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METHOD DEVELOPMENT AND VALIDATION STUDIES FOR ESTIMATION OF APIGENIN IN *ABIES WEBBIANA* AERIAL PARTS USING TLC DENSITOMETRY

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ABSTRACT :

According to the literature review, the plant has not yet been standardized based on its primary bioactive marker compound. Therefore, the goal of the current studies is to validate the established method in accordance with ICH requirements and standardize the plant based on apigenin, which has been scientifically separated from the plant, using a TLC densitometer. After defatting with n-hexane using a Soxhlet system, the methanol extract of plant aerial parts was made. The presence of flavonoids is confirmed by the Shinoda test of methanol extract. Using the toluene:ethyl acetate:methanol (10:7:3) mixture, the TLC densitometric method was created to achieve the best resolution of apigenin in methanol extract. Apigenin was discovered to be 0.4532 ± 0.0003% w/w in plant aerial parts. The created strategy for assessment of apigenin in *A. webbiana* aerial parts was approved according to the rules of ICH. The created method's validation metrics, including instrumental accuracy, intra- and inter-day precision, robustness, ruggedness, and repeatability, showed a percentage coefficient of variation that was within the specified range. In accuracy studies, apigenin recovery was greater than 98%. Additionally, there was no difference between the sample and standard's thin layer chromatograms and ultraviolet spectra. These findings suggest that the established method for estimating the amount of apigenin in the aerial sections of A. webbiana is specific, accurate, repeatable, and precise. The online scientific reports suggested that apigenin show their pharmacological activities through various related mechanisms. Thus it is suggested from the observations that apigenin may be one of the bioactive compound of *A. webbiana.* Therefore, it is selected as a marker to standardize *A. webbiana* aerial parts.

Key words: Abies webbiana, TLC densitometry, Apigenin, Flavonoid.

Introduction

A. webbiana Lindl. (Pinaceae family) Syn. *Abies spectabilis* (D. Don Spach.), commonly referred to as the West-Himalyan important level fir in English, Badar in Kashmir, Talispatra in Hindi and Bengali, and Talispatram in Sanskrit, is a massive, tall, evergreen tree that can grow up to 50 meters in height (Pullaiah, 2002; Khare, 2007). *A. webbiana* is utilized as society drug in ayurvedic arrangement of medication for the treatment of different problems, for example, cough, cancer, vomiting, chronic obstructive pulmonary sicknesses and mouth issue (Anonymous, 1976). The plant has been reported to contain various phytomolecules such as alkaloids – taxine (Nadkarni, 1976), 1,(4-methoxy phenyl) aziridine (Ghosh *et al*., 2010), 2-(*o*-tolylamino) ethanol (Ghosh and Bhattacharya, 2010); phytosterols - β-sitosterol, betuligenol (Rao *et al.,* 2012); glucosides – betuloside, methyl betuloside (Sarkar *et al*.,1987); bioflavonoid – abiesin (Chatterjee *et al*., 1984) and lignans – olivil (Yang et al., 2008). Numerous pharmacological actions, including sedative, anti-inflammatory, and antibacterial properties, have been described for the plant (Nayak et al., 2004; Vishnoi et al., 2007a; Kumar et al., 2006; Vishnoi et al., 2007a). According to the literature review, the plant has not yet been standardized based on its primary bioactive marker compound. Thus, the present investigations will be designed to standardize the plant on the basis of apigenin using TLC densitometer and validate the developed method as per ICH guidelines.

Materials and Methods

Plant material

In December 2024, aerial parts for *A. webbiana* were purchased from D.G. Ayurvedic Sangrah, Andheri, Mumbai, India. Additionally, on

10/01/2025, Dr. Preet Kawal Kaur, Professor at the Institute of Pharmaceutical Sciences, Bhaddal, Mianpur, Ropar, Punjab, India, issued an identification certificate with reference number Pcog/Auth/06/2025.

Preparation of extracts

The aerial parts of *A. webbiana* were dried in hot air oven at a constant temperature of 35˚C. The dried material is crushed and changed into fine powder with help of mixer grinder. The powdered material (10 g) was successively extracted with solvents (50 ml) in order of increasing polarity viz., n-hexane and methanol through Soxhlet process at temperature of 80˚C. The solvents from crude extracts were recovered by distillation process. The percentage yield of methanol extract was calculated and stored in a desiccator for further phytochemical and TLC densitometric studies (Sujata et al., 2017).

