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RESEARCH ARTICLE

Determination and quantification of stigmasterol and kaempferol from *in vitro* and *in vivo* plant parts of *Merremia aegyptia* and *Merremia dissecta*

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Abstract:*Merremia aegyptia* and *Merremia dissecta* are annual climbing herbs showing high larvicidal and antimicrobial activities. Stigmasterol and Kaempferol were identified and quantified from methanolic extracts of *in vivo* (leaves, stem, seed) and *in vitro* (callus) parts using HPTLC densitometric method of both the species. The separation was first performed on TLC aluminium plates precoated with silica gel 60 F_{254} . Good separation of stigmasterol was achieved in mobile phase using toluene: methanol (9:1, v/v) at (254, 310nm) and (650nm) and kaempferol using Toluene-acetone-formic acid (4.5: 4.5: 1, v/v) at (254, 366 nm). The instruments CAMAG TLC Scanner LINOMAT- V were used for the present analysis. Determination and quantitation were performed by densitometric scanning at 530 nm for stigmasterol and 254 nm for Kaempferol in reflection/absorbance mode. This method gave compact spots at Rf- 0.92 corresponding to stigmasterol and Rf 0.68-0.71 corresponding to Kaempferol. Highest amount of stigmasterol was calculated from *M. aegyptia* seed extract (59ng/µl).,Stem extract(9.8 ng/µl) and callus extract (9.0 ng/µl) concentration and Kaempferol concentration was found highest in *M. aegyptia* callus (4.9 ng/µl) and *M. dissecta* seeds (3.6 ng/µl). The present study to screen important bioactive constituents (stigmasterol and kaempferol) can be further utilized for analysis and routine quality control of drugs and formulations prepared from *M aegyptia* and *M. dissecta.*

Keywords: Densitometric, HPTLC, Kaempferol, *M. aegyptia ,M. dissecta,* Stigmasterol.

Introduction

Natural resources have benefitted human existence since prehistoric times and the knowledge has been accrued and jotted down after constant experimentations to identify and quantify these products. In each practicing traditional system of medicine, the medicinal plants play a major role and constitute backbone. Indian *Materia Medica* includes information on about 2000 drugs of natural origin almost all of which are derived from different traditional systems and folklore practices. According to the World Health Organization, 25% of prescribed drugs are based on plant-derived chemicals and about 50% of the top selling chemicals have been derived from natural resources (Terryn *et al.* 2006). Thousands of chemical structures have been identified so far and still there remains immense possibility to explore the untapped reservoir of natural products.

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Higher plants since their inceptions are producing an impressive and extraordinarily diverse array of chemical compounds called secondary metabolites or natural products at ease. These natural products have been used as template for formulation of many valuable modern drugs in the past and remain as a potential source for future drug discoveries. Analysis or isolation of active ingredients from plants using modern analytical tools provides the basis for development of novel, effective and high quality products for consumers (Julsing *et al.* 2007).

HPTLC is an offline technique in which the subsequent steps are relatively independent, allowing parallel treatment of multiple samples during chromatographic derivatization and detection. Other advantages include simultaneous processing of sample and standard, Lower analysis time and less cost per analysis, Low maintenance cost of instrument, Simple sample preparation, No prior treatment of solvents like filtration and degassing, Low mobile phase consumption per

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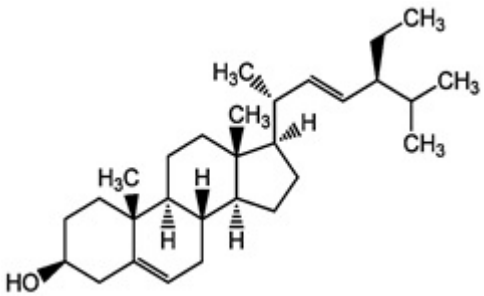
sample, No interference from previous analysis - fresh stationary and mobile phases used for each analysis and Visual detection is possible.

