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Research Article

A NEW METHOD OF EXTRACTION, ISOLATION AND DETERMINATION OF SOLANESOL FROM TOBACCO WASTE (*NICOTIANA TOBACUM* L.) BY NON-AQUEOUS RP-HPLC

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ABSTRACT

Objective: Isolation of solanesol of maximum purity from tobacco waste and rapid identification by RP-HPLC and structural characterization by FTIR, LC-MS, ^{1}H NMR spectrometry.

Methods: A method of cold extraction for isolation of solanesol from tobacco waste was developed. The crude extract containing solanesol (14.26% HPLC purity) was subjected to a series of steps, viz., saponification and solvent crystallization. The pure solanesol (92.14% HPLC purity) was characterized by FTIR, LC-MS, ^{1}H NMR spectrometry. The analysis was carried out by a simple and rapid RP-HPLC method on a Phenomenex Luna C_{18} column (250 mm x 4.6 mm i.d., particle size 5 µm) with methanol-isopropanol (60:40, v/v) as mobile phase and UV detection at 215 nm.

Results: A novel competent and inexpensive protocol was developed for isolation of solanesol from tobacco waste. The RP-HPLC method was linear over the range 10-50 µg/mL and the retention time was 6.127 min. Accuracy, precision and repeatability, were within the required limits. The mean recoveries measured at the three concentrations were higher than 98.6% with RSD < 2%.

Conclusion: It is a convenient and efficient method for the extraction of solanesol from tobacco waste and shows great potential for the large-scale industrial applications.

Keywords: Solanesol, *Nicotiana tobacum* L., Cold extraction, Isolation, Non-aqueous reversed phase HPLC.

INTRODUCTION

In present years, it becomes important to know that even in commonly cultivated crop species; there are significant levels of medicinal compounds that would form the basis of therapies. The best example is isoprenoid family, known as terpenoids representing the largest and oldest class of natural products known, consisting of more than 40,000 different molecules [1].

Solanesol a naturally occurring trisesquiterpenoid (C_{45}) alcohol, all–trans-nonaprenol, of tobacco is one of the important precursor of the tumorigenic polynuclear aromatic hydrocarbons (PAHs) of tobacco smoke. It is also the starting material for many high-value biochemicals, including coenzyme Q10 and Vitamin K analogues [2]. Coenzyme Q10 is well known not only to reduce the number and size of tumors but also improve cardiovascular health [3]. Solanesol itself could be used as an antibiotic, cardiac stimulant and lipid antioxidant. There is a great demand for solanesol for production of Coenzyme Q10 and other uses. Its isolation not only reduces the risks of PAH from tobacco smoke but also makes use of it as a starting material in synthesis of several value added products such as Q10 and other analogues. Therefore, isolation of solanesol from tobacco is gaining a lot of importance in recent years. Previously it is recognized that byproducts of crop products may be utilized for the extraction of solanesol using tobacco waste however it is worth considering other sources, especially with the greater investments in biorefining [4].

During the processing of fruits and vegetables like tomato and potato products, up to 40% of the initial material entering the plant ends up as waste and also after harvest vegetative material must be disposed of. In general, vegetative waste material is treated chemically to stop the spread of disease prior to undergoing pulping, composing or incineration.

However, new legislation will soon be in place to prevent these practices. Till date there is a very little work performed to utilize this substantial biomass for co-product isolation but the potential for using this material for solanesol extraction from tobacco waste should be explored [5].

The major problem in extraction of solanesol from tobacco is the selection of a suitable solvent for maximum yield and also purification pose several problems because of the presence of closely related fatty acids, alcohols, alkaloids, tobacco pigments, tar and other organic impurities. Especially the food and pharmaceutical grade of solanesol has to be of highest purity. Therefore, it is quite important to develop processes that can selectively separate the pure solanesol from the crude extracts of tobacco leaves. Most of the methods involve multiple step procedures, which are non-specific, quite tedious and time consuming [6-7].

