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Analytical Standards for the Flowers of *Tupistra Nutans* Wall. -A Rare Medicinal Plant of Sikkim Himalayan Region

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ABSTRACT

Standardization of herbal drugs is the need of the hour as the use and practice of traditional herbal drugs has increased tremendously. The main objective of the present study is to standardize the flowers of Tupistra nutans as per pharmacopoeial testing protocol which includes powder microscopy, physico-chemical screening, TLC profile and chromatographic studies. Preliminary phytochemical screening ascertains presence of phytosterols in petroleum ether and triterpenoids in benzene extract. Alkaloid, phytosterols, glycosides & phenolics in chloroform extract. Carbohydrates, triterpenoids, glycosides, phenolics, tannins in methanol extract and proteins, glycosides, carbohydrates and flavonoids in aqueous extract of the plant flower extracts.TLC profiling of all plant extracts also give an idea about the presence of these phytochemicals. R_f (Retention factor) value of different phytochemicals provide valuable clue regarding their polarity and selection of solvents for separation of phytochemicals. Concentrating on results obtained in TLC, the column chromatographic studies were set. The fraction containing search here was flavonoids as, quercetin. Performing TLC of each individual fraction, same R_f value fractions were collected& concentrated during column chromatography.

Keywords: *Tupistra nutans*, phytochemical screening, TLC Profiling, & Retention factor (Rf.), Column chromatography

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INTRODUCTION

The genus *Tupistra* [Family Liliaceae] widely spread in eastern Himalayan region of the world with long strap shaped leaves borne on a rosette from stout rhizome. Inflorescence produced in late summer. The flowers are fleshy and last for some time in full bloom. Cool growing it likes moisture on its roots throughout the year. Inflorescence with buds and open flowers made into curry. Texture and taste of button mushrooms, slightly bitter. Plant is widely cultivated in village homes in the hilly regions and during the flowering season the inflorescences are sold in markets along with other vegetables. The plant exhibit a wide spectrum of folk and indigenous medical uses. Powdered root and flower decoction are taken to control diabetes [1].

Due to its high cost as well as unavailabity, the chance for adulterating flowers of *Tupistra nutans* with substandard products is high. Thus to avoid adulteration, standardization of this valuable herbal drug is the need of the hour. In the present study an attempt has been made to standardize the original and authenticated flowers of *Tupistra nutans* by physicochemical characterization, TLC fingerprinting and column chromatographic analysis.

MATERIALS AND METHODS

Sample collection

The flower buds of *Tupistra nutans* plant for the proposed study were collected from Kalimpong, West Bengal in February 2012 and authenticated from Botanical Survey of India, Gangtok, where herbarium voucher specimen No. (SHRC-5/02/2014-Tech) has been deposited. Care was taken to select healthy plants and normal organs.

The collected flowers were cleaned and shade dried. Fresh samples were used for anatomical studies and dried parts were powdered, sieved and stored in an airtight container for further use.

Powder microscopy

Powder studies were carried out by using reagents and stains like iodine, potassium iodide, ferric chloride, Sudan III, ruthenium red and phloroglucinol with Con. HCl (1:1) [2, 3, 4]. Safranin (4%) and toludine blue were used to double stain the transverse sections [5]. All the reagents of analytical grade were procured from Hi-Media, Mumbai, India. Organoleptic characters like colour, texture, odor and taste were determined for flower powder.

Evaluation of Physical Constants

Physical constants have a major role in identification and purity determination of crude drugs. In the present study, physical constants such as total ash, acid insoluble ash, water soluble ash, alcohol soluble extractive and water soluble extractive values were evaluated as per standard protocols [6, 7, 8].

Preparation of extracts and preliminary phytochemical analysis

Petroleum ether (40-60^{o}), benzene, chloroform, methanol extract obtained by successive extraction method, and water extract by maceration method. All the extracts were subjected to proximate chemical analysis [9].

Preparation of extract for Thin Layer Chromatography [10]

200 g of powdered flowers was extracted with 2.5 liter of methanol by Soxhlet apparatus for 4 hrs. The TFME (*Tupistra nutans* flowers methanolic extract) was concentrated on rotary vacuum evaporator (ROTEVA EQUITRON, Mumbai) and further dried in vacuum dryer. 37.27 g yield obtain from 200 g powdered material i.e. 18.63 % w/w TFME.