Phytochemical screening

The methanol extract was screened through various chemical tests for the confirmation of the flavonoid presence in the plant (Farnsworth, 1996)

Shinoda test

To the alcoholic test solutions, magnesium turnings and concentrated hydrochloric acid were added. An appearance of red color indicates presence of flavonoids.

TLC densitometric method development

Test solutions

After being defatted by n-hexane, 10 g of coarsely powdered plant material was extracted completely using methanol in a Soxhlet system. Lower pressure was used to recover the solvent. After drying the extract, methanol was added to a volumetric flask to get the volume down to 25 ml.

Preparation of standard plot

For the TLC densitometric investigations, a stock solution containing a concentration of 1 mg/ml of the marker was made in methanol. Methanol was added to the marker stock solution to create six dilutions with varying concentrations (5, 7.5, 10, 12.5, 15, and 17.5 µg/ml). On a TLC plate that had already been coated, ten microliters of each dilution were applied in triplicate. The plate was developed at a distance of 8 cm in a chamber saturated for 10 minutes using the solvent system toluene:ethyl acetate:methanol (10:7:3). After being dried in a hot air current, the produced plate was scanned at 336 nm using a TLC scanner. For every track, the AUC of the peak that corresponded to the marker compound was recorded.

Estimation of marker compound

On a pre-coated TLC plate (5 × 10 cm), test solutions (10 µl) of methanol extract were applied in triplicate. The same process used to prepare a normal plot was followed to develop and scan the plate. The concentration of the marker compound was determined using the standard plot, and the average AUC of the peak corresponding to it was recorded in the test sample at 336 nm.

Validation of TLC densitometric method

The following criteria were used to validate the developed TLC densitometric approach in accordance with ICH recommendations (Randhawa et al., 2015).

RESULTS AND DISCUSSION

Confirmation of flavonoids presence in methanol extract of plant aerial parts

To find out whether flavonoids were present, a phytochemical assay called the Shinoda test was performed on a methanol extract of plant aerial parts. Flavonoids are present in the methanol extract, according to the phytochemical test.

Estimation of apigenin in plant aerial parts using TLC densitometer

The quantity of apigenin in plant aerial parts was calculated with help of standard plot between amount of apigenin (ng) loaded vs. area under the curve obtained (Figure 1). The content of apigenin in plant aerial parts was found to be 0.4532 ± 0.0003% w/w. Figure 2 displays the thin layer chromatogram overlay of the apigenin and methanol extract of plant aerial parts. Figure 3 displays the methanol extract of plant aerial parts and the ultraviolet overlay of apigenin.

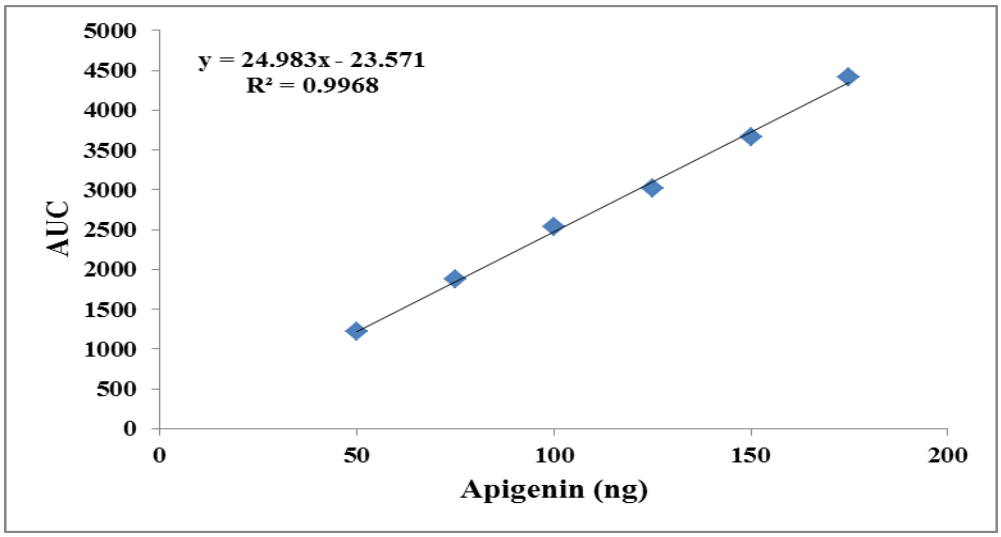

Figure 1: Standard plot of apigenin.