M. aegyptia- important plant contains high medicinal value, has been analysed for various usage in the past. Leaves of the plant species are being used to treat common skin diseases and jaundice (Areces-Berazain 2016). Oliveira *et al.,* (2010) reported activity of leaf extract of *M. aegyptia* against *Aedes aegypti* larvae with 100% mortality. Azam *et al.* (2010) reported that seeds of the plant are important sources of biodiesel. In earlier study phytochemicals responsible for anti-oxidant, anti-osteoarthritic, analgesic, antidiabetic, anti-microbial activity of *M. aegyptia* and *M. dissecta* plants were identified using advanced techniques (Joshi *et al.* 2018). In the present investigation stigmasterol and kaempferol constituents were quantified in both the species growing in Jaipur, Rajasthan (Longitude-75.78 º; Latitude- 26.91º; 433m above sea level).

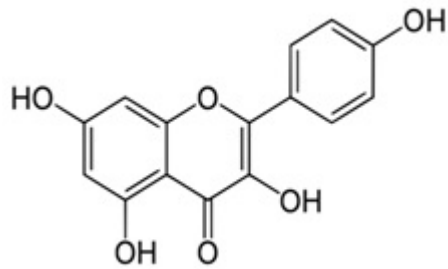
Stigmasterol



Molecular formula of stigmasterol is $C_{29}H_{48}O$ with mass of 412.69 g mol^{-1}. The phytochemical is an unsaturated phytosterol present in various medicinal herbs in the form of fats or oils. It is present in higher quantity in soybean, rape seed and in Chinese herbs *Ophiopogon japonicus* in *Mirabilis jalapa* and American Ginseng (Siddiqui *et al.* 1990).

Stigmasterol is used as precursor for manufacturing of semisynthetic progesterone (Sundaraman and Djerassi 1977) and act as an intermediate in the biosynthesis of androgens, estrogens, and corticoids. It is also used as the precursor of vitamin D_3 (Maestro *et al.* 2019). It has been reported to be preventive against certain cancers (ovarian, prostate, breast, and colon). Panda *et al.* (2009) recorded stigmasterol to possess thyroid inhibiting, hypoglycemic and potent antioxidant properties.

Kaempferol



Kaempferol (Molecular formula- $C_{15}H_{10}O_6$; Molar Mass- 286.23g mol^{-1}) is a tetrahydroxy flavone preventive against some chronic diseases like cancer. It may modulate apoptosis, angiogenesis, inflammation, and metastasis (Singh *et al.* 2009). It also has a role as an active antibacterial agent.

For the isolation and quantification of Stigmasterol and Kaempferol from *in vivo* and *in vitro* (mature callus) plant parts of *M. aegyptia* and *M. dissecta* through HPTLC, the given protocol was followed.

Materials and method

Chemicals

For the present investigation, chemicals were purchased of analytical grade from Qualigens, Fine Chemicals, Mumbai, India. Standards for the analysis Stigmasterol (97% w/w Pure) was purchased from Sigma Aldrich ltd and Kaempferol from TCI chemicals.

Plant Sample

In vivo and *in vitro* dried plant parts of *M. aegyptia* and *M. dissecta* (5 gm) were extracted using methanol in Soxhlet apparatus for approximately 24 hrs. Extract was cooled, filtered and concentrated using vacuum flash evaporator. Again the extract was diluted with 1ml methanol and centrifuged at 3000 rpm for 5min. This solution was used as test solution for HPTLC analysis.

TLC conditions

Precoated Silica gel 60 F_{254} TLC plates (20 × 10 cm; Merck) of 0.2 mm thickness with aluminium sheet support were used. The spotting device was a CAMAG Linomat V Automatic Sample Spotter (Camag Muttenz, Switzerland); the syringe (100 µL; Hamilton); the developing chamber was a CAMAG glass twin trough chamber (20 × 10 cm); the densitometer consisted of a CAMAG TLC Scanner 3 linked to winCATS software; the experimental condition temperature 25 ± 2 °C, relative humidity 40%.

The methanolic extract obtained after filtration was concentrated under vacuum and the volume was made upto 25 ml in a volumetric flask. This extract was used for further quantification of stigmasterol and kaempferol.

