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Comparative assessment of extraction methods and quantitative estimation of luteolin in the leaves of *Vitex negundo* Linn. by HPLCLubna Abidin<sup>1</sup>, Mohd Mujeeb<sup>1\*</sup>, Showkat Rasool Mir<sup>1</sup>, Shah Alam Khan<sup>2</sup>, Aftab Ahmad<sup>3</sup><sup>1</sup>Department of Pharmacognosy & Phytochemistry, Faculty of Pharmacy, Jamia Hamdard, New Delhi-110062, India<sup>2</sup>Department of Pharmacy, Oman Medical College, Muscat, Oman<sup>3</sup>Health Information Technology Department, Jeddah Community College, King Abdulaziz University, Jeddah, Kingdom of Saudi Arabia

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## ABSTRACT

**Objective:** To find out the ideal organic solvent and extraction technique for the isolation of luteolin from the leaves of *Vitex negundo* Linn. (*V. negundo*) by quantitative estimation of luteolin through high performance liquid chromatography (HPLC) method.**Methods:** The leaves of *V. negundo* were identified by a botanist, cleaned, dried under shade and powdered. Maceration, reflux, Soxhlet and ultrasound assisted extraction techniques were used for the extraction of luteolin from the leaves by using four different solvents of varying polarity such as methanol, ethanol, chloroform, and dichloromethane. A simple HPLC method was used to determine the quantity of luteolin in each sample extract.**Results:** The calibration plot of standard luteolin showed a linear relationship in the concentration range of 100–500 µg/mL with a correlation coefficient,  $r^2$  of 0.998. The methanolic extract was found to contain highest amount of luteolin and among various techniques employed for extraction and isolation of luteolin, reflux technique was observed to be the most efficient.**Conclusion:** Based on the HPLC results, it can be concluded that reflux technique using methanol is better than the other extraction techniques and should be preferred for the extraction and isolation of luteolin from *V. negundo* leaves extract in research labs or industries.

## 1. Introduction

The genus *Vitex* consists of about 250 species including trees and shrubs, which are mainly found in tropical and subtropical regions. About 14 species are found in India that have been reported to possess specific medicinal and commercial properties[1,2]. Plant *Vitex negundo* L. (*V. negundo*) is popularly known as nirgundi and belongs to family Verbenaceae. In Sanskrit language, the word “nirgundi” word is used for plant or any substance that protects the body from the disease(s). The usefulness of the

plant has been enumerated in De Materia Medica as well as in the fundamental texts of Ayurveda (Indian traditional medicine), Charaka Samhita. The plant is mainly known for its utility in female reproductive disorders like premenstrual syndrome[3], menopause[4], hyperprolactinemia[3,5], etc.

Plethora of therapeutic activities are possessed by *V. negundo* owing to phytoconstituents present in it, namely friedelin, carotene, casticin, artemisin, luteolin, vitexin, nigunduside, vitexicarpin, linalool, stearic acid, behenic acid, negundin, agnuside, vanillic acid, p-hydrobenzoic acid, vitexoside, stigmasterol, sabinene, gardenin, ursolic acid, betunilic acid, spathulenol, p-cymene, globulol, virdifloral, hentriacontane, lagundinin, vitedoamine, nishandaside and so on[6]. *V. negundo* is an extensively studied medicinal plant and is known to possess an extensive range of pharmacological activities like

\*Corresponding author: Dr. Mohd Mujeeb, Department of Pharmacognosy & Phytochemistry, Faculty of Pharmacy, Jamia Hamdard, New Delhi-110062, India.

Tel: +91-9212050090

E-mail: mmujeebzaidi@gmail.com; mohdmujeeb72@gmail.com

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analgesic and anti-inflammatory[7–9], hepatoprotective[10], antidiabetic[11], anticonvulsant[7,12], antioxidant[13,14], etc. In Indian traditional system of medicine (Ayurveda), *V. negundo* is used as an antihelmintic and for treating skin disorders. The Chinese Pharmacopoeia prescribes the fruit of *V. negundo* in the treatment of reddened, painful, and puffy eyes; headache and arthritic joints[15]. There is a big list of ailments for which *V. negundo* is used as a remedy in Ayurvedic, Unani, Sidhha and Chinese system of medicines[16,17].

