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Quantification of LUPEOL in *Excoecaria agallocha* Leaf, Stem and Root by HPTLC

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Abstract

An attempt to augment to quantify Lupeol, pharmacologically bioactive triterpenoid in the Excoecaria Agallocha mangrove (methanol extracts of leaf, stem, root parts), has been made. A HPTLC method was developed with the combination of n-Hexane: Ethyl acetate: Methanol (9:1:1 v/v) as solvent system and TLC silica gel 60 F_{254}plates as stationary phase under constant room temperature and relative humidity of 26.1 ± 0.4 ^{0}C and 76 ± 2 % respectively. Detection and quantification were performed by densitometry scanning at 540 nm after derivatization with Anisaldehide Sulfuric Acid Reagent (ASR). This method gave compact spots at R_F 0.31 corresponding to Lupeol. As a result, the content of Lupeol in the leaf, stem and root were found 492.42 ng, 704.23 ng and 728.15 ng respectively as well as % amount (w/w) of Lupeol in the leaf, stem and root were found 0.09948, 0.14085 and 0.14568 respectively.

Keywords: *Excoecaria agallocha*, HPTLC, Lupeol and Quantification.

Introduction

Mangroves are gigantic source of various phytochemicals[1-3]. Different mangroves including *Excoecaria agallocha* (also known as Thillai mangrove or blind your eye mangrove) having precious biological, pharmacological[2-5] and toxicological activities[6-7] as well as a rich platform of new and already existing bioactive compounds used as drug in different disease prevention strategies and systems[2,5,8]. The Lupeol, an active principle triterpenoid, is one of them, has voluminous biological activities like Antiprotozoal, anti- tumor (Anti-prostate cancer, Anti-melanoma, Anti-head and neck squamous cell carcinoma, Anti-pancreatic cancer), anti-inflammatory, antimicrobial, Nutraceutical or chemopreventative agent (Cardioprotective, Hepatoprotective and cancer chemoprotective etc.)[9-11].

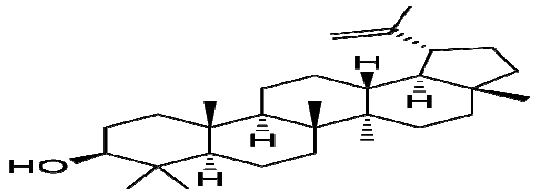

Figure-1: Chemical structure of Lupeol.

The identification and quantification these bioactive compounds from herbal origins has always been a difficult job for the researcher because of a variability of compounds, absence of working standards, small amount of compounds and unknown status in lots of times as well as many other influencing genetic and non-genetic factors associated with them[12]. For these reasons many analytical techniques are present to address and solve these issues like HPLC, GC, and HPTLC etc.[13]. Among them HPTLC has grown into a strong and indispensable analytical technique because of its simplicity, quickness of results and cheapness as compared with other techniques and the combinations with other advanced techniques made HPTLC, a most useful and applicable technique for herbal drug analysis[14-16].

In this present research work we used HPTLC as analytical method to quantify Lupeol in methanol extracts of *Excoecaria agallocha* leaf, stem and root parts and compared the presence of this bioactive compound among the different parts. This HPTLC method found as very cheap, extremely fast and less solvent consuming method for Lupeol detection - quantification and can be used as a routine method for Lupeol related analysis in the *Excoecaria agallocha* plant parts as well as serve as a base study for quantification of Lupeol in other herbal formulations.

Materials and methods

The *Excoecaria agallocha* mangrove sample was collected in October 2014 from the S.P. Godrej Marine Ecology Centre, Vikhroli, Mumbai city of Maharashtra, India. The plant materials were identified and authentication certificate issued by the same institute.

Extraction of the plant material and sample preparation: The leaf, stem and root are shed dried for 21 days and grounded by mechanical grinder into coarse particles. For the extract preparation 500 mg of power (leaf/stem/root) was mixed with 10 ml methanol. All three extracts were sonicated for 2 hours until the extracts were clear or colorless. High temperature and

directsun light were avoided to protect heat sensitive phytochemicals. Finally extracts were filtered (Whatman No.1 filter) to get the sample ready for HPTLC analysis.

Standard solution preparation: Stock preparation - 10 mg standard was mixed with 10 ml of n – Hexane in the 15 ml reagent bottle for making 1mg/ ml stock solution.

Working preparation - 1 ml of stock solution was taken in the 15 ml reagent bottle and mixed with 10 ml n – Hexane for making 0.1 mg/ml working solution.

Stationary Phase: HPTLC Aluminum plate silica gel $60F_{254}$ precoated 20 x10 cm.