Standard solutions

Standard solutions of stigmasterol and kaempferol were prepared by dissolving 100 mg of each in 100 ml methanol (1mg/ml).

Solvent system

Toluene:methanol (9:1, v/v) for chromatography with stigmasterol and Toluene-acetone-formic acid (4.5: 4.5: 1,v/v) for chromatography with kaempferol.

Determination of Stigmasterol

10µl each sample solutions and standard solution was applied on precoated plate developed in toluene: methanol (9:1, v/v) solvent system about 10 mm above the bottom edge, band length 6 mm, distance between each track and from sides was kept 10 mm and 15 mm respectively. Measurement was made through UV absorbance/reflectance mode. Total no. of tracks was 8 , Scan start position on X axis= 15 mm, Scan start position Y axis= 5.0 mm, Scan end position on Y axis = 75.0 mm, Micro with scanning speed 20 mm/s and data resolution 100µm/step. Detector was used as an automatic mode with sensitivity 33%,

The plates were developed in mentioned solvent system in CAMAG glass twin trough chamber (Dimension: 20*10 cm) with S.S. Lid up to 80mm. Developed plate was dried with air drier and subjected for TLC scanning using TLC Scanner 3 (CAMAG Switzerland). Spots were visualized under UV Light at 254 nm and 310 nm. Deuterium and tungsten lamps were the radiation source.

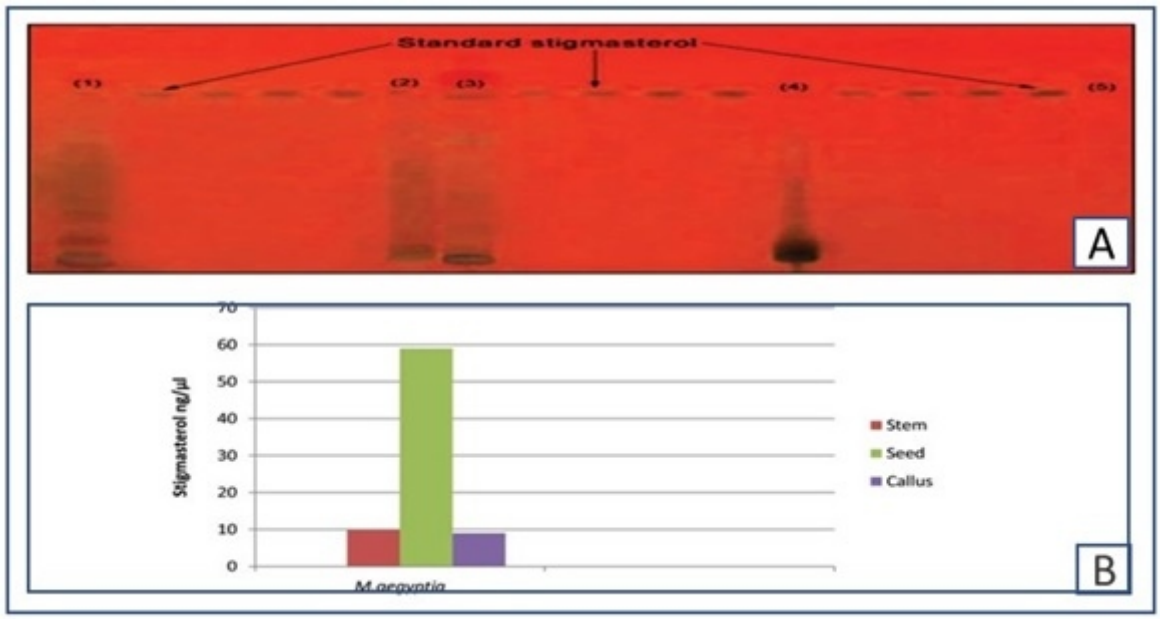
The plates were derivatized with anisaldehyde–sulphuric acid reagent and heated at 105 °C for 5 minutes and scanned densitometrically at 525 nm in absorbance mode using tungsten lamp. The area of the resolved peaks was recorded. The Rf values and color of the resolved bands were noted.

