ORIGINAL ARTICLE

Phyto-physicochemical and high performance thin layer chromatography investigation of *Melilotus officinalis* Linn.

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Abstract

Context: *Melilotus officinalis* belongs to family Leguminosae (Fabaceae), historically has been used for a variety of medicinal purposes. Despite its popular medicinal utilization, still no conclusive study has been reported so far regarding the pharmacognostical standardization. **Aim:** Thus, the present study was focused to scientifically establish a standard monograph of *M. officinalis* on the basis of physicochemical and phytochemical parameters. **Materials and Methods:** The various physicochemical parameters such as ash values, extractive values, volatile oil content, moisture content, and fluorescence analysis of *M. officinalis* were determined for ascertaining the quality of crude drug. The preliminary qualitative and quantitative phytochemical analysis and high performance thin layer chromatography (HPTLC) of *M. officinalis* were performed. **Results:** The physicochemical parameters were established. The various phytochemicals such as carbohydrates, sugar, sterols, triterpenoids, anthraquinone glycosides, saponin glycoside, flavonoids, tannins, and phenolic compounds were detected in various extracts of *M. officinalis*. The methanolic and aqueous extract of leaves was found to contain high amount of total phenols and flavonoids compared to stem. The HPTLC fingerprinting of various phytochemical in methanol and aqueous extract was done. **Conclusion:** The obtained data would serve as a useful guide toward establishing pharmacognostic standards, identification, assessing purity, standardization, and preparation of monograph of *M. officinalis*.

Key words: Fabaceae, high performance thin layer chromatography, *Melilotus officinalis* Linn., yellow sweet clover

INTRODUCTION

The plant *Melilotus officinalis* belongs to family Leguminosae (Fabaceae), known as yellow sweet clover in English and aspurk in Hindi. It is a tall, robust biennial herb, about 1 meter in height. *M. officinalis* have trifoliolate leaves, the leaflets is obovate, oblong or oblanceolate in shape. The flowers are in lax racemes, yellowish in color, ovoid pods, transversely rugose, compressed brown when ripe. The seeds are oval in shape, 2-3 mm in diameter, yellowish green and smooth.[1] *M. officinalis* believed to be native to Pakistan, Kashmir (Nubra Valley and Ladakh at high altitude of about 3000-4000 m), Tibet, Russia, China, Turkey, and Middle, Southern Europe and it was introduced in America and Tropical Africa.[2] The earlier claims showed that *M. officinalis* has iron chelating,[3] antibacterial, antitumor,[4] anti-inflammatory,[5] antihypertensive,[6] and astringent activity.[7] The plant is aromatic, emollient, carminative. It relieves flatulence, externally applied as poultice for pains and aches. The small fruits are used as demulcent, maturant, tonic, aphrodisiac, and useful in leukoderma.[8] It was reported that *M. officinalis* contains flavonoids and various phenolic compounds, melilotin, volatile oil, mucilage, tannin, fatty acid, triterpenes, coumarin, bishydroxycoumarin, choline, and glycosides.[9] Previously, we had reported the morphological and microscopical character of *M. officinalis*[10] but still the detail pharmacognostical standardization of *M. officinalis* is lacking. Hence, the present work was focused to investigate the phyto-physicochemical properties of *M. officinalis*.

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MATERIALS AND METHODS

Plant Collection and Authentication

The plant *M. officinalis* was collected in the flowering stage from the fields of Choglamsar, Leh, Jammu and Kashmir, India during August 2013. The plant was authenticated at Curator, Centre for Biodiversity and Taxonomy, Department of Botany, University of Kashmir, Jammu and Kashmir, India. A voucher specimen (1915-KASH) has been deposited in the institute.

Instruments

The UV-Visible spectrophotometer, model UV-1800, Shimadzu, Japan and HPTLC, Camag, Muttenz, Switzerland were used for the study.

Chemicals and Reagents

All chemicals and reagents used for the investigation of physicochemical and phytochemical property of *M. officinalis* were of analytical grade.