TLC pattern for TFME [11]

The Tupistra nutans Flowers methanol extract shows presence of flavonoids in preliminary phytochemical study.

Materials and methods for TLC study [12]

Plate dimensions	: 15 x 5 cm (length x width).
Plate material	: Glass material
Stationary phase	: Silica gel GF_{254} slurry prepared in distilled water
Chamber material	: Glass material
Chamber dimensions	: 25 x 10 x 30 cm (length x width x height).
Solvent system	: Toluene: Ethyl acetate: Formic acid **(6:2:0.8)**
Saturation time	: 30 minutes.
Visualization	: 1. UV 365nm 2. Spray Aluminum Chloride (1% in ethanol); observed under 365nm
Sample	: TFME
Reference standard	: quercetin
Observation	: Flavonoid gives fluorescence

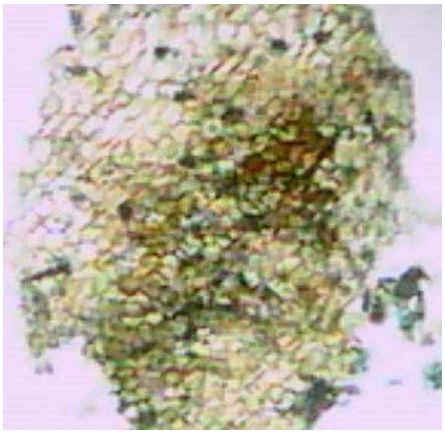
Column Chromatography (CC) of Methanol Extract of *Tupistra nutans* Flowers

The flavonoids are soluble in methanol solvent which was used for the isolation [13] [14]. The methanolic extract was suspended in small portion of water, extracted with ethyl acetate and then resulting solution were concentrated to provide ethyl acetate soluble parts. These parts were subjected to silica gel (60-120 mesh) column chromatography for the isolation of individual phytoconstituents. Ethyl acetate soluble part was eluted gradiently using chloroform, chloroform: ethyl acetate, ethyl acetate, ethyl acetate: methanol and methanol to give a compound which shown a single spot on TLC plate developed in Tolune: ethyl acetate: formic acid (6:2:0.8) and sprayed with 1% Aluminium chloride reagent.

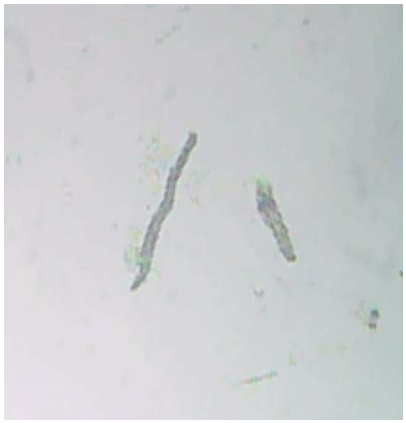
Column : Glass
Dimensions:
Diameter : 20 mm
Length : 550 mm
Stationary phase : Silica gel 60-120 #
Flow rate : 5 ml/min.
Fraction volume : 10-12 ml
Elution mode : Gradient
Elution : Chloroform (100 %)
Chloroform: Ethyl acetate (75:25)
Chloroform: Ethyl acetate (50:50)
Chloroform: Ethyl acetate (25:75)
Ethyl acetate (100)
Ethyl acetate: Methanol (90:10)
Ethyl acetate: Methanol (80:20)
Ethyl acetate: Methanol (70:30)
Ethyl acetate: Methanol (60:40)
Ethyl acetate: Methanol (40:60)
Ethyl acetate: Methanol (20:80)
Methanol (100)
No. of fractions collected : 41 for TFME

RESULTS AND DISCUSSION

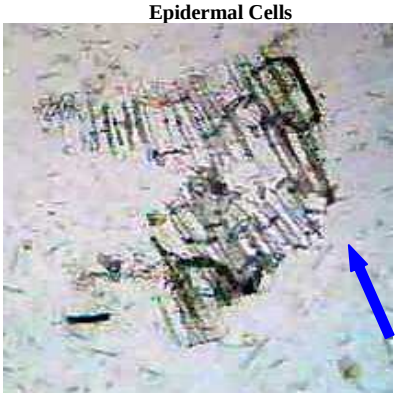
Figure 1. Powder characteristics of *Kalimpong* Flower