Luteolin is an important constituent of *V. negundo* L. Chemically it is 3',4',5,7-tetrahydroxyflavone (Figure 1) and possesses various therapeutic activities. Though many flavonoids are present in this medicinal plant but due to luteolin's small content in the drug and high therapeutic efficacy, its quantitative analysis becomes indispensable. The study was aimed to determine the most suitable solvent to optimize the extraction method for the isolation of luteolin from the leaves of *V. negundo* L. by quantitative determination of luteolin in different solvent extracts through high performance liquid chromatography (HPLC).

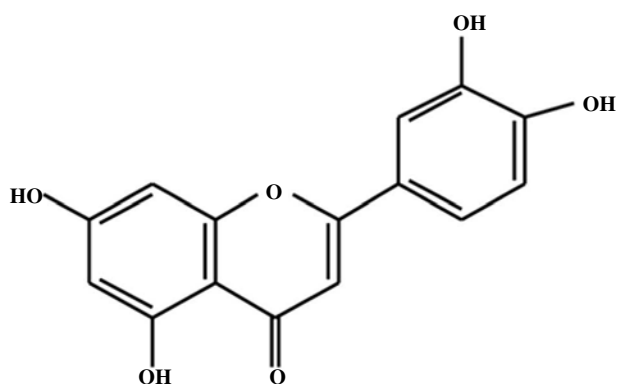


Figure 1. Chemical structure of luteolin.

## 2. Materials and methods

### 2.1. Plant material

The leaves of *V. negundo* Linn. were obtained from the Herbal Garden of Jamia Hamdard University, New Delhi and authenticated by a taxonomist, Department of Botany, Faculty of Science, Jamia Hamdard, New Delhi. A voucher specimen was deposited in the herbarium of Department of Pharmacognosy and Phytochemistry, Faculty of Pharmacy, Jamia Hamdard, India for future reference.

### 2.2. Chemicals

Standard luteolin was purchased (Sigma, USA). HPLC

grade methanol and water were obtained from S.D Fine Chemicals, India. All other reagents used were of analytical grade and purchased from S.D. Fine Chemicals, India.

### 2.3. Extraction of luteolin

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 (Usha Lexus 2753, India). The grounded leaves were then used for extraction of luteolin. Extraction was carried out by four methods *viz.* maceration, reflux, soxhlet and ultrasound assisted extraction (UAE) with four different solvents of varying polarity including ethanol, methanol, chloroform, and dichloromethane. Extract yield (mass of extract/mass of dry powder) was used as an indicator to compare the effectiveness of the extraction techniques. All the prepared extracts were subjected to Shinoda chemical test to ascertain the presence of flavonoids in them. The scheme of extraction by all four methods is briefly described below.

#### 2.3.1. Extraction through maceration

In total, 5 g of leaf powder was soaked in 50 mL of solvent [solvent: drug ratio=10:1 (mL/g)] for 72 h at room temperature. The menstruum was then filtered and the filtrate was evaporated to obtain the residue using rotary evaporator (HAHN SHIN, HS-2005V-N) at 40 °C. The residue was weighed and appropriate dilutions were made for quantitative estimation of luteolin in the extract.

#### 2.3.2. Extraction through reflux

Hot solvent extraction was done using a reflux apparatus for 2 h at 50 °C using 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.

#### 2.3.3. Extraction through soxhlet

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

#### 2.3.4. Extraction through (UAE)

Ultrasound assisted extraction was done for 20 min with solvent:drug ratio of 10:1 (mL/g) at 50 °C in a sonicator (TOSCHON, SW7). Further processing of the extracts was done in the similar manner discussed above.

## 2.4. Analysis of luteolin by HPLC

### 2.4.1. Luteolin standard solution preparation

Stock solution of luteolin was prepared in HPLC grade methanol at a concentration of 1 mg/mL. Then working solutions of 100, 125, 175, 200 and 500 µg/mL were prepared in HPLC grade methanol and stored at –20 °C. Before further use, the solutions were brought to room temperature. Each standard solution was filtered through 0.2 µm membrane filter (Axiva) and then subjected to HPLC analysis to obtain a peak height at a static retention time for each standard solution. 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 luteolin in test samples.