A number of gas chromatographic methods for determination of solanesol in tobacco were reported [8-12]. The other methods also reported include gravimetry, thin layer chromatography and coulometry [9-13]. HPLC with various detectors including UV, RID, ELSD and MS was used to determine solanesol. Most of the methods reported before 2006 were in normal phase mode with UV detection [14-16]. However, reversed phase HPLC with UV detection is often preferred not only because of its higher sensitivity but also wide availability and suitability. In particular, RP-HPLC with UV and ESI-TOF/MS determination of solanesol in the crude extracts of tobacco leaves also reported. The major drawback of this method lies in detection i.e., the analytes were monitored at 202 nm by PDA where the acetonitrile used as one of the mobile phase solvents generally interferes [17].

In the present investigation, a protocol involving the use of petroleum ether cold extraction, fractionation with methanol followed by saponification, solvent crystallization for isolation of solanesol from waste of *Nicotiana tobacum* L. was developed. Further, a simple non-aqueous RP-HPLC with UV method for determination of purity of solanesol in tobacco waste (crude extract), saponified extract, and isolated/purified solanesol of *Nicotiana tobacum* L. was described [18] and compared to the standard solanesol. The objective of this innovation is: (a) to avoid denicotinization, (b) to avoid ether as it is volatile and inflammable, (c) to avoid molecular distillation which requires high temperatures (d) to avoid the use of column chromatography which involves use of lot of solvents and time consuming (e) to avoid synthetic route for preparation of solanesol where yields are very low, making the process industrially unviable and (f) to provide an economical, safe, ecofriendly and industrially viable process for the isolation,

separation and purification of solanesol from tobacco waste with maximum possible purity.

MATERIALS AND METHODS

Chemicals and Reagents

All the reagents were of analytical-grade unless stated otherwise. HPLC-grade isopropanol (IPA) and methanol (MeOH) (Ranbaxy, SAS Nagar, India) were used. Dried tobacco waste received from local farmers in Ongole, Andhra Pradesh, India was used. Solanesol as reference standard (94% HPLC purity) was purchased from Laila Impex Ltd., Vijayawada, Andhra Pradesh, India.

Extraction procedure

Dried *Nicotiana tobacum* waste leaves (25 kg) were pulverized and macerated in hydrocarbon solvent (petroleum ether) by cooled extraction and worked up to give crude solanesol while concentrating the petroleum ether solution under reduced pressure using rotary vacuum evaporator. The residue obtained was fractionated with methanol for a period of preferably 25 to 30 minutes at a temperature of 50-55°C. This process (i.e., extraction with methanol) was repeated 3 to 4 times. Methanol was removed under vacuum and the residue (14.26% HPLC purity) was saponified with 10 % alkanolic-alkali metal hydroxide. Preferably the saponification was carried out using 10% ethanolic potassium hydroxide at a temperature of 55-60°C. The reaction mass was cooled and the saponified material separated by decantation. The unsaponified mass, which was settled down, washed with methanol 2 to 3 times. The moisture from the unsaponified mass was removed by azeotropic distillation using toluene to gave crude solanesol (18.75% HPLC purity) thus obtained, needed purification. The solanesol thus obtained was recrystallised with common laboratory solvents like methanol/ethanol/isopropanol/ acetonitrile/acetone or mixture of solvents in different ratio was tried. Methanol or a mixture containing methanol, acetone and acetonitrile (7:2:1) gave good quality product. However, an excellent purity of solanesol was achieved by crystallization from acetone or acetonitrile or preferably a mixture of methanol and acetone (9:1) (92.14% HPLC purity).

Apparatus

The HPLC system was composed of Water 1500 series HPLC binary pumps, a Waters 2487 Dual λ UV absorbance detector, a manual injector (all from Waters, Milford, MA). A reverse-phase Phenomenex Luna C_{18} column (250 mm x 4.6 mm i.d., particle size 5 µm) was used for separation and determination. Empower 2 soft ware used for data acquisition.

The **IR** spectra were recorded in Bruker FT-IR spectrophotometer (alpha) range 4000-400 cm^{-1} and resolution 4 cm^{-1} using KBr disc method. The number of scans was 20. The instrument was calibrated by using a polystyrene standard. **^{1}H NMR** spectra were recorded in the indicated solvent on a Bruker AMX 400 MHz with tetramethylsilane (TMS) as internal standard (chemical shifts in δ ppm). **LC-MS** [API/ESI-MS (80 eV)] spectra were recorded on Agilent HPLC 1100 series. Analytical TLC was performed on Silica Gel F_{254} plates (Merck) with visualization by UV (254 nm) chamber with protective filters.

