Journal of Pharmacognosy and Phytochemistry
Available online at www.phytojournal.com

E-ISSN: 2278-4136
P-ISSN: 2349-8234
https://www.phytojournal.com
JPP 2024; 13(2): 806-809
Received: 26-02-2024
Accepted: 03-04-2024

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Fast and simple HPTLC chemical fingerprinting and anti-oxidant analysis of medicinal herbs

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DOI: https://doi.org/10.22271/phyto.2024.v13.i2f.14927

Abstract
Medicinal plants are reservoirs of therapeutic phytochemicals with potential to become a lead molecule for the development of novel drugs. ICH based HPTLC analysis is fast and simple method for developing chemical profile of herbals. The isolation and profiling of bioactive phytochemicals depends upon the extraction method.
A comparative HPTLC fingerprint of hot and cold methanolic extracts of aromatic medicinal herbs *viz*. Rosemary (*Rosmarinus officinalis* L.), Oregano (*Origanum vulgare*) and Thyme (*Thymus vulgaris* L.) was developed on the CAMAG HPTLC system. The extracts were prepared by maceration, digestion, soxhlet and Ultrasound Assisted extraction methods. HPTLC studies were performed on 20 * 10 Silica gel 60 F 254 plates using Ethyl acetate: Formic Acid: GAA: Water (10:0.5:0.5:1.3 v/v/v/v) as solvent system to analyse the separation of phytochemicals under different extraction condition.
Preliminary phytochemical analysis confirmed the presence of flavanoids in the extract. Quercetin was isolated and identified by using modified solvent system of Chloroform: Acetone: Formic Acid (76:16.5:8.5 v/v). The chromatogram was derivatized with ASR for isolating standard Quercetin.
Among various extracts the soxhlet extract of Rosemary and Oregano showed better separation of phytochemicals. While all the four extracts of Thyme showed more or less similar separation of phytochemicals. Soxhlet is better method for separation of Quercetin in all the plants under study. Antioxidant activity of the extract was also evaluated by estimating DPPH radical scavenging assay. Oregano shows maximum antioxidant activity followed by Thyme and Rosemary.

Keywords: Rosemary, oregano, thyme, HPTLC, quercetin, antioxidant

Introduction
Fast food culture has introduced new exotic herbs in the diet of middle class Indians. In recent times many such herbs have become a part of household spices in India. They are now grown locally and are available in nearly all departmental stores in urban India. Rosemary (*Rosmarinus officinalis* L.), Oregano (*Origanum vulgare*) and Thyme (*Thymus vulgaris* L.) belongs to the group of such aromatic culinary herbs commonly used in the Mediterranean diet [1].
These culinary herbs exhibits multiple health benefits including anti-microbial, digestive, stimulant, anti-oxidant, anti-inflammatory, and anti-carcinogenic properties [2-5]. Apart from the aromatic oils these herbs also shows the presence of significant content of bioactive polyphenolic compunds especially the flavanoids. They exhibits wide range of biological activities including the antioxidant properties like free radical scavanging and metal ion chelation [6-8].
The present study deals with HPTLC fingerprint of three aromatic herbs *viz*. as per the ICH guidelines [9-11]. Identification, isolation and quantification of quercetin by HPTLC and evaluation of antioxidant activity in the plant under study.

Material and Methods
The plant material was procured from a local mall in south Mumbai and was identified in the Department of Botany, The Institute of Science, Mumbai. The plant material was air dried, macerated and stored in air tight glass containers for further investigations.

Preparation of extracts
Maceration
1 g of dried leaves powder was placed in a flask and mixed with 20 ml methanol. After placing the mixture for 1 day at room temperature with occasionally shaking, it was filtered, solvent evaporated at reduced pressure and the extract was stored at 4 ºC [11].

Digestion
1 g of dried leaves powder was mixed with 20 ml methanol and heated at 35-40 ºC for 1 hr. The extract was then filtered, concentrated under vacuum and stored at 4 ºC [11].