Physicochemical Analysis

The various physicochemical parameters of the powdered drug such as ash value, extractive value, volatile oil content, and loss on drying were performed.[11]

Fluorescence Analysis

The fluorescence characteristics of powdered *M. officinalis* with different chemicals were observed in daylight, short light (254 nm), and ultraviolet long (365 nm). The powdered *M. officinalis* was treated with the various neutral solvents (methanol and water), acidic (1 N hydrochloric acid, 50% hydrochloric acid, 50% sulfuric acid, and 50% nitric acid), and alkaline solvents (1 N sodium hydroxide and alcoholic 1 N sodium hydroxide). The various extracts of *M. officinalis* were also subjected to daylight, short light (254 nm), and ultraviolet long (365 nm) for determination of its fluorescence characteristics.[12]

Preparation of Extracts

The aerial part of *M. officinalis* was shade dried and powdered coarsely. The powdered material was successively extracted by soxhlet extraction method with petroleum ether, benzene, chloroform, acetone, methanol, and water as per increasing order of their polarity. The extracts were concentrated and dried to obtain residue. The dried extracts were weighed and the required quantity of the same was dissolved in appropriate solvents for further investigations. The shade dried leaves and stem of *M. officinalis* were also extracted in the same manner for quantitative estimation of total phenol and flavonoids contents.[13]

Phytochemical Investigation

The various extracts of *M. officinalis* were subjected to the preliminary qualitative phytochemical investigation.[14]

Quantitative Estimation of Constituents

The total phenol and flavonoid contents of methanol and aqueous extracts of leaves and stem of *M. officinalis* were determined by Folin-Ciocalteu reagent and aluminum chloride method, respectively.

Determination of Total Phenols

The total phenol contents were estimated by Folin-Ciocalteu reagent method.[15] A dilute extracts of *M. officinalis* (0.5 mL of 1:10 g/mL) or gallic acid (standard phenolic compound) were mixed with Folin-Ciocalteu reagent (5 mL, 1:10 diluted with distilled water) and aqueous Na_2CO_3 (4 mL, 1 M). The mixtures were allowed to stand for 15 min and total phenol contents were estimated using UV-Visible spectrophotometer at 765 nm. The calibration curve of gallic acid was prepared (50 to 450 µg/mL) in methanol and water (50:50 v/v). Total phenol contents were expressed in terms of gallic acid equivalent (mg/g).

Determination of Total Flavonoids

The total flavonoid contents were achieved by spectroscopic method using the reagent aluminum chloride.[16] The extracts of *M. officinalis* (0.5 mL of 1:10 g/mL) in methanol were mixed separately with 1.5 mL of methanol, 0.1 mL of 10% aluminum chloride, 0.1 mL of 1 M potassium acetate, and 2.8 mL of distilled water. The mixture was kept at room temperature for 30 min; the absorbance of the reaction mixture was measured at 415 nm with the help of UV-visible spectrophotometer. The calibration curve of quercetin was prepared (12.5-100 µg/mL) in methanol. Total flavonoid contents were expressed in terms of quercetin equivalent (mg/g).

High Performance Thin Layer Chromatography (HPTLC)

The HPTLC is the better means to separate the various components present in extract.[17] The methanolic and aqueous extracts of *M. officinalis* were found to have a number of phytoconstituents. Hence, further attempt was taken to separate the individual components with the help of HPTLC. The condition was maintains as follows:

Sample:
1. The methanol extract (20 mg) was dissolved in 10 mL ethanol
2. The aqueous extract (20 mg) was dissolved in 10 mL of ethanol.

Stationary phase: Silica gel 60 F_{254} coated TLC Aluminum Sheets (E. MERCK KGaA).

Mobile phase:
1. Methanol extract - Chloroform:methanol (9:1, v/v)
2. Aqueous extract - Toluene: chloroform: methanol (2:7:1 v/v/v).

Sample concentration:
1. Methanol extract - 2-16 µL
2. Aqueous extract - 2-16 µL.

Sample applicator: Camag Linomat - 5.
Size of the plate: 10 cm × 10 cm.
Developing chamber: Twin trough glass chamber, 20 cm × 10 cm.
Mode of application: Band.
Band size: 6 mm.
Separation technique: Ascending.

Temperature: Room temperature.
Saturation time: 15 min.
Scanner: CAMAG TLC scanner.

Scanning wavelength:
1. Methanol extract - 366 nm
2. Aqueous extract - 408 nm.

Scanning mode: Absorbance.

RESULTS AND DISCUSSION

Physicochemical Analysis

Physicochemical constants such as ash value, extractive value, volatile oil, and loss on drying are shown in Table 1. The results revealed that *M. officinalis* was having 11.25% ± 0.25% w/w total ash, 2.11% ± 0.12% w/w acid insoluble ash, and 6.86% ± 0.19% w/w water soluble ash. The aqueous extractive value was higher (13.27% w/w) as compared to methanol extractive value (3.96% w/w). The volatile oil content was found to be 0.59% ± 0.02% v/w and the moisture content was 6.69% ± 0.12% w/w.