Epidermal Cells



Lignified fibres in pink color



Stomata in peduncle tissues

Ground tissues of the corolla

Anther and Pollen grains

Lignified Xylem Tissue

Ca Oxalate crystal

Lignified tissues in pink color

Non lignified fiber

Oil globule

Table 1. Physicochemical analysis

Sr. No.	Physical Standard		Results (%W/W)
1	Ash Values (As per Ayurvedic Pharmacopoeia)	Total Ash Acid Insoluble Ash Water Soluble Ash	09.33 ± 0.22 01.63 07.46
2	Extractive values (As per Ayurvedic Pharmacopoeia)	Pet. Ether Water soluble alcohol soluble	01.05 ± 0.07 08.10 ± 0.31 37.00 ± 1.03
3	Moisture content (As per Ayurvedic Pharmacopoeia)	Standard value {NMT 10% W/W}	9.35 ± 0.12

Table 2. Phytochemical analysis showing successive Extractive Values of *Tupistra nutans* flowers

Extractives	*Tupistra nutans* **flowers**	
	% w/w	Consistency
Pet. ether (40-60°)	0.29	Sticky
Benzene	0.73	Sticky
Chloroform	2.69	Sticky
Methanol (95 %)	17.83	Very Sticky
Water	2.91	Solid

Table 3. Preliminary Phytochemical Investigation of *Tupistra nutans flowers*

Solvent Extract	**Alkaloid**	**Carbohydrate**	**Phytosterols**	**Triterpenoids**	**Glycoside**	**Phenolics**	**Tannins**	**Proteins**	**Mucilage**
Petroleum ether [40-60°]	-	-	+	-	-	-	-	-	-
Benzene	-	-	-	+	-	-	-	-	-
Chloroform	+	-	+	-	+	+	-	-	-
Methanol	-	+	-	+	+	+	+	-	+
Water	-	+	-	-	+	+	-	+	+

The Methanol extract shows presence of Glycosides, so the specific tests of Glycosides for *Tupistra nutans flowers* were performed given in **Table 4**.

Table 4. Specific Tests of Glycosides *Tupistra nutans flowers*

Glycosides	*Tupistra nutans* ***flowers***
	Methanol extract
Cardiac	-
Antraqinone	-
Cynogenetic	-
Coumarin	-
Flavonoid	+
Saponins	+