### 2.4.2. Sample solution preparation

Ten milligram of the various solvent extracts of the leaves of *V. negundo* L. prepared by different extraction methods, was weighed and dissolved in HPLC grade methanol to obtain a final concentration of 1 mg/mL. The solutions were then filtered through 0.2 µm membrane filter and 20 µL of the resulting solution was subjected to HPLC analysis. Final concentration of luteolin in the extracts was calculated by using linear equation for the calibration curve.

### 2.4.3. Chromatographic conditions

HPLC analysis of the extracts was performed on HPLC Quaternary System (Shimadzu, Japan) consisting of LC–10AT VP pumps (Shimadzu, Japan), a single wavelength programmable UV–visible detector, and a system controller. Samples were injected by using a rheodyne injector fitted with a 20 µL fixed loop. Standard and sample solutions were filtered through 0.2 µm syringe filter (Axixa) before injection. The separation was achieved by using column with 25 mm ×4.6 mm, particle size 5 µm, Lichrospher C18 reverse phase column (Merck, Germany). Determination of luteolin was carried out with the mobile phase composed of methanol and 1% aqueous acetic acid solution (99:1 v/v) at a flow rate of 1.0 mL/min. The optimum separation in HPLC was achieved at 30 °C and absorbance was measured at 289 nm.

## 3. Results

### 3.1. Phytochemical testing

All the prepared extracts *viz.* methanol, ethanol, chloroform

and dichloromethane showed the presence of flavonoids by Shinoda test<sup>[18]</sup>.

### 3.2. Extraction of luteolin by various extraction techniques

Luteolin was extracted from the leaves by four different extraction techniques such as maceration, soxhlet, reflux and UAE using ethanol, methanol, chloroform and dichloromethane as solvents. During the experiment, extraction time, extraction temperature and drug: solvent ratio was kept constant for all the solvents. The results of % extract yield in different solvents by various extraction techniques have been presented in Table 1. As it was expected, varying amount of extract yields was obtained with different solvents and extraction techniques. The maximum and minimum yield for ethanol extract (14.5% and 5.2%) was obtained by soxhlet and UAE techniques respectively. However the yield of methanol extract was found to be the highest by UAE technique. Appreciable amount of chloroform and dichloromethane extracts were obtained by reflux and soxhlet technique respectively. In general, the yields of the extracts with reflux and soxhlet extraction methods (hot solvent systems) were found to be greater than the maceration and UAE techniques.

**Table 1**

Luteolin content in different extracts of *V. negundo* Linn.

Extraction technique	Solvent	Extract yield (%)	Luteolin content (% w/w)
Maceration technique	Ethanol	6.2	0.576 7
	Methanol	9.4	1.020 0
	Chloroform	5.2	0.584 5
Soxhlet technique	Dichloromethane	4.0	0.583 8
	Ethanol	14.5	0.618 0
	Methanol	8.8	1.075 0
Reflux technique	Chloroform	8.6	0.583 6
	Dichloromethane	14.6	0.582 5
	Ethanol	9.6	4.811 0
UAE technique	Methanol	4.8	6.340 0
	oroform	15.4	0.576 0
	Dichloromethane	14.0	0.591 4
UAE technique	Ethanol	5.2	0.640 0
	Methanol	11.6	0.661 3
	Chloroform	4.1	0.584 5
	Dichloromethane	3.2	0.592 2

### 3.3. Quantitative estimation of luteolin by HPLC method

The standard solutions of luteolin and sample extracts were subjected to HPLC technique using methanol and 1% aqueous acetic acid (99:1 v/v) as mobile phase, in isocratic mode (1 mL/min). Calibration curve of luteolin was made with five dilutions of standard solution at a concentration ranged from 100 to 500 µg/mL. The linear regression equation was obtained in the form of  $y=mx+c$ , where  $y$  and

$x$  corresponds to area under the curve and concentration respectively (Figure 2). The regression equation and correlation coefficient were as follows:  $y=18.78x-1072$ ;  $r^2=0.998$ . The relative quantity of luteolin in the prepared extracts was then calculated from this equation and the results are shown in Table 1. The retention time of luteolin in HPLC chromatogram was observed at 3.883 min (Figures 3 and 4).