Chromatographic conditions

Analytical HPLC was performed with Phenomenex Luna C_{18} (PX Advanced Technology Solutions Pvt. Ltd.) column (250 mm x 4.6 mm i.d., particle size 5 µm) using Water 1500 series HPLC binary pumps. Before delivering in to the system, the mobile-phase consisting of MeOH:IPA (60:40, v/v) was filtered through 0.45 µm membrane filter (Millipore) and deaerated ultrasonically prior to use. The analysis was carried out under isocratic conditions using a flow rate and injection volume was 1.0 mL/min and 10 µL, respectively. All chromatographic operations were carried out at room temperature (28°C) and chromatograms were recorded at 215 nm using Waters 2487 Dual λ UV absorbance detector.

Preparation of standard stock solution

10 mg of reference standard solanesol (94% pure) was dissolved in methanol (1 mg/1mL). Aliquots of standard solutions (0.1 to 0.5 mL) were diluted with methanol to get working standard solutions at concentrations 10 to 50 µg/mL range.

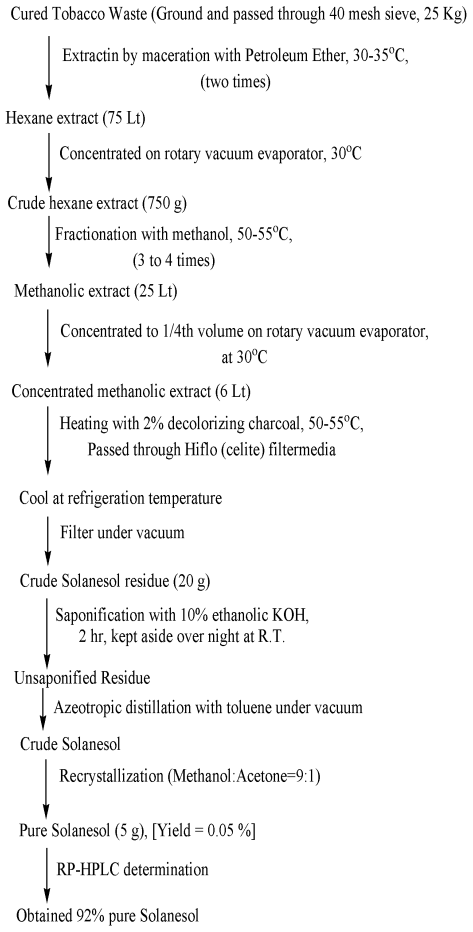
Preparation of sample solutions

10 mg of each solanesol crude extract, solanesol saponified extract and solanesol purified were dissolved in methanol (1 mg/mL) separately. These sample solutions were further diluted with methanol to get working sample solutions at concentrations 10 to 50 µg/mL range.

RESULTS AND DISCUSSION

Extraction of solanesol

Preparing the solanesol crude extract is always economical in a cold extraction by petroleum ether followed by fractionation with methanol at 50-55°C and the solvent was concentrated under high vacuum. The crude extract containing (14.26% HPLC purity) solanesol was purified by three different methods: (i) silica gel column chromatography; (ii) saponification followed by silica gel column chromatography; (iii) saponification followed by recrystallization with different solvents, viz., acetone, methanol and acetonitrile. From the obtained results it was found that method (iii) is the best for its purification.



Scheme 1. Isolation and purification of Solanesol from the tobacco waste.

In the third method the saponified fraction was subjected to recrystallization with common laboratory solvents gave the yield of 0.05%. The preferred solvent system for recrystallization is methanol-acetone = 9:1 (92.14% HPLC purity). This would be due to the fact that a substantial portion of the solanesol in tobacco exists in the form of esters of fatty acids rather than free solanesol. Therefore, the saponification of the crude extract increased the

solanesol content and in turns the yield. Hence, it is a best choice for isolation and purification of solanesol from tobacco waste **(Scheme 1)**.

Spectral data

The isolated solanesol structure was characterized by the following spectral data. IR (KBr, cm^{-1}): 3420, 2955, 2924, 2854, 1631, 1443, 1380, 1150, 1016, 876. ^1H NMR (400 MHz) (solvent: $CDCl_3$) δ ppm: 1.24-1.27 (m, 32H), 2.04 (21H, 7CH3), 1.65 (s, 3H), 1.68 (s, 3H), 1.59 (3H, 1CH3), 5.09-5.13 (t, 2H, O-CH2), 4.09-4.14 (m, 9H). LC-MS (*m/z*): 654 ($M + Na^+$). The chemical structure of solanesol is shown in **Fig. 1**.