Table 1: Physicochemical parameters of powdered *M. officinalis*

Parameters	% w/w
Total ash	11.25±0.25
Acid insoluble	2.11±0.12
Water soluble	6.86±0.19
Extractive value	
Methanol	3.96
Aqueous	13.27
Volatile oil	0.59±0.02#
Loss on drying	6.69±0.12

The results were expressed as mean±SD (*n*=04).
#Data expressed in % v/w. SD: Standard deviation,
M. officinalis: Melilotus officinalis

Fluorescence Analysis

The result of fluorescence analysis of powdered and various extracts of *M. officinalis* is summarized in Tables 2 and 3 which showed the presence of various chemical constituents.

Phytochemical Investigation

The various extracts of aerial part of *M. officinalis* were carried out and percentage yield was calculated [Table 4]. The preliminary phytochemical tests were carried out, which revealed the presence of various phytoconstituents such as carbohydrates, sugar, sterols, triterpenoids anthraquinone glycosides, tannins, phenolic, flavonoids, and saponin glycoside [Table 5].

Table 2: Fluorescent analysis of powder of *M. officinalis*

Treatments	Daylight	Short UV light (254 nm)	Long UV light (365 nm)
1 N HCL	Green	Fluorescent green	Dark green
50% HCL	Green	Fluorescent green	Green
50% H_2SO_4	Brown	Fluorescent green	Dark green
50% HNO_3	Light brown	Fluorescent green	Green
1 N NaOH	Green	Fluorescent green	Dark green
Alcoholic NaOH	Green	Green	Dirty green
Methanol	Light green	Green	Green
Aqueous	Buff	Green	Fluorescent green

M. officinalis: Melilotus officinalis

Table 3: Fluorescent analysis of various extract of *M. officinalis*

Treatments	Daylight	Short UV light (254 nm)	Long UV light (365 nm)
Petroleum ether extract	Dark green	Green	Dark brick red fluorescent
Benzene extract	Dark green	Green	Dark orange fluorescent
Chloroform extract	Green	Green	Light brick red fluorescent
Acetone extract	Green	Green	Brick red fluorescent
Methanol extract	Brownish green	Dark green	Green fluorescent
Aqueous extract	Brown	Green fluorescent	Dark green fluorescent

M. officinalis: Melilotus officinalis

Table 4: Percentage yield of various extracts of *M. officinalis*

Extracts	Color	Consistency	% yield (w/w)
Petroleum ether	Dark green	Sticky	1.12
Benzene	Dark green	Semi-solid sticky	2.09
Chloroform	Green	Semi-solid sticky	1.28
Acetone	Green	Semi-solid sticky	0.64
Methanol	Brownish green	Semi-solid sticky	2.66
Aqueous	Brown	Sticky	11.94

M. officinalis: Melilotus officinalis

Quantitative Estimation of Total Phenol and Total Flavonoids

Total phenol contents of methanol and aqueous extracts were measured by Folin-Ciocalteu reagent in terms of gallic acid equivalent (mg/g). The calibration curve of gallic acid was satisfactorily liner over the concentration ranges from 50 to 450 µg/mL as shown in Figure 1. The standard curve equation was y = 0.096x + 0.002, r^2= 0.998. The methanol and aqueous extract of leaves of *M. officinalis* contains high total phenols 287.10 ± 1.00 mg/g and 263.63 ± 1.53 mg/g, respectively, compared to stem, which were 243.89 ± 1.32 mg/g and 163.14 ± 0.99 mg/g, respectively, in methanol and aqueous extract [Table 6].

The total flavonoid contents of the methanol and aqueous extracts in terms of quercetin equivalent (mg/g) were determined by aluminum chloride method. The calibration curve of quercetin was satisfactorily liner over the concentration ranges from 12.5 to 100 µg/mL as shown in Figure 2. The standard curve equation was y = 0.001x + 0.003, r^2 = 0.997. The result revealed that the leaves contains high amount of total flavonoids in methanol and aqueous extracts 70.67 ± 5.13 mg/g and 60.50 ± 2.95 mg/g, respectively, as compared to stem which were 32.83 ± 4.71 mg/g and 26.83 ± 2.64 mg/g, respectively, in methanol and aqueous extract of *M. officinalis* [Table 6].