'+' test is positive; '-' test is negative

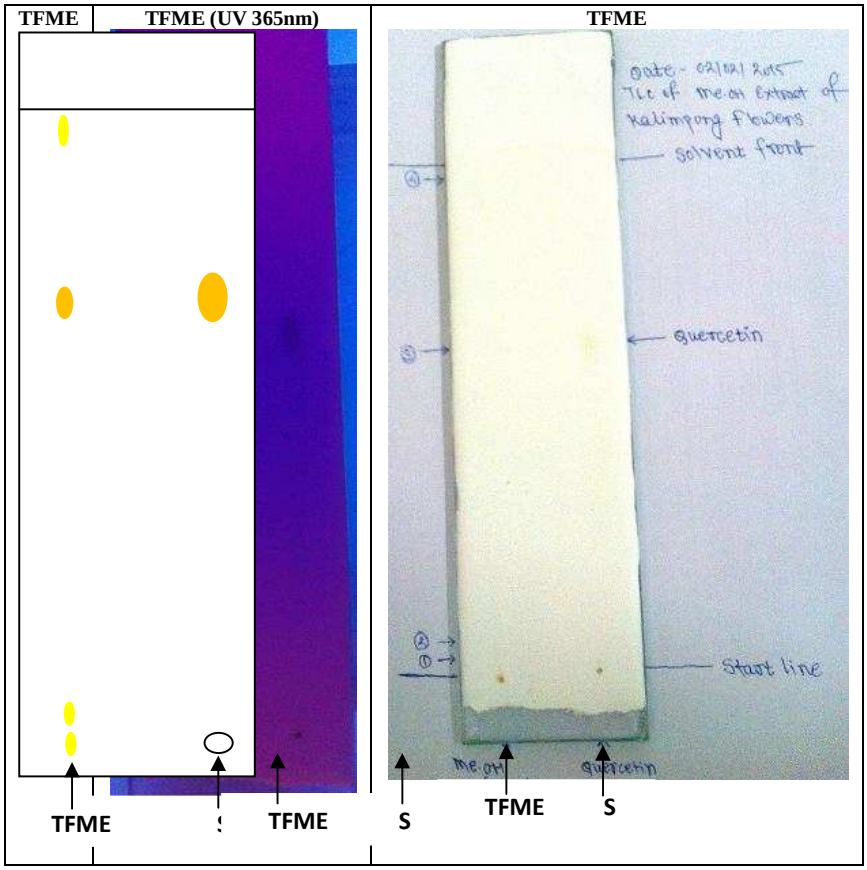
TLC Analysis

After adjusting the solvent system best results were arises as, R_f value of standard quercetin shows 0.64 match with the obtained R_f value of TFME.

Table 5. TLC Pattern for TFME

Fluorescence (Under UV 365nm)	**R_f Value**	
	TFME	**Std Quercetin**
Yellow	0.03	--
Yellow	0.07	--
Greenish Yellow	0.64	0.64
Yellow	0.98	--

The TLC profile has revealed the presence of four spots in KFME after observing under UV light 365nm. The intense spot observe after spraying 1% Aluminum Chloride solution, the screening was carried out targeting the Flavonoid viz., Quercetin.



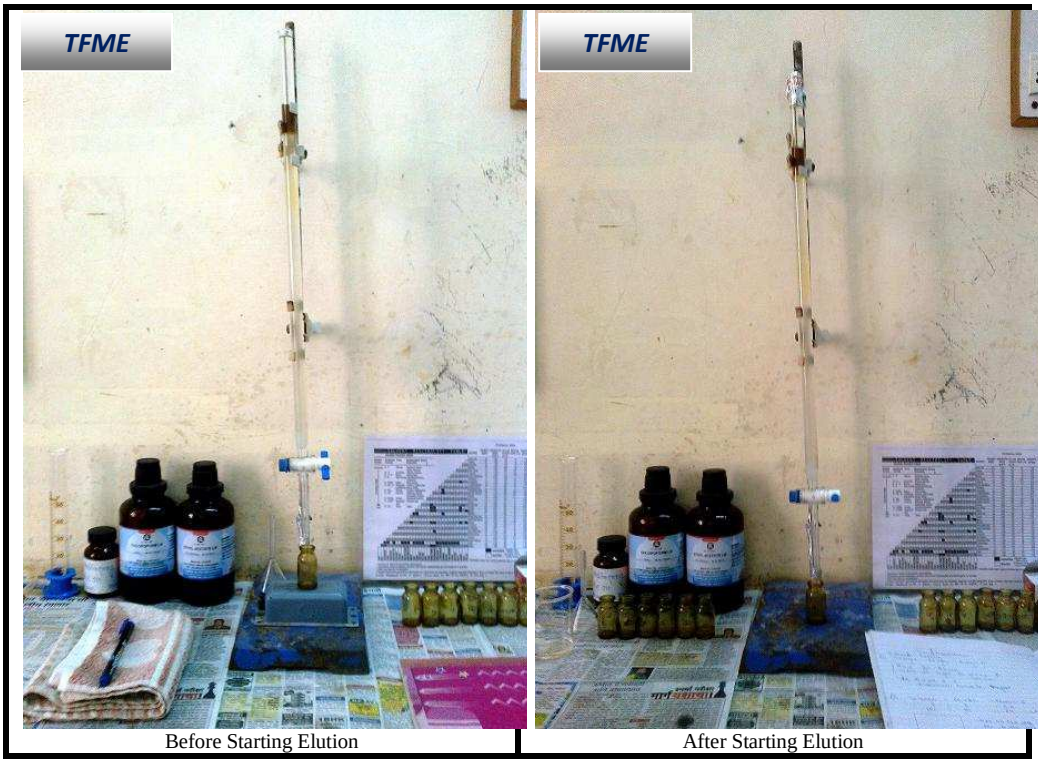
Photograph-1: TLC Plate of KFME after Spraying 1% Aluminum Chloride Solution
Abbreviation: TFME- Tupistra nutans Flowers methanolic extract; S- Quercetin.

Column Chromatography (CC) of Methanol Extract of Kalimpong Flowers
TLC pattern of fractions obtained from the column chromatography of KFME given in **Table 2**.

