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Original Research Article

Extraction Efficiency of Agnuside from *Vitex Negundo* Leaves Using Different Techniques and its Quantitative Determination by HPLC

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ABSTRACT

Objective: To investigate an efficient extraction technology and HPLC method for quantification of agnuside from *Vitex negundo* methanolic extract.
Methodology: Different extraction methods have been compared that includes reflux, ultrasonication, maceration and microwave extraction techniques which were evaluated for their efficiencies. For reliable quantification of agnuside, HPLC was done using solvent system, acetonitrile and water containing 0.5% OPA at wavelength 254nm and flow rate of 1ml/minute. Based on HPLC analysis the best extracting method was selected. The developed method was applied for evaluation of extracts as well as formulations like tablets and capsules.
Results: Among the four conventional methods microwave assisted technique, showed highest extraction efficiency for agnuside hence it was further evaluated. The calibration plot of standard agnuside showed a linear relationship in the concentration range of 20-100 µg/mL with a correlation coefficient, r^2 of 0.999.
Conclusion: The maximum yield for agnuside was obtained by microwave assisted extraction technique. The HPLC method was found to be linear having high precision, accuracy and good recovery of the compound.

Key Words: Agnuside, HPLC, Microwave, Reflux, Validation, *Vitex.*

1. INTRODUCTION

The genus *Vitex* (family Verbenaceae) has 80 genera and about 800 species. *V.negundo, V. trifolia, V.glabrata, V.rotundifolia V.cymosa. V. agnus* are some of the most common and important species of *Vitex*. [1] *Vitex negundo* Linn., is a shrub which is quite abundant in India with major applications in folk and traditional medicine. [2] The leaf extract of *V. negundo* has been reported to reveal a wide range of biological actions including mosquito-repellent activity, anti-angiogenic, hepatoprotective, analgesic, anti-inflammatory, anti-arthritic, [3-5] antimicrobial, antihistaminic, CNS depressant, anti-filarial activities etc. These actions may be due to the various phytoconstituents present in the plant, which include iridoids, flavonoids, [6] polyphenolic compounds, alkaloids, terpenoids [7] etc. Owing to these various phytochemicals this plant has a crucial role in phytomedicine. The decoction of various parts of this plant part is used against toothache, ulcers, sinusitis, gonorrhoea, bronchitis, eye disease, leucoderma etc. [8]

Iridoids are cyclopentane monoterpenoids produced as secondary metabolites in plants and have many therapeutic effects. [9-11] *Vitex* species along with other chemical constituents shows the presence of numerous iridoids like agnuside, negundoside, nishindaside, aucubin that are

responsible for the different pharmacological activities. Among these iridoids, agnuside is an important chemotaxonomic marker that can be used in standardization of *Vitex* extract and formulations containing it. Agnuside is also called Buddhlejoside found in *Vitex* and *Rhinanthus* species. It is an ester of aucubin and p-hydroxy benzoic acid. It reveals pharmacological actions like anti-arthritic, anti-inflammatory [12] and in pre-menstrual syndrome [13] etc. This study envisages the extraction efficiency of the iridoid agnuside from the plant using various extraction technologies like maceration, reflux, microwave and ultrasonication. [14] Also a HPLC method was used with a few modifications of the reported method [15] and validated for quantitative evaluation of agnuside from plant extract as well as its formulation.

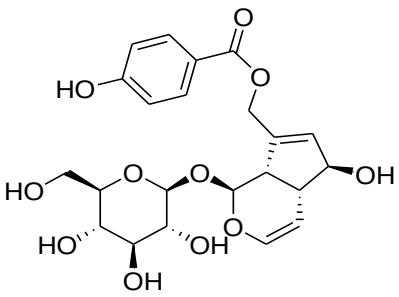

Fig.1. Structure of Agnuside

2. MATERIALS AND METHODS

2.1 Plant Material

The leaves of *V. negundo* were collected from Gadhinglaj, Kolhapur, India and identified. A voucher specimen (ICT/MNPRL/VN/02) was deposited at Medicinal Natural Product Research Laboratory, Department of Pharmaceutical Sciences and Technology, Institute of Chemical Technology, Mumbai.

2.2 Chemicals

Agnuside reference standard was isolated with purity about 95%. All chemicals and solvents used for extraction and isolation were of laboratory grade. Solvents used for HPLC analysis were of analytical grade obtained from Merck, India.

