoprotective activity [6, 8]. Many methods have been reported for isolation of negundoside from leaves extract of *Vitex negundo* [7-9].

No method has been reported for validation of HPLC method for negundoside in leaves extract of *Vitex negundo*. Thus, the present work has been aimed to develop and validate an HPLC method for the quantitative determination of negundoside in the methanol extract of leaves of *Vitex negundo* for succeeding application in standardization of extracts and formulations containing *Vitex negundo.*

MATERIALS AND METHODS

Reagents and standards

All the solvents and purified water were of HPLC grade from S. D. Fine chemicals, Mumbai, India. All the solutions were filtered through 0.2 µ PTFE filter (Goettingen, Germany). Standard of negundoside (Purity 95.0 %w/w) was procured from Natural Remedies Pvt. Ltd., Bangalore, India.

Plant materials

Different samples of leaves of *Vitex negundo* were obtained from three geographical sources of India, Tirunelvelli district of Tamilnadu, campus of C. U. Shah College of Pharmacy, Mumbai, Maharashtra and Valsad district of Gujarat in November 2011. Leaves samples were authenticated at Botanical Survey of India, Pune, India under voucher specimen names VNTSOD13, VNMSOD14 and VNGSOD15, respectively. The leaves were dried in oven at 50 ^{0}C for a week, pulverized, sieved and used for further study.

Preparation of methanol extract

Methanol extracts of leaves obtained from three geographical sources were prepared by macerating 10 g of powdered raw material with 100 ml of methanol for 24 h and then filtered. To ensure complete extraction, the process was repeated thrice and all the filtrates were pooled together. The combined methanol extracts were concentrated under vacuum using rotary evaporator (Buchi, Switzerland), and kept in an amber container until used.

HPLC analysis

The RP-HPLC analysis was performed on Dionex-Ultimate 3000 HPLC system (Germany), equipped with auto-sampler, quaternary pumps, column oven and diode array detector (DAD). The injection volume was 20 µl. Chromatographic separation was achieved using Eclipse XBD column (150 mm X 4.6 mm I. D., 5 µm) (Agilent Technologies Ltd). Chromeleon software (version 6.80) was used to evaluate all data. Initially, various proportions of methanol–water or acetonitrile–water were tried as a mobile phase in both isocratic and gradient modes but satisfactory separation was not achieved. In progression, gradient elution with methanol, acetonitrile and 0.1 % TFA in different ratios were tried. Eventually, it was found that the mobile phase comprising of solvent A: 0.1% TFA and solvent B: acetonitrile in gradient mode gave optimum separation of components than any other mobile phase used. The gradient used was as follows:

Time/% A: 0/90, 15/40, 20/10, 21/90, 25/90

Detection was carried out at 254 nm and the column oven was equilibrated at 35 ^{0}C. The total run time for both standard and test was 25 min. Six individual injections of standard solution were performed prior to all measurements to evaluate the suitability parameters, including resolution, theoretical plates, asymmetry and repeatability of the peak area.

Preparation of standard solution

Standard solution of negundoside was prepared by dissolving 10 mg of negundoside in 100 ml of methanol by sonication for 10 min. From this, different aliquots were taken and diluted with suitable volume of methanol to produce different concentrations for further study. Prepared solutions were filtered through a 0.2 µ PTFE filter.

Preparation of sample solution

About 0.1 g methanol extract was dissolved in 10 ml of methanol, sonicated for 10 min and filtered through a 0.2 µ PTFE filter.

Sample preparation for analysis of formulations

Tablet (Brand I): Twenty tablets were weighed individually; their average weight was determined and the tablets were triturated. Accurately weighed 10 g of tablet triturate was transferred to 250 ml volumetric flask containing 100 ml of methanol and stirred at ambient temperature for 24 h. The solution was filtered and the solvent was evaporated to obtain dry residue. Methanol extract thus obtained (20 mg) was dissolved in 5 ml of methanol, sonicated for 10 min, filtered and injected in HPLC.

