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PharmTech

International Journal of PharmTech Research
CODEN (USA): IJPRIF ISSN : 0974-4304
Vol.1, No.4, pp 1129-1135, Oct-Dec 2009

Development and Validation of HPTLC method for determination of 3-hydroxy androstane [16,17-C](6'methyl, 2'-1-hydroxy –isopropene-1-yl) 4,5,6 H pyran in Jambul seed (*Syzygium cumini*)

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Abstract: A new, simple, sensitive, accurate and precise high-performance thin-layer chromatographic method for quantification of 3-hydroxy androstane [16,17-C](6'methyl, 2'-1-hydroxy –isopropene-1-yl) 4,5,6 H pyran, a marker compound in *Syzygium cumini* was developed and validated. This marker compound was isolated from the ethanol extract and identification was confirmed by using melting point and IR, NMR spectroscopy. An ethanol extract of the seed powder was chromatographed on silica gel 60F-254 plate with toluene : ethyl acetate (8.5:1.5 v/v) as mobile phase. Detection was performed by scanning in fluorescence mode at 366 nm. The method was validated for linearity, accuracy, recovery, precision, limit of detection, limit of quantification and specificity. The linear regression analysis data for the calibration plots for 3-hydroxy androstane [16,17-C](6'methyl, 2'-1-hydroxy –isopropene-1-yl) 4,5,6 H pyran showed good linear relationship with $r^2 = 0.999$, in the concentration range of 1000-5000 ng/spot. The limit of detection and limit of quantification were 131and 430 ng/spot, respectively. The amount of 3-hydroxy androstane [16,17-C](6'methyl, 2'-1-hydroxy –isopropene-1-yl) 4,5,6 H pyran found in seed powder extract was 7.38% . This method can be used as quality control method for checking the purity of *Syzygium cumini* seed powder, extract and its formulation.

Keywords: *Syzygium cumini* , HPTLC, Validation.

Introduction

Plants have provided mankind with herbal remedies for many diseases for many centuries and even today. They continue to play a major role in primary healthcare as therapeutic remedies in developing countries. In India, herbal medicines have been the basis of treatment and cure for various diseases in traditional methods practiced such as Ayurveda, Unani and Sidha. *Syzygium cumini* (Linn) Skeels syn. *Eugenia jambolana* Lam ; *E.cumini* Druce. (fam Myrtaceae); is a large evergreen tree up to 30 m height and a girth of 3.6 m with a bole upto 15 m found throughout India upto an altitude of 1,800 m.[1] Most of the plant parts of *E. jambolana* are used in traditional system of medicine in India having promising therapeutic value with its various phytoconstituents such as glycosides (jamboline), ellagic acid, gallic acid, tannins,[1] fatty oil, steroids, flavonoids, triterpenes[2], phenolics, monoterpenoids,[3] minerals, proteins, calcium and vitamins. [4] Its pharmacological actions like hypoglycaemic,[5,8]. anti-diarrhoeal[6], anti-inflammatory[7], antibacterial,[8] radioprotective[9], antiallergic [10] and antioxidant[11] proven on animal models. Most of the studies of *Syzygium cumini* as antidiabetic agent with its possible mechanism of action and delaying complications of diabetes such as cataract, neuropathy have been conducted but detailed research on isolation of bioactives and its standardisation is seriously required to know the potential of the plant . HPTLC, is a valuable tool for the investigation of herbal products with respect to different aspects of their quality. HPTLC analysis is comparatively short and many samples can conveniently be compared side by side on the same plate. This is particularly important for screening and inspection / selection of raw materials and for process control during manufacturing. With HPTLC, the same analysis can be viewed using different wavelengths of light thereby providing a more complete

profile of the plant and extreme flexibility of detection ,the convenience of specific derivatization , and the possibility of multiple detections without repeating the chromatography. HPTLC results are not only reported as peak data but can also be presented and communicated as images.[12]

Experimental

Reagents and Chemicals

All chemicals were AR grade and purchased from S. D. Fine chemicals, Mumbai. Chemicals such as ethanol, toluene, ethyl acetate were used.

Plant material

The seeds of *Syzygium cumini* were procured from local market and authenticated for their correct botanical identity by scientist of Agharkar Research institute Pune. Seeds were washed with water, dried at room temperature, powdered and defatted with petroleum ether (60- 80º C), stored in air tight stoppered bottle before subjected to physical and chemical evaluation of different properties.