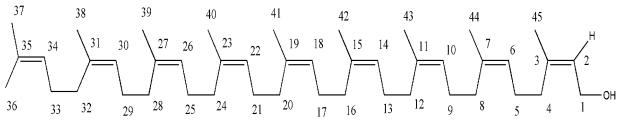


Fig. 1: Chemical Structure of Solanesol

HPLC method development

Solanesol is lipid soluble fraction of tobacco constituted with C_{45} terpenoid and is soluble in polar solvents but insoluble in water. The preferred stationary phase for analytes separation is silica. Low polar solvents must be used to achieve ample retention in normal phase silica. Except the trace amounts of water in the solvents are carefully controlled it becomes absolutely difficult to maintain reproducibility in such systems. By using reversed-phase chromatography, the problems encountered in the separation of hydrophobic compounds by normal phase chromatography are generally conquered.

To achieve the elution in acceptable time should be used non-aqueous solvents such as methanol, acetonitrile and tetrahydrofuran because hydrophobic compounds exhibited large retention time on chemically bonded C_{18} phases. For example fats, carotinoids and sterols are usually separated by non-aqueous reversed phase chromatography [19-20].

An additional advantage of reversed-phase separation is enhanced resolution of homologues/isomers under such conditions. Hence solanesol was separated effectively from the other components of tobacco by using non-aqueous reversed phase HPLC. Phenomenex Luna C_{18} column (250 mm x 4.6 mm i.d., particle size 5 µm) with a mixture of MeOH:IPA (60:40 v/v) used for better separation and resolution. The total run time was 15 min and the solanesol has been eluted with the retention time was found to be 6.127 min. Solanesol was identified on co-injection and comparison of retention time with that of reference standard. The HPLC chromatograms of solanesol standard, crude extract of solanesol, saponified extract, isolated/purified solanesol and over lined chromatogram are shown in **Fig. 2, 3, 4, 5** and **6,** respectively.

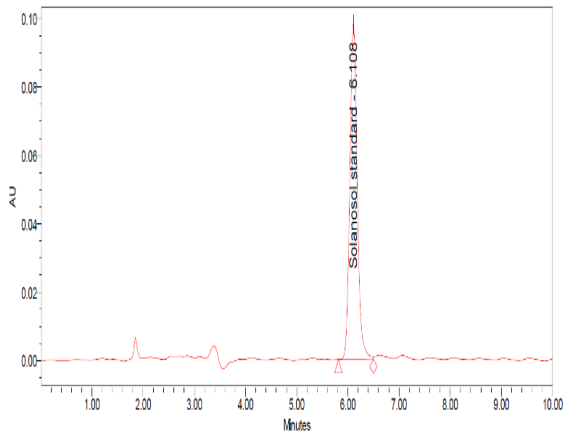


Fig. 2: HPLC Chromatogram of solanesol standard.

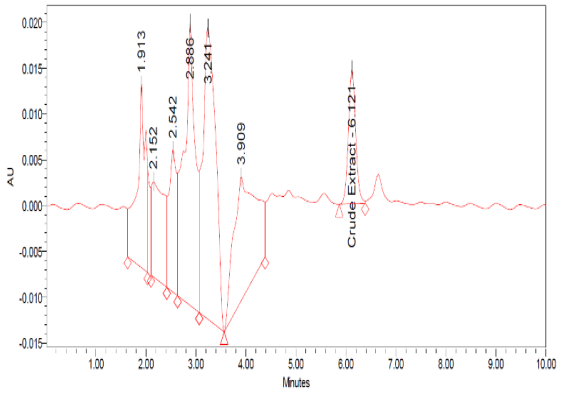


Fig. 3: HPLC Chromatogram of solanesol crude extract.