Soxhlet Extraction (SE)
2 g of dried leaves powder was placed individually in a soxhlet apparatus and were extracted with 200 ml methanol for 12 hr. The extracts were then concentrated under reduced pressure using a rotary evaporator and stored at 4 ºC [11].

Ultrasound Assisted Extraction (UAE)
An ultrasonic apparatus was used to extract 1 g of dried leaves powder with 20 ml methanol for 45 mins with 15 min interval [11].

HPTLC fingerprinting:
The HPTLC analyses was performed on aluminum plates pre-coated with silica gel 60 F254, Merk, Germany (No. 20190320). 1 µl of soxhlet extract and 2 µl of Ultrasound Assisted Extract (sonicated), digested and macerated extract was applied on the plate of 20 X 10 cm as bands of 8 mm width with the help of CAMAG Linomat V sample applicator (S/N: 240353). The plates were developed in a CAMAG twin-trough chamber previously equilibrated with a mobile phase for 20 minutes. The solvent system of Ethyl acetate: Formic Acid: GAA: Water (10:0.5:0.5:1.3 v/v/v/v) was used to develop HPTLC fingerprint profile. The plate was developed up to 8 cm, air dried, viewed and scanned at wavelength of 254 & 366 nm using CAMAG TLC Scanner 4 (S/N:241066) and CAMAG Visualizer 2 (S/N:241042). The plate was then derivatized with anisaldehyde sulphuric acid in Automated CAMAG Derivatizer using a green nozzle at level 3. The plate was heated at 105 ºC on CAMAG Plate heater till the development of colour. Derivatized chromatogram was again scanned at 254, 366 and 540 nm [12].

HPTLC profile of flavonoids
The profile for flavonoids was developed by using a modified solvent system of Chloroform: Acetone: Formic Acid (76:16.5:8.5 v/v). Quercetin was used as the standard while retaining the rest of the chromatographic conditions as mentioned above.

Antioxidant activity
The anti-oxidant activity was evaluated by estimating DPPH radical scavenging assay (Brand-Williams *et al.*, 1995). The radical scavenging activity against the stable DPPH radical was measured at different time interval *viz*. t=0 min and t=30 min. 2 mM methanolic DPPH solution and various plant extract were incubated for 30 min in the dark at room temperatures, and the decrease of absorbance at 515 nm was measured. All determinations were performed in quadraplicates. The anti-oxidant activity of the plant extract was calculated as the 'inhibition percentage' according to the following equation

$$\text{Inhibition percentage (\%)} = [(A_{C(0)} - A_{C(t)}/A_{C(0)}] \times 100$$

where $A_{C(0)}$ is an absorbance of blank DPPH solution at 0 min and $A_{C(t)}$/is an absorbance of plant extracts at 30 min.

Results and Discussions
HPTLC fingerprints
The HPTLC fingerprint of various extracts of Rosemary, Oregano and Thyme gives a comparative profile of separation of phytochemicals under different extraction conditions. The solvent system of Ethyl acetate: Formic Acid: GAA: Water (10:0.5:0.5:1.3 v/v/v/v) was found to be good for the development of fingerprint profile in the plants under study, in the given conditions (Plate 1 and 2). Soxhlet extract was very dark in colour as compared to other extracts so the dosage volume was selected as 1 µL while dosage for all other extracts was selected as 2 µL.
Soxhlet extract of Rosemary and Oregano showed better separation of phytochemicals than the other extracts. While all the four extracts of Thyme showed more or less similar separation of phytochemicals. The HPTLC fingerprint profile can be employed for the purpose of identification of the plant under study.