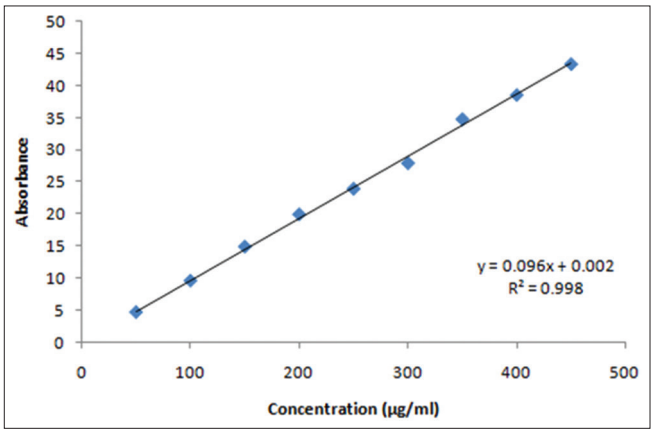

Figure 1: Calibration curve of gallic acid

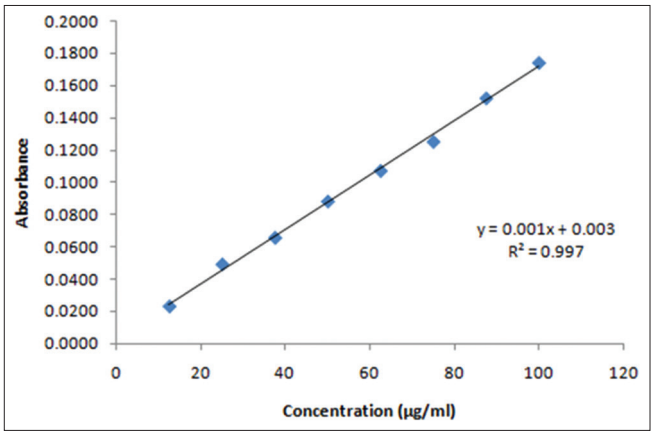

Figure 2: Calibration curve of quercetin

HPTLC

Both the methanol and aqueous extract of *M. officinalis* were subjected for HPTLC screening for the qualitative analysis of phytoconstituents. The numbers of solvent systems from lower to higher polarity were tried, but the solvent system which showed good resolution was used. The results are shown in Table 7 and Figures 3-6. The results of HPTLC fingerprint scanned at 366 nm for methanol extract of *M. officinalis* revealed 7 spots at R_f value 0.02, 0.10, 0.32, 0.43, 0.48, 0.72, and 0.78 with percentage area 62.39, 2.50, 9.24, 1.51, 5.63, 17.19, and 1.55 in solvent system of chloroform:methanol (9:1, v/v). The HPTLC fingerprint for aqueous extract of

Table 5: Qualitative chemical tests of different extracts of *M. officinalis*

Class of compound	Petroleum ether extract	Benzene extract	Chloroform extract	Acetone extract	Methanol extract	Aqueous extract
Carbohydrates and sugar						
Molisch test	-	-	-	-	+	+
Fehling's test	-	-	-	-	+	+
Benedicts test	-	-	-	-	+	+
Sterols and triterpenoids						
Salkowski test	+	+	+	+	+	-
Liebermann Buchard test	+	-	-	+	+	-
Anthraquinone glycoside						
Borntrager test	-	-	-	+	+	+
Modified Borntrager test	-	-	-	+	+	+
Tannins/phenols						
$FeCl_3$ 5%	-	-	-	-	+	+
Lead acetate test	-	-	-	+	+	+
Flavonoids						
Shinoda test	-	-	-	-	+	+
Saponin glycoside						
Foam test	-	-	-	-	+	+

M. officinalis: Melilotus officinalis

Table 6: Quantitative estimation of constituents of *M. officinalis*

Extracts	Constituents	Total phenol content (mg/g)	Total flavonoid content (mg/g)
Methanol	Leaf	287.50±1.00	70.67±5.13
	Stem	243.89±1.32	32.83±4.71
Aqueous	Leaf	263.63±1.53	60.50±2.95
	Stem	163.14±0.99	26.83±2.64

The results were expressed as mean±SD (*n*=04). *M. officinalis: Melilotus officinalis,* SD: Standard deviation

M. officinalis scanned at 408 nm which showed the 9 spots at R_f value 0.01, 0.09, 0.16, 0.28, 0.35, 0.46, 0.49, 0.87, and 0.93 with percentage area 37.00, 2.78, 15.43, 6.70, 2.18, 21.92, 6.68, 2.37, and 4.93 using toluene:chloroform:methanol (2:7:1, v/v/v) as solvent system.