2.3 Instrumentation

The MAE was performed using a digitally-controlled microwave extractor (CATA 2R) manufactured by Catalyst Systems (Pune, India) equipped with a magnetron of 2450MHz with a maximum power of 700W (100%), a reflux unit, 10 power levels 140W (20%) to 700W (100%)), time controller, temperature sensor, exhaust system, beam reflector and a stirring device. UV spectrum was recorded on Jasco V-530 spectrophotometer. IR spectra were recorded on Shimadzu instrument., HPLC analysis was performed with a Jasco (Hachioji, Tokyo, Japan) system consisting of an intelligent pump (PU-1580, PU-2080), a high-pressure mixer (MX-2080-31), a manual sample injection valve (Rheodyne 7725i) equipped with a 20-µL loop, and a UV–visible detector (UV-1575). Compounds were separated on a 250 mm × 4.6 mm i.d, 5-µm particle, Hibar LiChrocart Purospher Star RP-18 endcapped column (Merck, Darmstadt, Germany). Mass spectrum was recorded on Micromass Q-TOF MS Mass spectrometer.^{1}HNMR spectra was recorded on JOEL 400-MHz instrument with an internal standard of Tetramethylsilane (TMS).

3. Experimental

3.1. Extraction and isolation

The leaves of *V. negundo* were properly cleansed to remove any adhering foreign matter and then washed with water. The leaves were air-dried under shade and powdered with the help of a domestic grinder. The ground leaves were then used for extraction of agnuside. Powdered leaves (1kg) was extracted with methanol (5L) & concentrated and dried. Dried methanolic extract (350g) was suspended in water (500ml). Flavonoids and tannins were precipitated with lead acetate and the aqueous fraction was partitioned with ethyl acetate. The ethyl acetate layer was concentrated and precipitated with Petroleum ether to give crude agnuside (10.20g). This was subjected to column chromatography using chloroform and

methanol solvent mixture. Pure agnuside was first isolated using chloroform: methanol ratio of 100:8. TLC and HPLC studies were then carried to determine the purity of the isolated compounds and structure was elucidated and confirmed by UV, IR, MS and 1HNMR spectral analysis. Extraction was carried out by four methods viz. maceration, reflux, microwave and ultrasound assisted extraction (UAE) using methanol as a solvent. The scheme of extraction by all four methods is briefly described below.

Extraction using maceration

5g of leaf powder was soaked in 50 ml methanol (solvent: drug ratio =10:1 (mL/g)) for 24 h at room temperature. The mixture was then filtered and the filtrate was evaporated to obtain the residue. The residue was weighed and appropriate dilutions were made for quantitative estimation of agnuside in the extract.

Extraction using reflux

Hot solvent extraction was done using a reflux apparatus with 5g of leaf powder in 50 ml methanol for 1 h at 50 °C, solvent: drug ratio of 10:1 (mL/g). Further processing of the extracts was done in the same manner as discussed above in the case of maceration.

Extraction using ultra-sonication (UAE)

Ultrasound assisted extraction was done for 20 min using 1g of leaf powder soaked in 10 ml methanol with solvent: drug ratio of 10:1 (mL/g) at ambient temperature in a sonicator (Spectra lab). UAE was carried out at various extraction times 10,20,30,40 and, 50 min. Further processing of the extracts was done in the similar manner discussed above

Microwave using extraction (MAE)

Microwave assisted extraction (MAE) was done at 350 W for 10 min with solvent: drug ratio of 10:1 (mL/g). MAE was carried out at various extraction times 1, 5, 10,20 and 30 min. Further processing of the extracts was done in the similar manner discussed above.

Soxhlet extraction for formulation

Tablets were crushed to powder form and 5g was weighed. Continuous hot solvent extraction of the powder was done using a soxhlet apparatus for 2 h at 50 °C using methanol and solvent: drug ratio of 10:1 (mL/g). Further processing of the extracts was done in the same manner as described earlier.

3.2. Analysis of Agnuside by HPLC

Agnuside standard solution preparation

Stock solution of agnuside was prepared in HPLC grade methanol at a concentration of 1 mg/ml. Then working solutions of 20,40,60,80,100 µg/ml were prepared in HPLC grade methanol. Calibration plot was then made for concentration (µg/mL) versus peak area. The linear equation from the standard plot was used to determine concentration of agnuside in test samples.

Sample solution preparation

10 mg of leaf extract obtained by different extraction methods was dissolved in HPLC grade methanol and volume was made up to 10 ml to obtain final concentration of 1 mg/mL. The solutions were then filtered and the resulting solution was subjected to HPLC analysis. Final concentration of agnuside in the extracts was calculated by using a linear equation for the calibration curve.

Chromatographic conditions

Determination of agnuside was carried out with the mobile phase comprising of acetonitrile: water with 0.5% OPA (18:82) at 254 nm with a flow rate of 1.0 mL/min. The optimum separation in HPLC was achieved at 30°C and absorbance was measured at 254 nm. Standard and sample solutions were filtered before injection.