HPTLC profile of flavonoids
The fingerprint reveals the profile of flavonoids under different extraction conditions. Quercetin was separated in majority of extracts (Plate 3). Overall the soxhlet extraction method was found to be a better method for separation of flavonoids. Quercetin was found in all the extracts in Thyme. Although soxhlet extract shows better extraction than all other extracts of Thyme. It can be concluded that hot extracts help to reveal better separation of flavonoids and majority of flavonoids are heat stable.
The content of Quercetin was calculated in soxhlet extracts of *R. officinalis* (Rosemary), *T. vulgaris* (Thyme), & *O. vulgare* (Oregano) as it was showing maximum concentration of Quercetin in the chromatogram.
The content of Quercetin was found to be 1.14 µg/mg (0.114% of dry weight) in Rosemary, 1.32 µg/mg (0.132% of dry weight) in Thyme and 0.44 µg/mg (0.044% of dry weight) in Oregano (Table 1).

Anti-oxidant assay
The radical scavenging activity against the DPPH was calculated as the inhibition percentage (Table 2). Soxhlet extracts gives better performance among all the extracts. The anti-oxidant activity was found slightly more in Thyme extract as compared to Rosemary. The Quercetin content and anti-oxidant assay followed similar trend thus indicating the role of Quercetin as potent anti-oxidant agent.

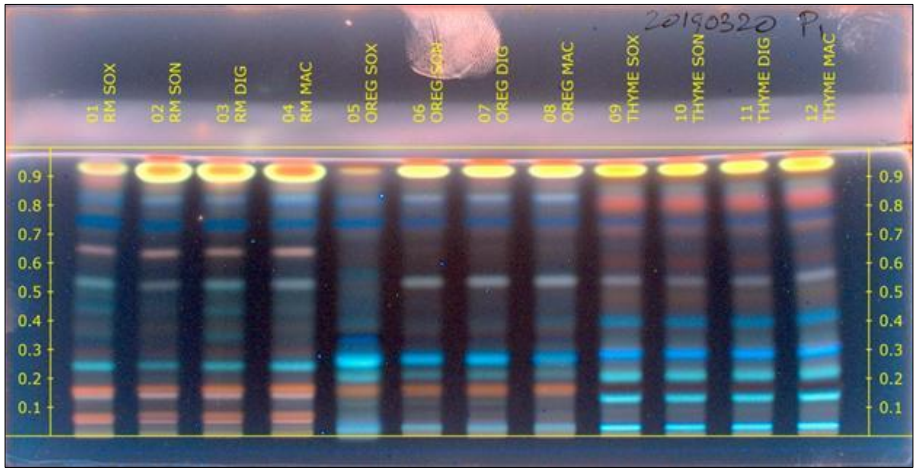

Fig 1: HPTLC fingerprint profile of Soxhlet, Sonicated, Digested & Macerated Extracts of *R. officinalis* (Rosemary), *T. vulgaris* (Thyme), & *O. vulgare* (Oregano) viewed at 366 nm after derivatization.

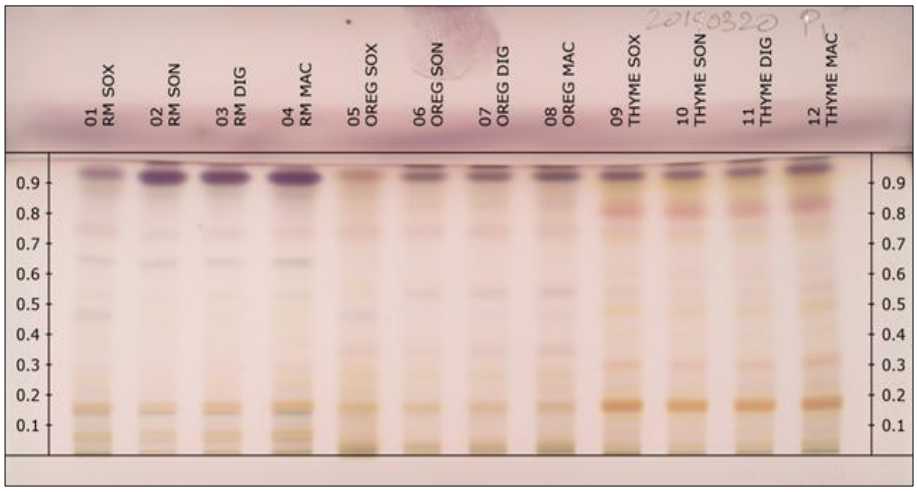

Fig 2: HPTLC fingerprint profile of Soxhlet, Sonicated, Digested and Macerated Extracts of *R. officinalis* (Rosemary), *T. vulgaris* (Thyme), & *O. vulgare* (Oregano) viewed at 540 nm after derivatization.