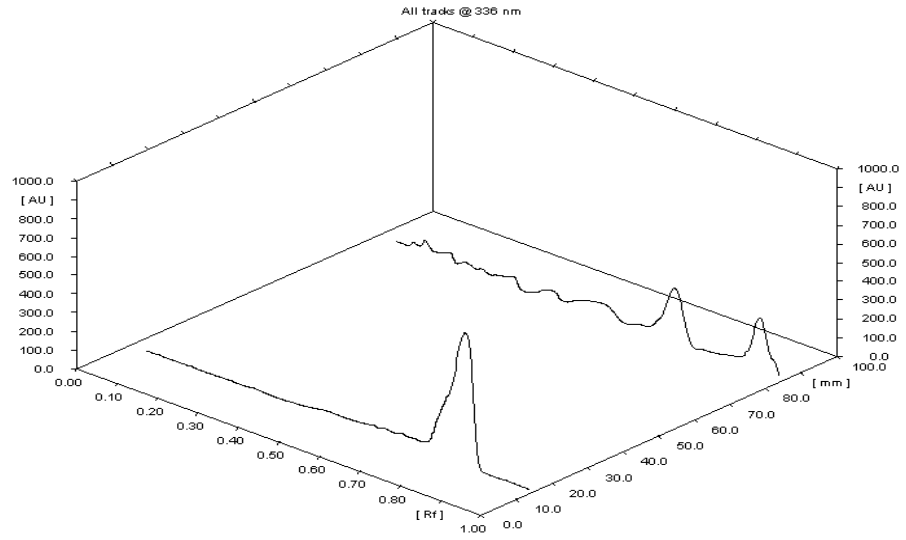

Figure 2: Shows the apigenin and methanol extract of plant aerial parts superimposed over a thin layer chromatogram.

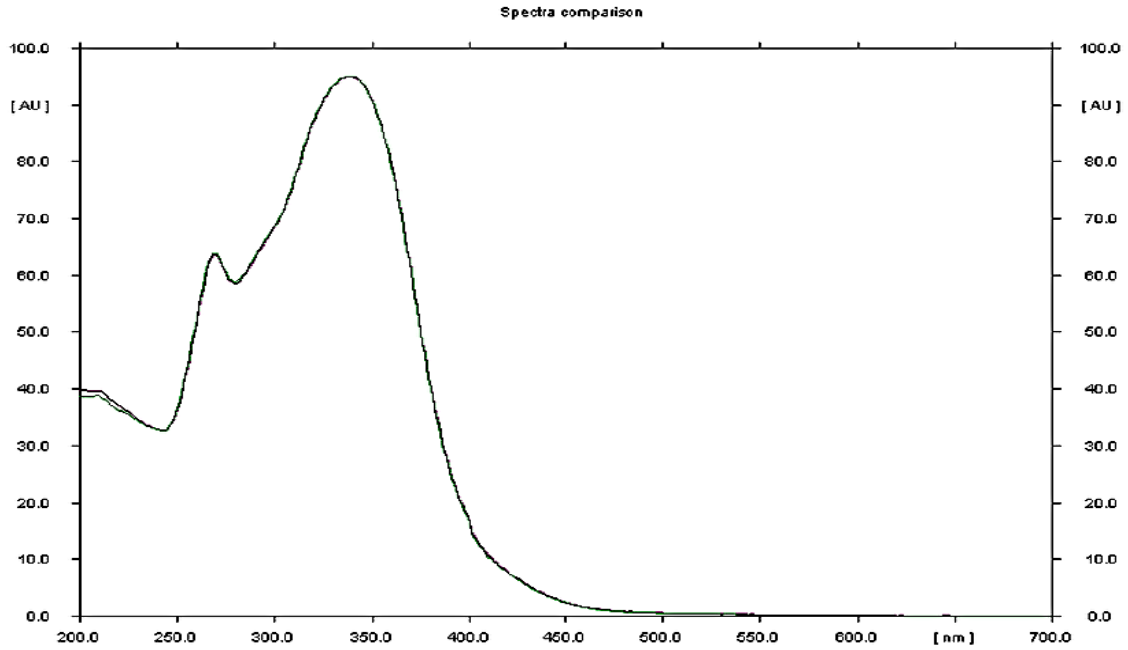

Figure 3: Apigenin and methanol extract of plant aerial parts thin layer spectrum overlay.