Pre – activation of plate: Heating the plate on CAMAG Plate Heater at 110 ^{0}C for 3 minutes.

Mobile phase: n – Hexane: Ethyl acetate: Methanol (9:1:1) respectively.

Development chamber: Twin – through chamber of 20 x 10 cm.

Chamber Saturation time: 20 minutes.

Plate development time: 10 minutes (Till solvent front reaches at 80 mm on the plate).

Room temperature: 26.1 ± 0.4 ^{0}C (constant).

Relative humidity: 76 ±2 % (constant).

Applicator parameters: CAMAG LINOMAT V was used for application. Spraying gas was inert nitrogen gas. 150 nl/s dosage speeds were automatic adjusted according to the solvent system, which was for methanol.

Sample application: Samples were applied with the use of CAMAG LINOMAT V sample applicator. 15 µl of samples in triplicate for each leaf/stem/root sample and 2 - 8 µl of Lupeol were applied on 20x10 cm TLC plate and the band lengths were 8 mm. Numbers of tracks were 10. The track number 1, 2 and 3, 4, 5, 6, 7, were assigned for ascending order of standards concentration and track number 8, 9, 10 were represented the applied sample in triplicate for any one among the leaf/stem/root of *Excoecaria agallocha* mangrove.

Development Distance – 80 mm from the bottom edge.

Visualization – Visible white light.

Derivatizating – Plate was dipped on Anisaldehyde Sulphuric Acid (ASR) solution for 1 minute and then heated on CAMAG Plate Heater at 110 ^{0}C for 10 minutes.

Photo documentation of detection – Only after derivatizating with ASR visible light.

Measurement mode–After derivatizating visible light.

Densitometry – Tungsten (W) lamp was used for visible light (540nm). 6.00 x 0.45 mm (Slit Dimension), Micro and 20 mm/s (Speed of Scan).

Procedure: A working program was generated by the use of winCATS software in the computer. The specific volume of samples were taken by the use of 100 µl Hamilton Syringe and applied) on the TLC plate as the predefined 8 mm band length by CAMAG LINOMATV sample applicator. After the completion of the sample applicator program the plate was subjected for drying with the use of a drier and then placed onto CAMAG plate heater for 5 minutes for elimination of any water and moisture content from the plate. The prepared specific mobile phase was subjected to the CAMAG Twin- Through chamber for 20 minutes for saturation. A filter papered rinsed with mobile phase was also exposed in the chamber to get a uniform vapour saturation scenario in the chamber prior to adding the sample applied plate.

After the 20 min saturation of the CAMAG Twin- Through chamber, the plates were placed in it, till the solvent front reached up to the distance of 80mm (previously marked). This process took 8 - 10 minutes to develop the plate depends on the interactions between the mobile phase molecules, stationary phase molecules and sample molecules.

After development, the plates were subjected to drying by hot air device (drier) and 10 minutes on CAMAG plate heater (110^0C) at room temperature and kept it for documentation in the CAMAG TLC visualize and images were taken after derivatization with ARS under white light. After these, the plated were subjected to densitometry evaluation under tungustan lamp, only visible detection was applied after derivatization with ARS and scanning was performed under 540 nm.

Results and discussion

Qualitative HPTLC plate results – The qualitative HPTLC plate results showed the presence of characteristic spot at the R_F – 0.31 under 540 nm after derivatization with ASR. This spots (7 spots of Lupeol standards and 3 – 3 spots of leaf, stem, root respectively in triplicated samples) were visible as purple – blue in the white light and can be detectable under 540 nm densitometrical (Figure-2).

Quantitative HPTLC plate results –The densitometry evaluation of plate under 540nm after derivatization with ASR showed the average R_F – 0.31 corresponding to Lupeol in the *Excoecaria agallocha* leaf and the 9420.2 AU average area of that Lupeol peak (Table-1). The same as leaf, the stem and root parts expressed the average area of 12249.6 AU and 5106.3 AU respectively for the Lupeol peak (Table-2 and 3). The volume of Lupeol on plate in ascending mode from 2 µl - 8 µl and their corresponding calculated amount in nano gram and the calculated area in AU, were also presented (Table-4).