Tablet (Brand II): Twenty tablets were weighed individually; their average weight was determined and the tablets were triturated. Accurately weighed 10 g of tablet triturate was transferred to 250 ml volumetric flask containing 100 ml of methanol and stirred at ambient temperature for 24 h. The solution was filtered and the solvent was evaporated to obtain dry residue. Methanol extract thus obtained (20 mg) was dissolved in 5 ml of methanol, sonicated for 10 min, filtered and injected in HPLC.

Tablet (Brand III): Twenty tablets were weighed individually; their average weight was determined and the tablets were triturated. Accurately weighed 10 g of tablet triturate was transferred to 250 ml volumetric flask containing 100 ml of methanol and stirred at ambient temperature for 24 h. The solution was filtered and the solvent was evaporated to obtain dry residue. Methanol extract thus obtained (20 mg) was dissolved in 5 ml of methanol, sonicated for 10 min, filtered and injected in HPLC.

Capsule: Twenty capsules were weighed individually, their average weight was determined. Accurately weighed 10 g of capsule content was transferred to 250 ml volumetric flask containing 100 ml of methanol and stirred at ambient temperature for 24 h. The solution was filtered and the solvent was evaporated to obtain dry residue. Methanol extract thus obtained (20 mg) was dissolved in 5 ml of methanol, sonicated for 10 min, filtered and injected in HPLC.

Vati: Twenty *Vatis* were weighed individually, their average weight was determined and triturated. Accurately weighed 10 g of *vati* triturate was transferred to 250 ml volumetric flask containing 100 ml of methanol and stirred at ambient temperature for 24 h. The solution was filtered and the solvent was evaporated to obtain dry residue. Methanol extract thus obtained (20 mg) was dissolved in 5 ml of methanol, sonicated for 10 min, filtered and injected in HPLC.

Gel: Accurately weighed 10 g of gel was transferred to 250 ml volumetric flask containing 100 ml of methanol and stirred at ambient temperature for 24 h. The solution was filtered and the solvent was evaporated to obtain dry residue. Methanol extract thus obtained (20 mg) was dissolved in 5 ml of methanol, sonicated for 10 min, filtered and injected in HPLC.

***Taila*:** Accurately measured 10 ml of *Taila* was extracted with methanol (3 X 50 ml). Combined methanol extracts were evaporated to obtain dry residue. 200 mg of dried methanol extract was dissolved in 5 ml of methanol, sonicated for 10 min, filtered and injected in HPLC.

Method validation [10]

The developed method was validated for various parameters such as linearity, LOD, LOQ, accuracy, precision, robustness and system suitability as per ICH guidelines. Linear relationship between concentration and area was evaluated between 1.0-100 µg/ml. Duplicate injections using 20 µl loop were made and detection was carried out at 254 nm. Average area for each concentration was calculated and graph of area versus concentration was plotted. The correlation coefficient (r^2) was determined. The LOD and LOQ were calculated based on signal to noise ratio method. The accuracy studies were carried out by spiking known amount of standard solutions to the sample at three different concentration levels (80%, 100% and 120%). Repeatability (intra-day) and intermediate precision (interday) were studied through triplicate injections of methanol extract at three levels that is 80 %, 100 % and 120 % of test concentrations and the % relative standard deviation (% RSD) was determined. The robustness of the method was evaluated by deliberately varying the mobile phase flow rate (0.9, 1.0 and 1.1 ml/ min), oven temperature (34, 35 and 36 ^{0}C) and detection wavelength (253, 254 and 255 nm) and its effect on retention time, area, and negundoside content in the sample (methanol extract) was evaluated.

The developed validated method was applied for quality control of three different extracts and marketed Ayurvedic formulations containing *Vitex negundo.* The three extracts were prepared from leaves obtained from three different geographical regions of India to identify content variation of negundoside. Different codes were given to the different extracts as given below:

Extract-I: Prepared from leaves obtained from Mumbai, Maharashtra, India

Extract-II: Prepared from leaves obtained from Valsad district of Gujarat, India

Extract-III: Prepared from leaves obtained from Tirunelvelli district of Tamilnadu, India

RESULTS AND DISCUSSION

In this work, new, sensitive and rapid HPLC-DAD method was developed and validated for quantitative estimation of negundoside in *Vitex negundo*. Acceptable separation of negundoside from other components present in methanol extract was achieved (Fig. 2 and fig. 3). Negundoside eluted at mean retention time of 4.853 min. Detection wavelength 254 nm was selected based on UV spectrum of the compound. The calibration curve for negundoside was found to be linear over the proposed range 1.0-100.0 µg/ml with linear regression coefficient (r^2) of 0.9990 for the compound studied, demonstrating an acceptable data fit to the regression line, (y = 0.3243x+ 0.3276). The method was found be sensitive as it could detect (LOD=0.33 µg/ml) and quantitate (LOQ = 1.0 µg/ml) low amount of the compound.