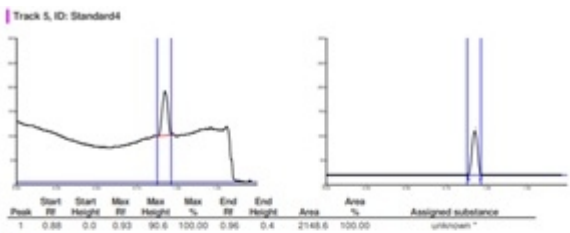
Determination of Kaempferol

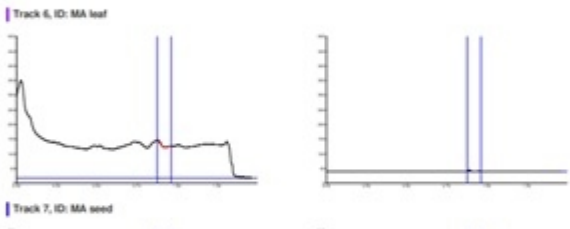
10 µl standard and test solutions were applied as 6mm band length with thickness: 0.2 mm on Aluminium plate precoated with silica gel G 60F_{254} Plate size used was 10×10 cms. Plates were developed in Toluene-acetone-formic acid (4.5: 4.5: 1,v/v) solution up to 80mm. The developed plate was dried by hot air. The plate was read through Photo documentation chamber (CAMAG REPROSTAR 3) and collected results with White light, UV 254nm and UV366nm. The developed plate was sprayed using respective spray reagent and dried at 100°C in oven. Before derivatization, the plate was fixed in scanner stage (CAMAG TLC SCANNER 3) and scanning was done at UV 254nm. Calibration curve of kaempferol was obtained by plotting peak areas *vs* concentrations of stigmasterol applied.

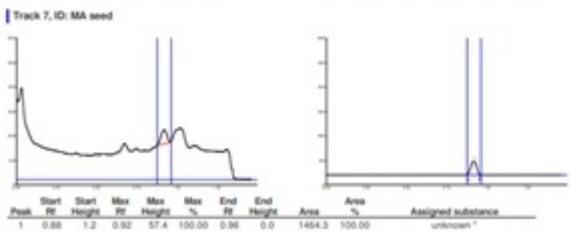
Result and discussion

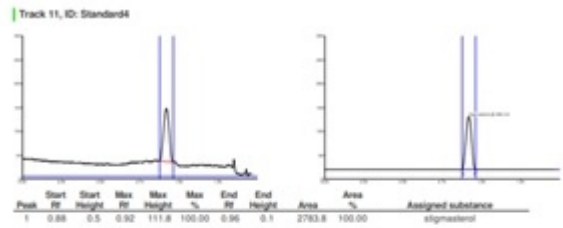
Various ethnomedicinal properties are exhibited by both the species, *M. aegyptia* and *M. dissecta.* Previous studies in genus *Merremia* such as biochemical studies have led to isolation of flavonoids diosmetin, luteolin, diosmetin-7-O-'β'-D-glucoside and luteolin-7-O-'β'-D-glucoside from the aerial parts of *Merremia tridentata*.(Neyanila *et al.* 2013). Other workers performed comparative study of Prasarini (*M. tridentata*) and *Paederia foetida* for their usefulness in Amavata or Rheumatoid arthritis.(Rajshekhara *et al.* 2009). Anti-diabetic activity in root extracts of *Merremia tridentata* has also been reported (Arunachalam and Parimelazhagan 2012). Qualitative and quantitative standardization of stigmasterol and

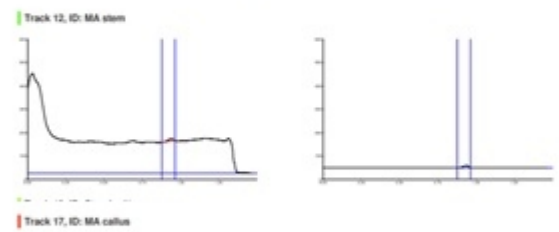

Standard stigmasterol
(1)
(2)
(3)
(4)
(5)
A
70
60
50
40
30
20
10
0
Stigmasterol ng/µl
Stem
Seed
Callus
M.aegyptia
B