Isolation of marker [13]

Dried seed powder of *Syzygium cumini* (75 gm) were exhaustively extracted with 500 ml ethanol in Soxhlet apparatus for 24 hrs and dark brown extract was concentrated in vaccum via rota vaccum drier. The dark brown residue was subjected to column chromatography by using 100 gm of silica gel F 256 and successive elution with toluene : ethyl acetate (8.5:1.5 v/v) . Fractions were collected in test tubes. The elute of column was tested by TLC on plate of silica gel F 254 using toluene : ethyl acetate (8.5:1.5 v/v) as mobile phase. The developed plates were observed under uv lamp at long wavelength. Some earlier fractions gave single spot and same Rf (0.50 ± 0.2) with fluorescence characteristic. Similar Rf test tubes were combined and filtered. The filtrate was evaporated under reduced pressure to obtain brown coloured amorphous compound having chemical name as 3-hydroxy androstane [16,17-C](6'methyl, 2'-1-hydroxy –isopropene-1-yl) 4,5,6 H pyran.

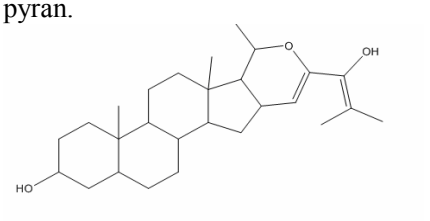


Fig. 1. Chemical structure of the isolated compound

Instrumentation

Melting point of isolated compound was determined by open capillary method using Veego's (VMP-D) melting point apparatus. Structural confirmation of the isolated compound was done by using IR and NMR spectroscopy. FTIR spectra of the isolated compound was recorded using KBr on a Shimadzu FTIR 8400S and characteristic absorption signals are reported in cm^{-1}. ^1H NMR spectra was done at IIT Powai, Mumbai.

High Performance Thin Layer Chromatography

Chromatographic separation was performed on Merck TLC plates precoated with silica gel 60 F254 (20cm ×10 cm with 200 µm layer thickness) from E. Merck, Germany. The samples were applied onto the plates as a band with 8 mm width using Camag 100 microlitre sample syringe (Hamilton, Switzerland) with a Camag Linomat 5 applicator (Camag, Switzerland). Linear ascending development was carried out in a twin trough glass chamber (20 x 10 cm) with the mobile phase toluene : ethyl acetate (8.5 : 1.5 v/v). Scanning was performed using Camag TLC scanner 3 at 366 nm through fluorescence mode and operated by winCATS software (version 1.4.1, Camag).

Preparation of Standard Stock Solution

Standard stock solution of marker: 10 mg of marker was weighed and dissolved in 10 mL of ethanol to obtain 1000 µg/mL stock solution of marker. Then dilution was carried out to get concentration of marker compound 100 µg/mL which was used for further analysis.

Preparation of sample solution: Extract of *Syzygium cumini* obtained from Soxhlet apparatus was weighed 25 mg exactly and dispersed in 50 mL of ethanol. It was sonicated for 10 minutes. The sample was filtered through Whatman qualitative No.1 filter paper, pore size 0.45µm.

Method Validation

This method was validated as per the ICH guidelines[14], the method validation parameters checked were linearity, accuracy and recovery, precision, limit of detection, limit of quantification, robustness and specificity.

Preparation of calibration curves

From the final standard solution of marker, a volume of 10-50µL was spotted in duplicate on the TLC plate to obtain final concentration in the range of 1000-5000 ng/spot. The solvent system, toluene: ethyl acetate (8.5:1.5, v/v) was used as the mobile phase. Chromatogram was developed in a twin trough glass chamber, using 20 minutes chamber saturation time. The length of chromatogram run was 80 mm. The developed plates were air-dried. Scanning was performed in the fluorescence mode at 366 nm. The slit dimension was kept at 6 x 0.45 mm at scanning speed of 100 nm/s. After completion of scanning, peak areas of marker compound were noted. Peak areas were plotted against corresponding concentrations and least square regression analysis was performed to generate the calibration equation for marker .

Precision

A stock solution containing marker compound was prepared in ethanol and six 10 µl (1000 ng /spot) bands were applied and analyzed by proposed method to determine instrument precision. Six different volumes of same concentration were spotted on a plate and analyzed by the proposed method to determine variation arising from method itself. To evaluate intra-day precision, six samples at three different concentrations (1000, 2000 and 3000 ng/ spot) were analyzed on the same day. The inter-

day precision was studied by comparing assays performed on three different days.