The accuracy of the standard in the spiked sample was evaluated at low, medium and high levels (80%, 100% and 120% respectively) with mean recoveries of 95.85 %, 98.27 % and 98.54 % respectively (Table 1).

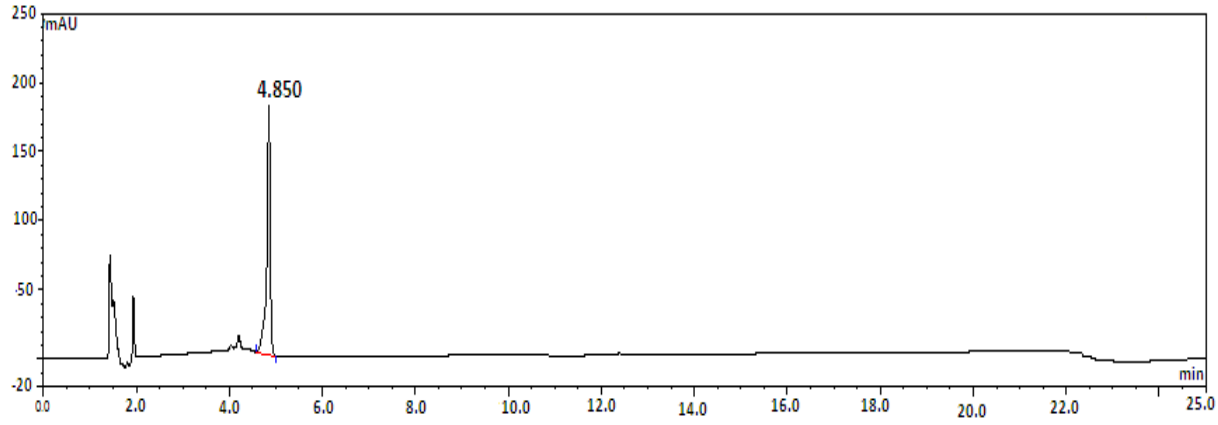

Fig. 2: HPLC chromatogram of standard negundoside

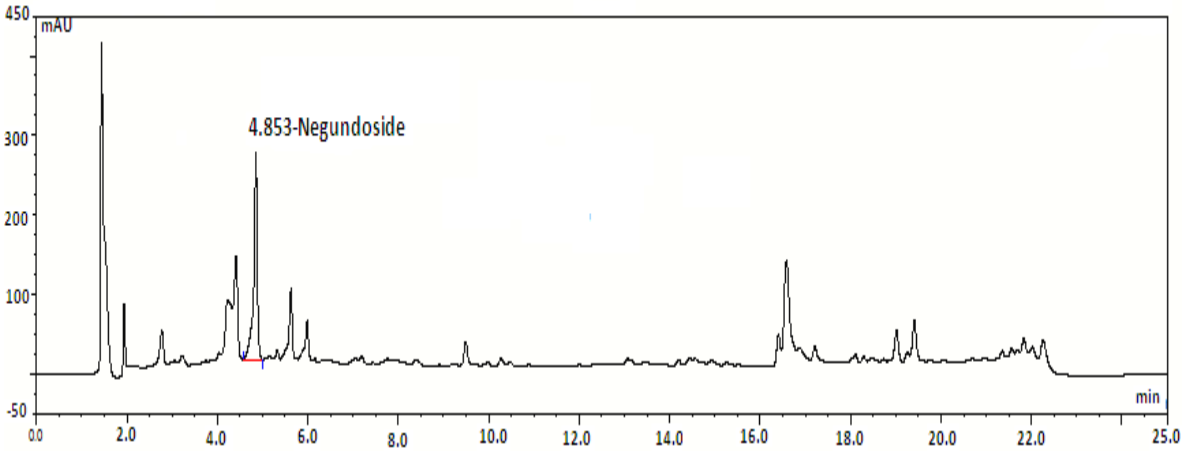

Fig. 3: HPLC chromatogram of methanol extract of *Vitex negundo*

Table 1: Percentage of recovery, to evaluate the accuracy of the method

Component	[a]Amount Present (µg/ml)±S.D.	[a]Amount Found (µg/ml)±S.D.	% Recovery± S.D.
Negundoside	42.1829±0.46	40.4331±0.22	95.8590±1.11
	49.5951±1.04	48.7204 ±0.95	98.2720± 3.15
	61.2915±1.79	60.3675±0.50	98.5404 ± 2.53

[a] n=3, triplicate injections

Table 2: Validation results for intra- and inter-day precision

Component	Amount level (g/10 ml)	Intra day (% RSD)	Inter day (% RSD)	
		Day 1	Day 1	Day 2
Negundoside	0.08	1.9829	0.6661	0.2053
	0.10	0.5687	0.3631	0.3076
	0.12	0.4906	0.2930	1.4754

[a] n=3, triplicate injections

The values of % RSD at all three levels of concentrations for both intraday and interday precision studies indicating that the method was precise (Table 2). The robustness of the developed method was checked by making small but deliberate changes in the method parameters. The low % RSD (< 2.0 %) was obtained for method variables such as detection wavelength, flow rate and column oven temperature indicates sufficient robustness of the method (Table 3). The present method was found to be robust for the content of negundoside present in the methanol extract. The developed method was also found to be suitable for analysis of negundoside with resolution greater than 2.0, peak symmetry 0.72, theoretical plates greater than 28,000 and % RSD less than 2.0.

The developed method was applied for quantitative determination of negundoside in three different extracts and marketed Ayurvedic formulations (Table 4).

Table 3: Chromatographic parameters from robustness studies