4. RESULTS

4.1. Extraction of Agnuside by Various Extraction Techniques

The results of agnuside content obtained by various extraction techniques have been presented in Table 1. The expected yields obtained varied with

different extraction techniques. The maximum and minimum yield for agnuside was obtained by microwave assisted extraction technique and reflux technique respectively. Quantification of agnuside in different extracts of *V. negundo* leaves through HPLC revealed that MAE is the best method for extraction of agnuside (Table 1). Hence to optimize extraction different durations were studied. Highest yield was obtained at 20 min time duration with gradual increase in yield from 1 to 20 min and a sharp decrease in agnuside yield at 30 min (Fig.2).

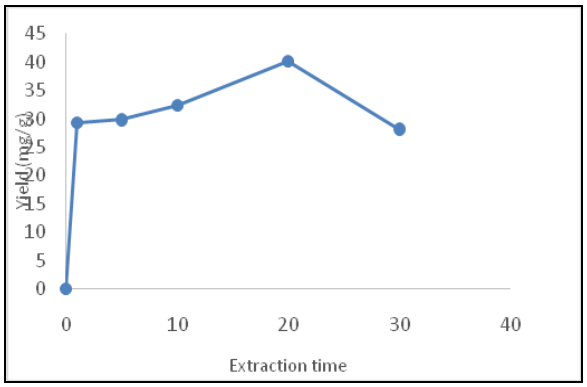

Fig. 2. Effect of Microwave extraction time.

UAE method was the second best method and hence studied under the different ultrasonic time (10, 20, 30, 40, 50 min) with the ratio of solid/liquid at 1:10 (g/mL) at ambient temperature. The results showed the extraction yield has a significant increase from 0 to 30 min (Fig.3). However, the extraction yield showed no change between 30 and 40 min suggesting the extraction efficiency has maximized. The extraction yield decreased after 40 minutes.

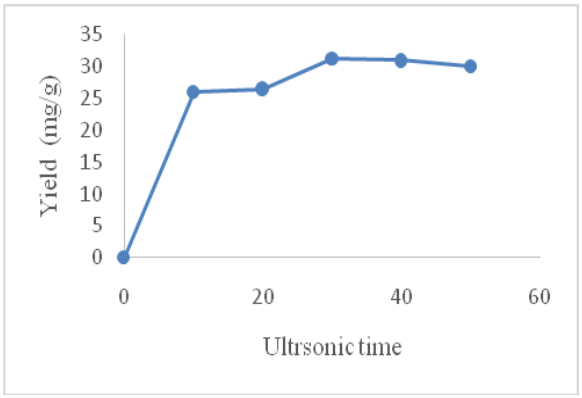

Fig. 3. Effect of ultrasonic time.

Table 1. Amount of Agnuside quantified by different extraction methods.

Extraction Technique	Amount of Agnuside quantified (mg/g)
Microwave	40.10
Ultrasonication	31.31
Maceration	20.90
Reflux	19.20

Table 2. Amount of Agnuside in tablet formulations

Formulation	Amount of Agnuside quantified (mg/g)
Formulation 1	0.012
Formulation 2	0.82

4.2. Quantitative estimation of agnuside by HPLC and method validation

The standard solutions of agnuside and sample extracts were subjected to HPLC technique using acetonitrile and 0.5% aqueous OPA (18:82 v/v) as mobile phase, in isocratic mode (1 mL/min). Calibration curve of agnuside was made with five dilutions of standard solution at a concentration range from 20 to 100 µg/mL. The regression equation and correlation coefficient were as follows: y=17581x-34979; r^2=0.9994. The relative quantity of agnuside in the prepared extracted solutions was calculated from the above equation and the results are shown in Table 1.

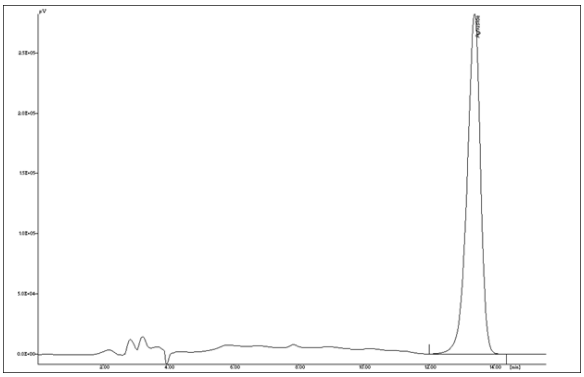

Fig. 4. HPLC chromatogram of isolated Agnuside

To ensure the accurate assessment of agnuside in *Vitex* extracts, the HPLC method was validated as per the ICH guidelines on the validation of analytical methods. [16] The LOD and LOQ was found to be 2.085 µg/mL and 6.318 µg/mL respectively, which indicated high sensitivity under the applied HPLC conditions. Calibration curves were linear over a concentration range: 20-100 µg/ml.

RSD values for repeatability of intraday and interday precision studies were less than 2.0%. Recovery studies were carried out to check the accuracy of the developed HPLC method. Three different quantities (low, medium and high) of the standards were spiked into blank samples.