Method validation studies of developed TLC densitometric method

In present examination, TLC-densitometric technique was produced for the assessment of apigenin in *A. webbiana* aerial parts. Further, the created strategy was approved for the boundaries depicted in ICH rules. The content of apigenin in plant aerial parts was discovered to be 0.4532 ± 0.0003% w/w. The created strategy for assessment of apigenin in *A. webbiana* aerial parts was approved according to the rules of ICH. The created method's validation metrics, including instrumental accuracy, intra- and inter-day precision, robustness, ruggedness, and repeatability, showed a percentage coefficient of variation that was within the specified range. In accuracy studies, apigenin recovery was greater than 98%. Additionally, there was no difference between the sample and standard's thin layer chromatograms and ultraviolet spectra. These findings suggest that the established method for estimating the amount of apigenin in the aerial sections of A. webbiana is specific, accurate, repeatable, and precise.

Table 1: Method validation parameters of TLC-densitometric method.

Parameter	Apigenin
Instrumental precision (% CV, n=7)	0.77
Repeatability (% CV, n=5)	0.61
Coefficient of determination	
Linearity (ng)	50-175 ng
LOD (ng)	8
LOQ (ng)	29
Intra-day precision (% CV, n=9)	0.85
Inter-day precision (% CV, n=9)	0.96
Accuracy (average % recovery)	98.26
Specificity	Specific
Robustness 334 nm / 338 nm	0.96 / 0.59
Ruggedness Analyst 1 / Analyst 2	0.94 / 0.80

The accuracy is the percentage of marker compound or analyte recovered by assay from known added amount. Solutions were prepared in triplicate at three levels 50%, 100% and 150% of marker compound. The result of the accuracy studies is displayed in table 2.

Table 2: Results of recovery studies.

Amount of apigenin (µg)	Amount of apigenin added (µg)	Amount of apigenin found (µg) (Mean^n ± S.D.)	Recovery (%)	Average Recovery (Mean ± S.D.)
100	50	146.90 ± 1.25	97.93	
100	100	195.14 ± 1.06	97.57	98.26 ± 0.91
100	150	248.25 ± 1.18	99.30	

n = 3

The TLC densitometry is extremely valuable subjective examination strategy; it consolidates the craft of chromatography and with quickness at a moderate expense. It is a significant progression of TLC principle with less time consuming and better separation of phytoconstituents. TLC densitometry is a refined instrumental procedure with benefits of simple strategy improvement and approval, validation, filtering, full advancement, specific recognition of marker compound, less requirement of sample, and so forth, empower it to be an incredible analytical apparatus for quantitative assurance of specific compound(s) in complex combinations of inorganic, natural and biomolecules (Randhawa et al., 2015).

Conclusion

The apigenin is one of the significant dietary flavonoids related to class of flavonols. The ethyl ether-induced hypnosis test (Gazola et al., 2015) demonstrated that apigenin had sedative qualities at 0.6 mg/kg, p.o.; the actophotometer showed mild locomotor activity at 25 mg/kg, p.o. (Kumar et al., 2008); the EPM showed anxiolytic activity at 2 mg/kg, p.o. (Kumar and Sharma, 2006); the FST showed antidepressant activity at doses of 12.5, 25, or 200 mg/kg, i.p. (Nakazawa et al., 2003); and the tail immersion test revealed analgesic activity at 10 mg/kg, p.o. (Kumar et al., 2008). The online scientific reports suggested that apigenin show their pharmacological activities through various related mechanisms. Thus it is suggested from the observations that apigenin may be one of the bioactive compound of *A. webbiana.* Therefore, it is selected as a marker to standardize *A. webbiana* aerial parts.

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