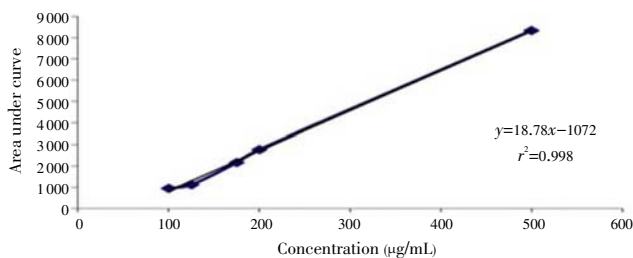


Figure 2. Calibration plot of luteolin.

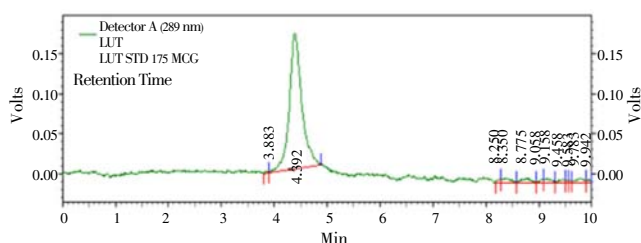


Figure 3. HPLC chromatogram of standard luteolin (175 µg/mL).

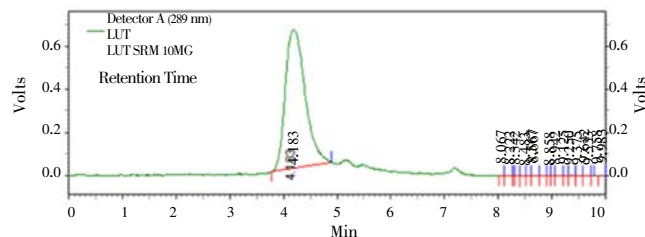


Figure 4. HPLC chromatogram of methanolic *V. negundo* leaf extract by reflux technique.

Quantification of different extracts of *V. negundo* L. through HPLC revealed that methanol is the best solvent for the extraction of luteolin. Luteolin content in methanol extract obtained through maceration, soxhlation, reflux and UAE techniques was found to be 1.020%, 1.075%, 6.340% and 0.640% respectively (Table 1). In ethanol, luteolin content extracted by various techniques was not as high as in the case of methanol. As evident, in other solvents like, chloroform and dichloromethane the content was very less in the ranges of 0.5%–0.6%. Moreover, it was also observed that among various techniques employed for extraction and isolation of luteolin, reflux technique was found to be the most efficient. Clearly, the results of our study indicated that methanol is the most suitable solvent for luteolin extraction.

## 4. Discussion

Over the last few decades many new extraction techniques have been developed for the isolation of bioactive components of medicinal plants. But several studies have reported that biological activities of extracts are affected by the extraction techniques employed. Therefore, it is important to select a suitable solvent as well as extraction method, which is least influenced by the presence of interfering substances, based on the chemical and physical properties of sample matrix<sup>[19]</sup>.

Luteolin is an important constituent of *V. negundo* and is known to possess lots of therapeutic benefits. Therefore, its content in the plant needs to be known. Luteolin is present in very minute quantity in the leaves of this plant. Therefore HPLC technique was used for its quantification. HPLC is a commonly used chromatographic technique for qualitative and quantitative analyses of flavonoids in plant materials as it is considered to be the most convenient method<sup>[20]</sup>. Also the separations are far more rapid than classical methods and provide high resolution and sensitivity.

The extract yields varied with the solvents and/or extraction techniques but the maximum % yield was obtained with the reflux technique. The differences in the % yield of leaves extracts could be due to the differences in the availability of extractable components by different solvent/extraction techniques<sup>[21]</sup>. It can be inferred that hot solvent extraction methods are better choice for extract preparation from *V. negundo* leaves as compared to non thermal methods such as maceration or UAE. Also, HPLC analysis results showed that luteolin content was the highest in methanol extracts irrespective of the extraction technique employed, which implies that methanol is a favourable choice of solvent for the extraction of luteolin.

Thus it can be concluded that extraction procedure and solvent does affect the isolation and yield of luteolin from *V. negundo* leaves extract. The outcome of this study will surely help the researchers in selecting the suitable solvent and appropriate extraction technique for the isolation of luteolin from the leaves extract of *V. negundo*, which is methanol and reflux method. Also luteolin can be used as a biomarker in standardization and quality control of leaves extract of this drug 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.

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