Accuracy and Recovery :

For accuracy of method, recovery studies were carried out by applying the method to drug sample to which known amount of marker was added at level of 50, 100 and 150% (standard addition method). Three determinations were performed at each level, and their results were compared with the expected one.

Limit of Detection and Limit of Quantification

The detection limit (LOD) of an individual analytical procedure is the lowest amount of analyte in a sample which can be detected but not necessarily quantitated as an exact value. LOD was calculated using the following formula,

$$LOD = \frac{3.3 \times \text{Standard Deviation of the y-intercept}}{\text{Slope of calibration curve}}$$

The quantitation limit (LOQ) of an individual analytical procedure is the lowest amount of analyte in a sample which can be quantitatively determined with suitable precision and accuracy. LOQ was calculated using the following formula,

$$LOQ = \frac{10 \times \text{Standard Deviation of the y-intercept}}{\text{Slope of calibration curve}}$$

Robustness

By introducing small changes in the mobile phase composition, the effects on the results were examined in triplicate. Mobile phases having different composition of toluene : ethyl acetate (7.5:2.5 v/v) was tried at two different concentration levels of 1000 and 2000 ng/spot.

Specificity

The specificity of the method was ascertained by analyzing the Rf value and the spectra of the isolated marker and the seed extract of *Syzygium cumini*.

Determination of marker in extract

Extract solution (20µL) of *Syzygium cumini* seed were applied in triplicate as bands to the plate. The plate was developed and scanned as described above and peak areas were recorded. The amount of marker was calculated using the calibration plot, considering the isolated marker to be 100% pure.

Results and Discussion

Structural Elucidation of Isolated Compound

Melting point of isolated compound was found to be 122˚C. This matches with the reported value[13]. IR spectrum showed sharp peaks at 3413.77, 2927.74, 1687.6,1458.08, 1166.85, 817.76 cm^{-1} ,while result obtained from ^1H NMR spectrum showed signals given in Table 1. This matches with the reported values.[13]

Optimization of Solvent System and Chromatographic conditions

Chromatographic separation studies were carried out on the stock solution of marker. TLC procedure was optimised with varying ratios of toluene and ethyl acetate. The mobile phase toluene : ethyl acetate with ratio (8.5: 1.5 v/v) gave good resolution as well as a well defined peak at Rf value of 0.50 . Other chromatographic conditions like chamber saturation time, run length, sample application rate and volume, sample application positions, distance between tracks, detection wavelength, were optimized to give reproducible Rf values, better resolution, and symmetrical peak shape for the marker.

Linearity

Marker showed good correlation coefficient when peak area of the resolved spot was plotted against concentration in the range of 1000-5000ng/spot. The equation of the regression line was y = 2431.832 x + 269.127 (r^2 = 0.999). (Fig.2)

Precision

The proposed method was found to be precise as indicated by percent RSD (Relative Standard Deviation) not more than 1.5 (Table 2 and 3).

Accuracy and Recovery

The proposed method when used for quantification of marker from seed extract after spiking with standard afforded average recovery of 100.30 % (Table 4).

Limit of Detection and Limit of Quantification

The limit of detection was found to be 131 ng/spot while the limit of quantification was found to be 430ng/spot.

Robustness

The low values of % RSD obtained after introducing small changes in mobile phase composition indicated the robustness of the method (Table 5)

Specificity: The specificity of the method was ascertained by analyzing standard isolated marker and the seed extract. There were no interfering spots by the plant constituents at the Rf values of the marker. The absorption spectra of standard marker (Rf = 0.5) and the corresponding spot present in seed matched exactly, indicating no interference by the other plant constituents (Fig. 3 and 4).

Determination of marker in dry extract:

The amount of marker in dry extract was found to be 7.38 %. Chromatogram of marker compound and extract of *Syzygium cumini* seed are given in Fig. 5 and 6 respectively.