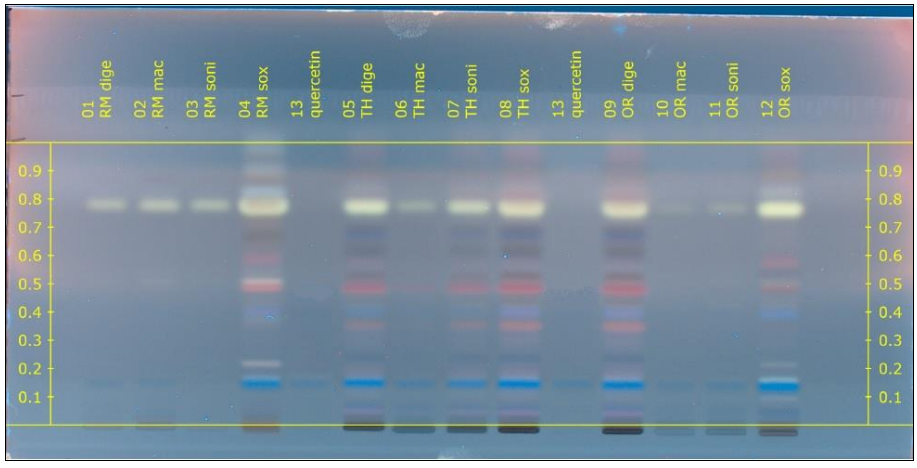

Fig 3: Flavanoids profile in digested, macerated, sonicated and soxhlet extracts of *R. officinalis* (Rosemary), *T. vulgaris* (Thyme), & *O. vulgare* (Oregano) viewed at 366 nm after derivatization

Table 1: Quercetin content in soxhlet extracts of Rosemary, Thyme and Oregano

Plant extract	Quercetin content
Rosemary	1.14 µg/g DW
Thyme	1.32 µg/g DW
Oregano	0.44 µg/g DW

Table 2: Anti-oxidant analysis of various extracts of Rosemary, Thyme and Oregano

Plant	Extract	DPPH (inhibition %age*)
Rosemary	Soxhlet extract	47.18±2.72
	Sonicated extract	46.72±3.14
	Digested extract	47.56±1.18
	Macerated extract	46.92±3.72
Thyme	Soxhlet extract	48.22±1.78
	Sonicated extract	47.26±2.66
	Digested extract	47.94±4.72
	Macerated extract	46.98±3.52
Oregano	Soxhlet extract	39.66±2.36
	Sonicated extract	37.02±2.88
	Digested extract	38.22±1.16
	Macerated extract	36.80±3.32

(Mean ± Standard deviation)

Conclusion

These plants proved to be of good source of Quercetin. They are regularly used in daily dietary consumption. The present study will establish their therapeutic value and justify the use of these plants in dietary consumption. HPTLC fingerprints of *R. officinalis* (Rosemary), *T. vulgaris* (Thyme), & *O. vulgare* (Oregano) will help in identification and authentication of the plant. The method for HPTLC fingerprinting yielded very good results under the given conditions and the same can be applied for simultaneous evaluation of herbals. The fingerprint also compared effectiveness four different extraction methods and found that soxhlet method is better as compared to other methods. The HPTLC fingerprint of Flavanoids has revealed the presence of huge diversity in the composition of this particular class of secondary metabolites in the plants under study, which can be further worked upon to evaluate the bioactivity potential of individual compound. Antioxidant activity will justify various pharmacological activities exhibited by the plant under study. The presence of strong antioxidant activity can be attributed to the presence of huge diversity of flavonoids highlighted during the present work.

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