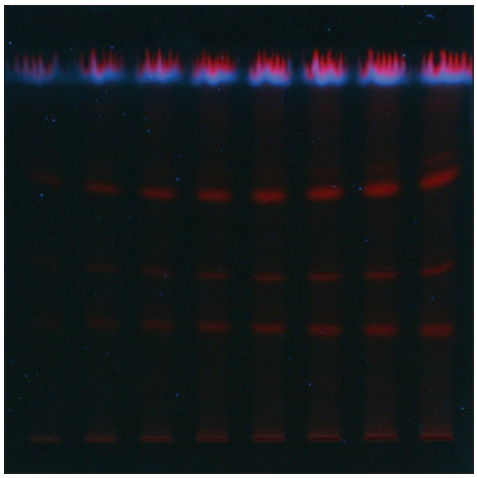

Figure 3: High performance thin layer chromatography of methanol extract of *Melilotus officinalis*

CONCLUSION

The various physicochemical parameters such as ash values, extractive values, volatile oil content, moisture content, and fluorescence analysis of powdered, and various extracts of *M. officinalis* were determined for ascertaining the quality of crude drug. The phytochemical analysis of the various extracts of *M. officinalis* revealed the presence of various phytoconstituents. The methanol and aqueous extract of leaves of *M. officinalis* contain high amount of total phenols and total flavonoids compared to stem. The various phytoconstituents were separated with the help of HPTLC and their respective R_f values have been accounted. The data revealed that aqueous extract gives better extraction of the phytochemicals than methanol extract since the aqueous extract resolved into a maximum number of bands as compared to methanol extract. Hence, the present study will provide useful information regarding correct identity, purity, and standardization of *M. officinalis*. The results of the present study could be useful for preparation of a plant monograph.

Table 7: High performance thin layer chromatography of extracts of *M. officinalis*

Extracts	Solvent system	Wavelength (nm)	Number of sports	R_f values	% area
Methanol	Chloroform:methanol (9:1, v/v)	366	7	0.02, 0.10, 0.32, 0.43, 0.48, 0.72, 0.78	62.39, 2.50, 9.24, 1.51, 5.63, 17.19, 1.55
Aqueous	Toluene:chloroform:methanol (2:7:1, v/v/v)	408	9	0.01, 0.09, 0.16, 0.28, 0.35, 0.46, 0.49, 0.87, 0.93	37.00, 2.78, 15.43, 6.70, 2.18, 21.92, 6.68, 2.37, 4.93

M. officinalis: Melilotus officinalis

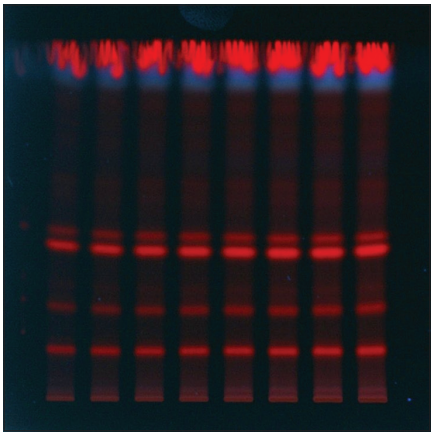

Figure 4: High performance thin layer chromatography of aqueous extract of *Melilotus officinalis*

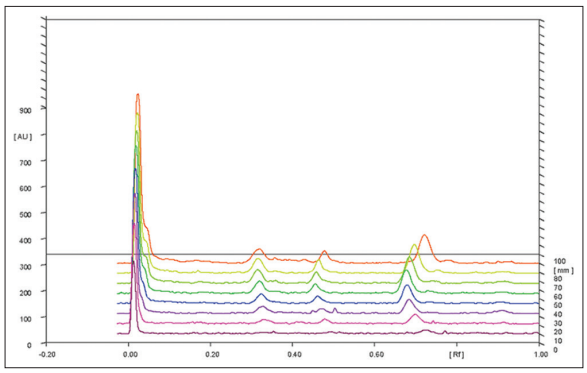

Figure 5: High performance thin layer chromatography fingerprint profile of methanol extract of *Melilotus officinalis*

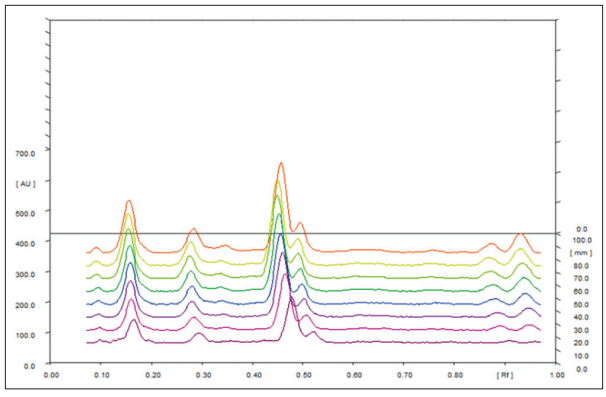

Figure 6: High performance thin layer chromatography fingerprint profile of aqueous extract of *Melilotus officinalis*

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