Conclusion

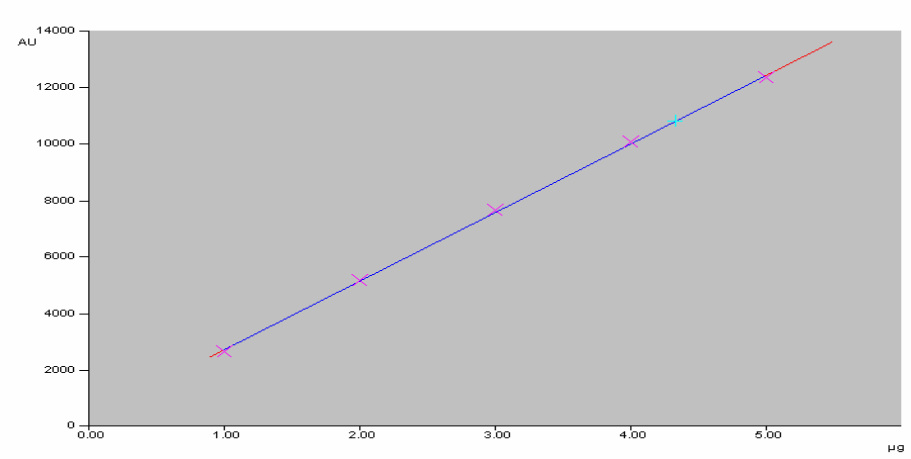
3-hydroxy androstane [16,17-C](6'methyl, 2'-1-hydroxy –isopropene-1-yl) 4,5,6 H pyran present in *Syzygium cumini* seed is one of the important marker compound. This marker compound has antidiabetic activity. In this work a HPTLC method has been developed for quantification of marker in dry seed powder extract of *Syzygium cumini*. This method can be used as quality control method for *Syzygium cumini*. The validated HPTLC method employed here proved to be simple, fast, accurate, precise and sensitive, thus can be used for routine analysis of 3-hydroxy androstane [16,17-C](6'methyl, 2'-1-hydroxy –isopropene-1-yl) 4,5,6 H pyran in *Syzygium cumini* seed extract.

Table 1. NMR Data of Marker compound isolated from *Syzygium cumini*

SR. NO.	δ VALUE	INTEGRATION	NO. OF HYDROGENS	
1	12.026	0.2	1H	Enolic alcohol
2	5.3	0.56	3H	Methine CH
3	2.7	0.27	1H	Alcohol OH
4	2.1-2.46 1.8-2.1 1.4-1.8 0.9-1.1 0.6-0.9 0.5	1.00 1.28 2.24 1.08 1.91 0.21	5H 6H 11H 5H 10H 1H	Hydrogen's attached to rings and of methyl groups attached.

Fig. 2. Calibration plot obtained by chromatography of marker compound

Regression via Area Regression mode = Linear



Y = 2431.832x + 269.127 r^2 = 0.99982

Table 2 : Method validation data

Instrument precision (CV%, n =6)	0.57
Method precision (CV%, n =6)	0.37
Limit of Detection (ng)	131
Limit of quantitication (ng)	430
Specificity	Specific
Linearity (correlation coefficient)	0.99982
Range (ng per spot)	1000 - 5000
Robustness	Robust

Table 3. Intra-day and inter-day precision of the method (n = 6)

Amount (ng/ spot)	Intra- day precision			Inter- day Precision		
	Mean area	SD	% RSD	Mean area	SD	% RSD
1000	2489.58	1.866	0.0749	2493.395	3.5284	0.141
2000	4901.485	2.849	0.0581	4907.68	5.9114	0.12
3000	7345.425	1.1667	0.0158	7341.09	4.9568	0.067

Table 4. Recovery studies (n = 3)

Amount of marker added (ng)	Amount of marker found (ng)	Recovery (%)	Average Recovery (%)
500	499.25	99.85	100.30
1000	1011.47	101.147	
1500	1498.66	99.91	

Table 5. Robustness of the method.

Amount (ng/ spot)	%RSD	
	Toluene: Ethyl acetate (8.5: 1.5)	Toluene: Ethyl acetate (7.5: 2.5)
1000	0.95	1.43
2000	0.98	1.47