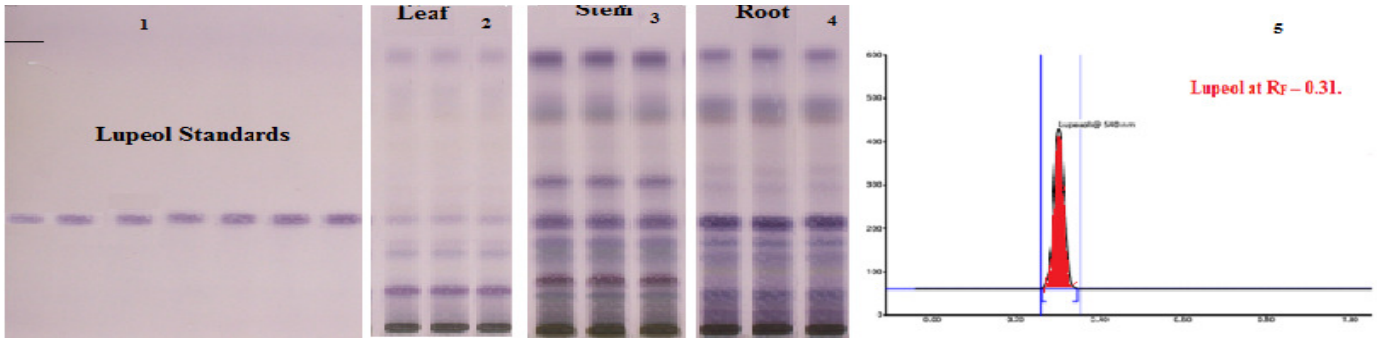

Figure-2: Quantification TLC plate of Lupeol and densitogram. Here 1 = Lupeol standards from 1 to 7 samples in increasing from (sample volume 2 µl to 8 µl). Number 2, 3 and 4 = triplicate samples on TLC plate of leaf, stem and root respectively (sample volume 15 µl). Number 5 = densitogram of Lupeol at R_F – 0.31 under 540 nm after derivatization with ASR.

Table-1: *Excoecaria agallocha* leaf triplicated densitogram results.

Track	Peak	Start Position (R_F)	Start Height (AU)	MAX Position (R_F)	End Position (R_F)	End Height (AU)	Area (AU)	Area %	Assigned Substance
A	1	0.28	0.71	0.31	0.34	3.8	9428.4	100	Lupeol
B	1	0.29	0.69	0.32	0.35	3.9	9396.5	100	Lupeol
C	1	0.26	0.70	0.30	0.33	3.6	9435.7	100	Lupeol
Total Average		0.27	0.7	0.31	0.34	3.76	9420.2		

Table-2: *Excoecaria agallocha* stem triplicated densitogram results.

Track	Peak	Start Position (R_F)	Start Height (AU)	MAX Position (R_F)	End Position (R_F)	End Height (AU)	Area (AU)	Area %	Assigned Substance
D	1	0.26	2.3	0.31	0.34	1.5	12279.1	100%	Lupeol
E	1	0.28	2.0	0.32	0.36	1.8	12241.3	100%	Lupeol
F	1	0.24	2.1	0.30	0.32	1.3	12226.6	100%	Lupeol
Total Average		0.26	2.1	0.31	0.34	1.53	12249.6		

Table-3: *Excoecaria agallocha* root triplicated densitogram results.

Track	Peak	Start Position (R_F)	Start Height (AU)	MAX Position (R_F)	End Position (R_F)	End Height (AU)	Area (AU)	Area %	Assigned Substance
G	1	0.24	9.9	0.30	0.32	0.5	12397.8	100%	Lupeol
H	1	0.27	9.8	0.32	0.35	0.7	12337.5	100%	Lupeol
I	1	0.25	9.9	0.31	0.33	0.6	12512.1	100%	Lupeol
Total Average		0.25	9.86	0.31	0.33	0.6	12415.8		

Standards curve fitting results – The standard curve fitting of the triplicated leaf, stem and root samples on standard Lupeol curve showed the specific place (Figure-3). For the leaf sample the value of Y = 3992 +11.03*X, the value of r = 0.99458 and the standard deviation = 2.21. For the stem sample the value of Y = 3205 + 12.81 *X, the value of r = 0.98706 and the standard deviation = 3.95. For the root sample the value of Y = 371.3 + 9.504 *X, the value of r = 0.99672 and the standard deviation = 4.34 (Table-5).

Table-4: Volume of Lupeol standards placed on TLC plate, their corresponding R_F, amount in nanograms on plate and calculated area in area units. The table is also showing the sample volumes, their R_F, their calculated amount in nanograms, calculated area and CV% in triplicated.