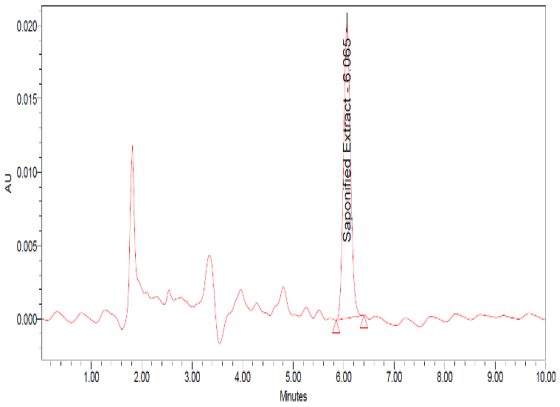


Fig. 4: HPLC Chromatogram of solanesol saponified extract.

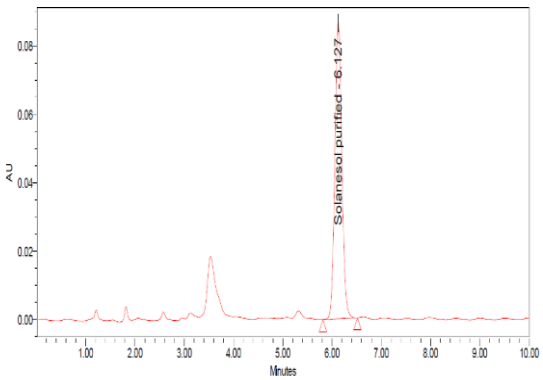


Fig. 5: HPLC Chromatogram of solanesol purified.

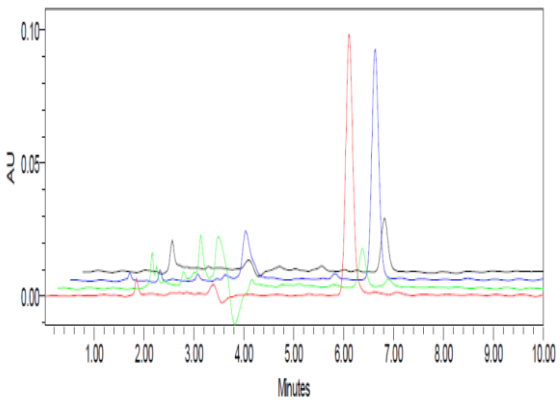


Fig. 6: HPLC Over lined chromatograms.

Validation

Linearity

Calibration curve for solanesol standard was carried out in the range of 10-50 µg/mL with five different concentrations. The mean equation of the calibration curve (n=6) obtained from six points was y = 21911*x* + 18054 with a regression coefficient of 0.9994.

Selectivity

The selectivity of the method was determined by the standard solanesol and samples. The peaks of crude extract of solanesol, saponified extract, and isolated/purified solanesol were identified by comparing their retention times in a UV spectrum with that of standard. Spiking of selected samples with the standard was also used to confirm peak identity.

Accuracy and precision

The precision was evaluated by repeated injections of the sample solution six times. The R.S.D. of peak area and retention time was 1.68%. Intra- and inter-day variability was determined by analysis of standard solutions at low, medium and high concentrations of isolated solanesol on three different days.

The acceptable intra- and inter-day precisions (R.S.D.) and accuracy (relative error, RE) were <1% and ±5%, respectively. The assay precision was < 5%, and the accuracy was > 98%.

Recovery

The three different concentrations diluted from the stock solution were added to an predetermined isolated/purified solanesol with a known content of solanesol standard and the recovery of solanesol was calculated.

The results of recovery of the tests (triplicate) were acceptable as the average recovery of solanesol was 98.6, 100.3 and 99.5.

Limits of detection (LOD) and quantification (LOQ)

LOD was defined as the lowest concentration of isolated solanesol at which the signal was larger than three times of the baseline noise S/N = 3 and LOQ as S/N = 10. The measured LOD and LOQ values were 0.25 and 0.65 µg/mL respectively.

CONCLUSION

A new competent and inexpensive protocol was developed for isolation of solanesol from tobacco waste using cold extraction followed by fractionation, saponification and recrystallization procedure. The purity of isolated solanesol was obtained 92-93% using common laboratory chemicals. The cold extraction with petroleum ether or n-hexane is better to isolate the solanesol on a large scale from tobacco waste also.

Additionally a simple and rapid method for separation and determination of solanesol from tobacco using non-aqueous RP-HPLC in an isocratic elution mode and using UV detector at 215 nm was developed. Therefore, it is a convenient, efficient method for the extraction of solanesol from tobacco waste and shows great potential for the large-scale industrial application in the near future.

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