Fig. 3. Spectra of Marker compound

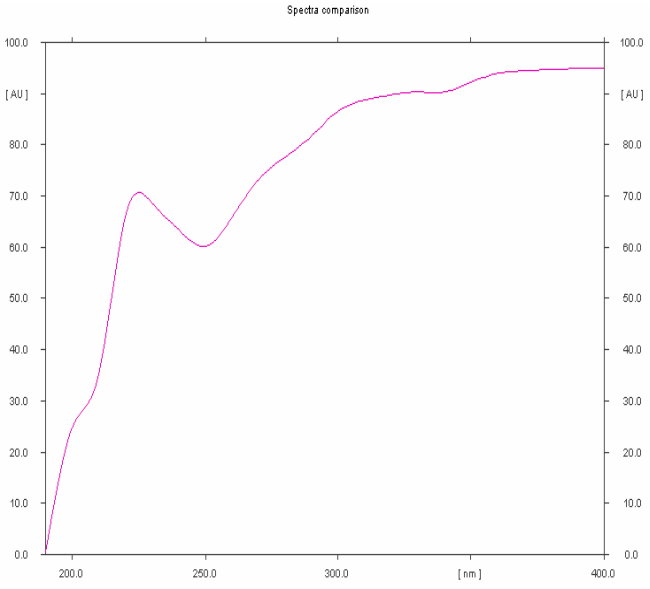


Fig. 4. Spectra of Ethanolic extract of *Syzygium cumini*

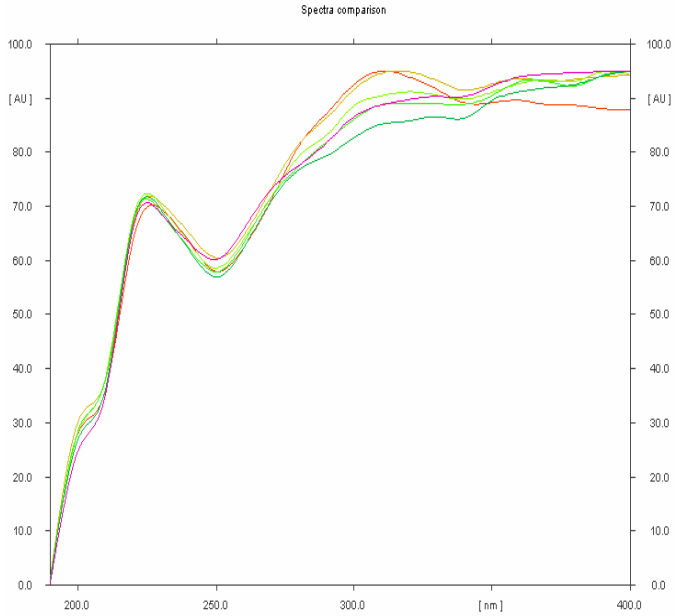


Fig. 5. Chromatogram of marker compound

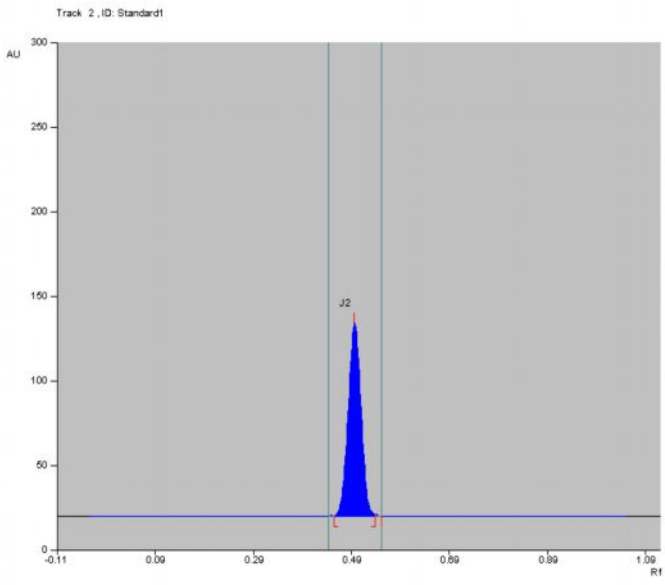
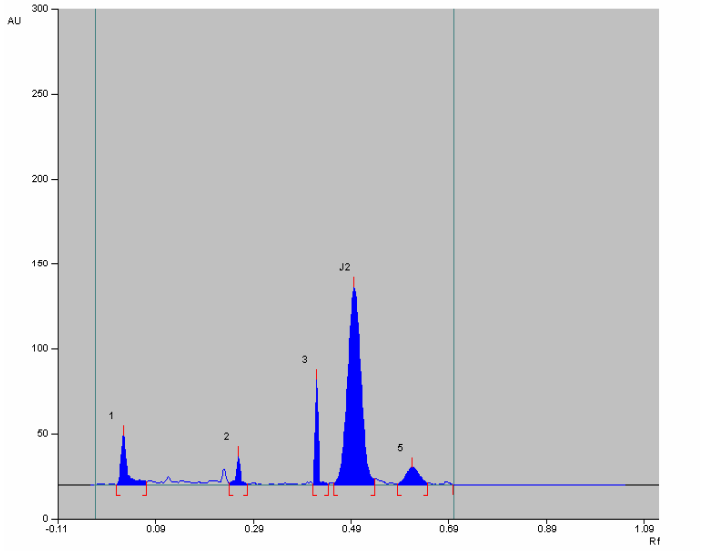


Fig. 6. Chromatogram of Ethanolic extract of *Syzygium cumini*



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