Track number	Peak	R_F	Sample volume	Amount (ng)	Calculated area (AU)	CV % calculated only for samples
1	Lupeol (standard)	0.31	2 µl	200	5311.62	NA
2	Lupeol (standard)	0.31	3 µl	300	6640.67	NA
3	Lupeol (standard)	0.31	4 µl	400	8678.53	NA
4	Lupeol (standard)	0.31	5 µl	500	9849.38	NA
5	Lupeol (standard)	0.31	6 µl	600	10985.05	NA
6	Lupeol (standard)	0.31	7 µl	700	11891.39	NA
7	Lupeol (standard)	0.31	8 µl	800	12885.97	NA
A,B,C (avg.)	LUPEOL in *Excoecaria agallocha* leaf	0.31	15 µl	492.42 ng	9420.2	1.425
D,E,F (avg.)	LUPEOL in *Excoecaria agallocha* stem	0.31	15 µl	704.23 ng	12249.6	0.567
G,H,I (avg.)	LUPEOL in *Excoecaria agallocha* root	0.31	15 µl	728.15 ng	12415.8	1.467

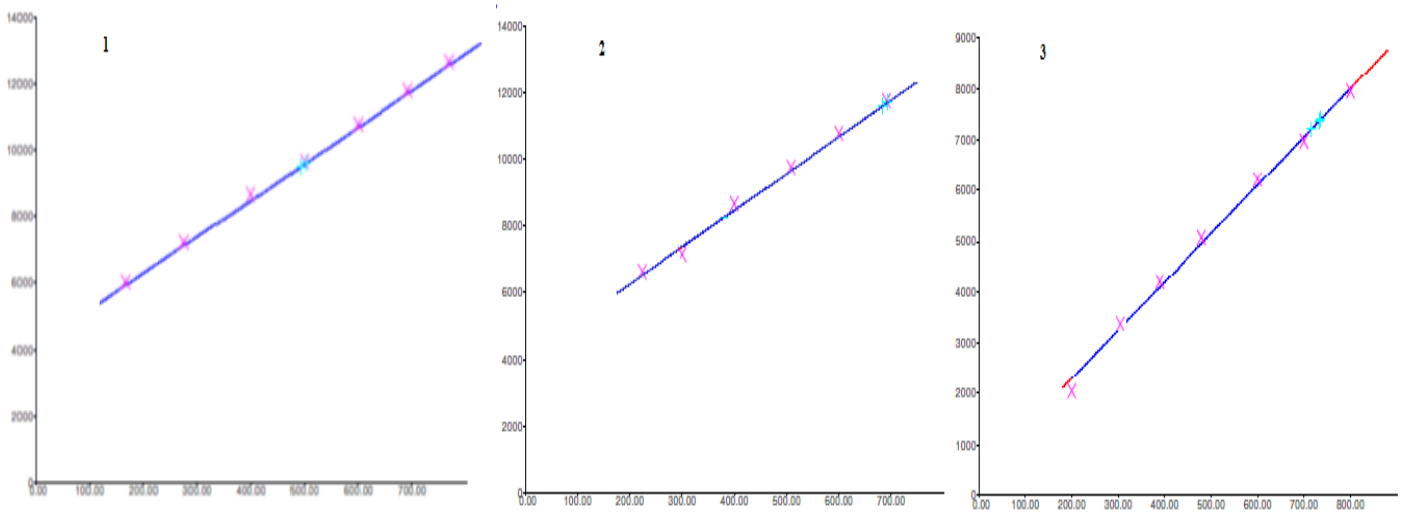

Figure-3: Lupeol standard curves between the calculated area unit (AU) vs concentration in nano gram (ng). The number 1,2,3 graph referees for leaf, stem and root respectively.

Table-5: The regression curve values and curve fitting for leaf, stem and root.

Graph number	Y (intercept)	r value	SDV
1. Leaf	3992 +11.03*X	0.99458	2.21
2. Stem	3205 + 12.81 *X	0.98706	3.95
3. Root	371.3 + 9.504 *X	0.99672	4.34

Calculation of amount of Lupeol –the initiation concentration of the sample (500 mg / 10 ml) was multiplied with the sample volume size (10 µl) for concentration and percentage area concentration calculations with the respect of Lupeol standards (0.1 mg/ml and sample size 2 µl - 8 µl)pulled on the TLC plate. The final calculations achieved by the calculate peak area (in area units) of the sample and respect to the linear line of the standards on the graph with the help of winCATS software.

Conclusion

In conclusion the Lupeol is present among all three *Excoecaria agallocha* methanol extracts in a significant level. This HPTLC method for quantification of Lupeol in *Excoecaria agallocha* methanol extracts is an extremely simple, very low-cost, enormously quick and tremendously fast method to assess the amount of Lupeol. The presence of this precious bioactive compound (Lupeol) made the leaf, stem and root parts of this mangrove an identified natural source and can be used for the making of isolation strategies for this bioactive compound from this plant as well as other herbal raw materials. The root showed the maximum amount of Lupeol followed by stem and leaf respectively. The root of this mangrove can be used for higher amounts of this pharmacologically active compound in the form of Lupeol isolation or other formulation of Lupeol.

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