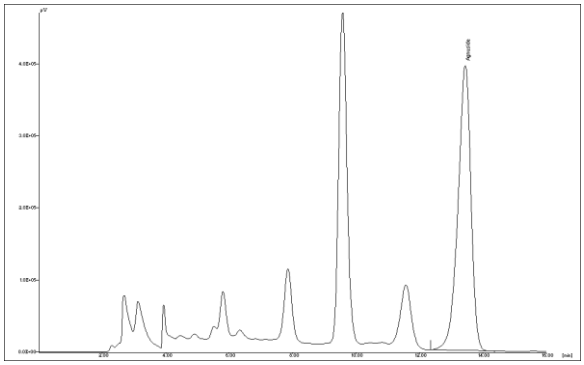

Fig.5. HPLC chromatogram of methanolic extract of *V.negundo*

5. DISCUSSION

There are many conventional and new extraction techniques for isolation of phytoconstituents. Selection of suitable extraction technique plays a phenomenal role to get good yield and to scale up at industrial level. The extraction process also has an effect on the biological activities of the phyto extracts. Agnuside is an important marker iridoid compound of *Vitex* species known to possess some very important therapeutic benefits. Therefore, its content in the plant needs to be investigated. HPLC is a very common method for qualitative as well as quantitative analysis of phyto compounds giving rapid separation of compounds with high accuracy. The agnuside yield varies with the extraction techniques but the maximum yield was obtained with the microwave extraction technique. The differences in the yield of agnuside could be due to the differences in the solubility and availability of extractable components by different extraction techniques. It can be inferred that microwave assisted extraction method is a better choice for extraction of agnuside from *V. negundo* leaves as compared to other conventional methods such as soxhlet, maceration and reflux. Solvent extraction at room temperature was carried out in order to compare MAE with a traditional extraction method (Table 2). We suggest that a relative decrease in MAE recoveries versus conventional method could be due to the slight thermal degradation of agnuside at high temperature. However, it is emphasized that agnuside degradation could be minimized by the use of a short extraction time (less than 15 min) even if the temperature was as high as150 ^{o}C. The main advantages of the use of MAE are the considerable reduction of time and solvent consumption when compared to conventional extraction. In terms of selectivity, MAE using aqueous methanol as solvent yields extracts that could be directly analyzed by HPLC without the need for further preliminary clean-up steps. MAE technique was studied at different extraction time to explore the maximum time required to extract agnuside from the plant material. From this study we discerned that maximum agnuside yield was obtained (40.10 mg per g) in a very short period of time (20 min) using insignificant amount of solvent (10ml).

Agnuside in *Vitex* extracts was estimated quantitatively using HPLC method. The HPLC method was validated as per the ICH guidelines on the validation of analytical methods. The validation results of the intraday and interday precision, average recovery, LOD and LOQ are shown in Table 2. This method was found to be precise, as the RSD values for repeatability of intraday and inter day precision studies were less than 2.0%, which is as per the ICH guidelines. These results established that this method was reproducible with good accuracy. Recovery studies were carried out to check the accuracy of the HPLC method. Three different quantities (low, medium and high) of the standards were spiked into blank samples.

UAE technique also showed a comparable extraction yield hence was further studied to know the best sonication time required for higher extraction efficiency. HPLC analysis showed that

agnuside content was obtained highest at 30 minutes and the yield decreased after 40 minutes which may be due to degradation occurring due to long term ultrasonic radiations. It is reported that ultrasound can rupture plant cells by induction of acoustic cavitation and promote solvent penetration into the cells. [17] Extension of extraction time increases the yield until certain level and then causes a drop in the yield. This may be due to the rupture of plant cells, releasing all the cell contents in extraction solvent, thus causing decrease in solvent permeability. Also, there is a possibility of desired phytocompounds getting adsorbed on the ruptured plant particles causing a lessening in yields. [18] Therefore, it is unfavorable to continue extraction when the maximum extraction yield has been achieved.

Alike MAE, UAE method also has similar advantages like speed, efficiency and less solvent usage but, ultrasound device is economically cheaper, and its operation is much easier in comparison to MAE. In comparison to other traditional extraction techniques like maceration, reflux, soxhlet extraction above two methods are very efficient and can be done conveniently for analytical and industrial scale. The results indicated that the time factor selected in this study had a significant function on the extraction of agnuside from *V. negundo*. The outcome of this study will surely help the researchers in selecting the appropriate extraction technique for the isolation of agnuside from the leaves extract of *V. negundo* that is microwave assisted extraction method. Also, agnuside can be used as a biomarker in standardization and quality control of leaves extract of this drug as well as its formulations in herbal industries as this HPLC method is simple, selective, sensitive and quick.

Conflict of interest statement: We declare that we have no conflict of interest.

6. ACKNOWLEDGEMENT

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