Concentrating on results obtained in TLC, the column chromatographic studies were set. The fraction containing search here was flavonoids as, quercetin. Performing TLC of each individual fraction (**Table 6)**, same R_f value fractions collected, concentrated during column chromatography.

Table 6. TLC Pattern of TFME Fractions

Fractions	TLC pattern	Qty (mg)
01-05	No any spot	--
06-21	Single Spot	25
22-27	Trace amount mixture	--
28-35	No any spot	--
35-41	Mixture of 2 compound	--



Photograph-2: Column Chromatography of TFME

Observation

TFME: In TLC fraction 06 to 21 shows compounds with similar R_f value for solvent system Tolune: ethyl acetate: formic acid (6:2:0.8) and sprayed with 1% Aluminium chloride reagent.

The fraction 06 to 21 prepared by mixing after evaporation of solvents it gives yellowish brown solid compound.

From this one pure compound (9mg) was obtained and labeled as; *Tupistra nutans* Flowers methanol fraction **(TFM-1)**.

TFM-1

Color	**:** Brownish yellow color
Solubility	**:** Soluble in chloroform, methanol, ethyl acetate and ethanol
Color	**:** Dull brown
Shinoda Test	**:** Pink color was obtained
λ_{max}	**:** 255, 373 nm

After studying the physical properties and chemical properties, we can predict that **TFM-1** is **quercetin.**

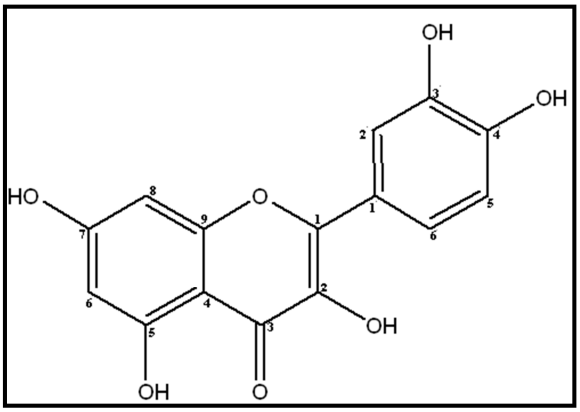


Figure 2. Predicted Structure of TFM-1 as, Quercetin

DISCUSSION

When a new drug is to be discovered, qualitative phytochemical analysis is a very important step as it gives information about the presence of any particular primary or secondary metabolite in the extracts of the plant which is having a clinical significance. In any case, if any significant bioactive natural product is present, it is necessary to separate that compound from the mixture of compounds by using suitable chromatographic technique.

The evaluation of a crude drug is an important diagnostic character useful in determining authenticity and identifying adulteration. As there is no phytochemical work recorded on this medicinally potent plant, the present work was undertaken to lay down the standards which could be useful for establishing its authenticity. Pharmacognostic parameters like microscopic features of flower have been studied. Preliminary phytochemical screening reveals the useful findings about chemical nature of drugs. Total ash values and extractive values are useful in identification and authentication of the plant material .Extractive values is useful to evaluate the chemical constituents of crude drug.

Preliminary phytochemical screening ascertains presence of phytosterols in petroleum ether and triterpenoids in benzene extract. Alkaloid, phytosterols, glycosides & phenolics in chloroform extract. Carbohydrates, triterpenoids, glycosides, phenolics, tannins in methanol extract and proteins, glycosides, carbohydrates and flavonoids in aqueous extract of the plant flower extracts.TLC profiling of extracts gives an impressive result that directing towards the presence of number of phytochemicals. Various phytochemicals give different R_f values indifferent solvent system. This variation in Rf values of the phytochemicals provide a very important clue in understanding of their polarity and also help in selection of appropriate solvent system for separation of pure compounds by column chromatography. Mixture of solvents with variable polarity in different ratio can be used for separation of pure compound from plant extract. The selection of appropriate solvent system for a particular plant extracts can only be achieved by analyzing the Rf values of compounds in different solvent system. Different R_f values of the compound also reflect an idea about their polarity. This information will help in selection of appropriate solvent system for further separation of compound from these plant extracts.

CONCULUSION

The results obtained in the present investigation indicated *Tupistra nutans* as a rich source of secondary metabolites. These findings suggested that *Tupistra nutans* could be a potential source of natural antioxidant having great importance as therapeutic agent and preventing oxidative stress related degenerative diseases

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