Parameters	Retention time (Rt)	Area	% RSD	% w/w of negundoside
Detection wavelength(nm)				
253	4.852	15.7307	0.3814	0.7270
254	4.852	15.7537	0.3826	0.7273
255	4.852	15.7490	0.3790	0.7275
Flow rate (ml/min)				
0.9	5.236	18.9565	0.6340	0.7132
1.0	4.852	15.7537	0.3826	0.7273
1.1	4.551	14.0956	0.4838	0.7166
Column oven temperature (^{0}C)				
34	4.891	16.0600	0.5379	0.7150
35	4.852	15.7537	0.3826	0.7273
36	4.836	16.2982	1.1620	0.7154

Table 4: Results of quantitative determination of negundoside in extracts and formulations

Sr. No.	Extracts /Formulations	% w/w of negundoside [a] ± S.D.
1	Extract –I	0.6793± 0.00
2	Extract –II	0.4557±0.01
3	Extract –III	3.7111±0.34
4	Tablet (Brand I)	0.6560±0.00
5	Tablet (Brand II)	0.6838±0.00
6	Tablet (Brand III)	nd[b]
7	Capsule	0.3893±0.10
8	*Vati*	0.3808±0.00
9	Gel	0.1038±0.00
10	*Taila*	0.0171±0.00

[a] n=3, triplicate injections, [b]nd=Not detected

Thus, the developed method can be utilized to find out the difference in the content of negundoside in the different extracts of different geographical origin. The amount of negundoside in extract-III was significantly higher than the other two extracts. In the analysis of formulations, tablet brand -III which claimed to contain *Vitex negundo* extract did not show peak corresponding to negundoside. Thus, this method has justified its use in the quality control of herbal medicines prepared from extracts of *Vitex negundo*.

CONCLUSION

A simple, sensitive, selective, precise and accurate HPLC-DAD method has been developed for quantifying negundoside in *Vitex negundo* extracts, and can be useful for quality control of herbal medicines prepared from extracts of *Vitex negundo*.

ACKNOWLEDGEMENT

This work was financially supported by National Medicinal Plants Board (NMPB), Dept. of AYUSH, New Delhi, Government of India (Grant no. F. No. Z.18017/187/CSS/R&D/MH-02/2009-10-NMPB).

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