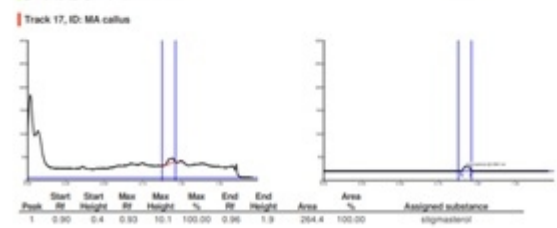

Track 5, ID: Standard4
Peak Start Rf Start Height Max Rf Max Height Max % End Rf End Height Area Area % Assigned substance
1 0.88 0.0 0.93 90.6 100.00 0.96 0.4 2148.6 100.00 unknown *

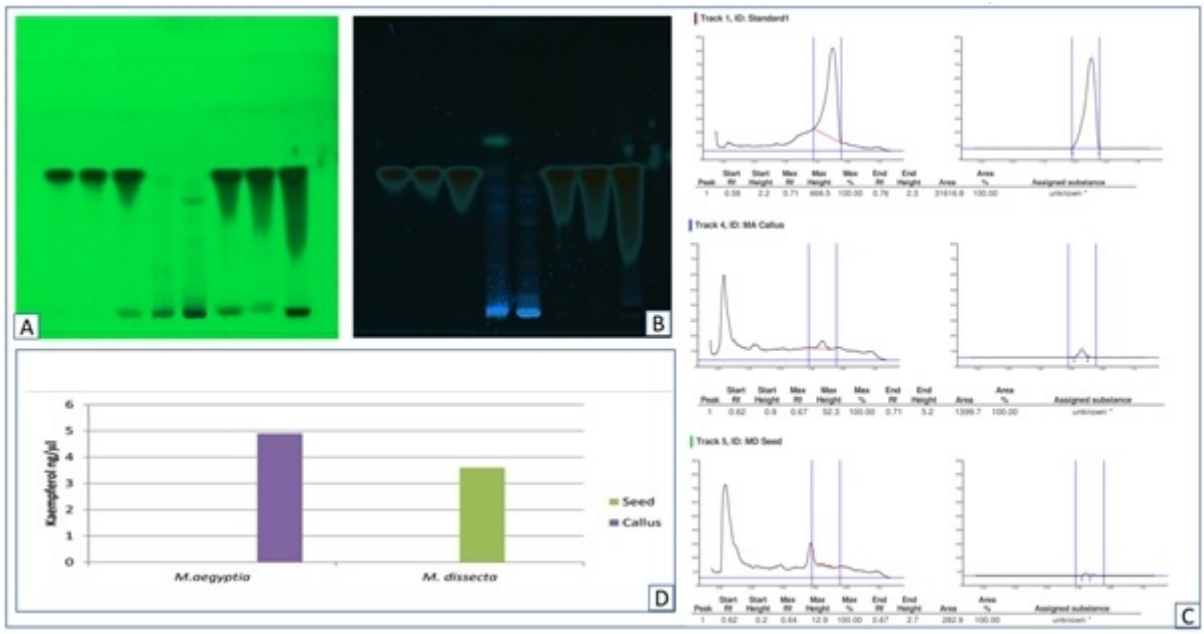

Track 6, ID: MA leaf


Track 7, ID: MA seed
Peak Start Rf Start Height Max Rf Max Height Max % End Rf End Height Area Area % Assigned substance
1 0.88 1.2 0.92 57.4 100.00 0.96 0.0 1464.3 100.00 unknown *


Track 11, ID: Standard4
Peak Start Rf Start Height Max Rf Max Height Max % End Rf End Height Area Area % Assigned substance
1 0.88 0.5 0.92 111.8 100.00 0.96 0.1 2783.8 100.00 stigmasterol


Track 12, ID: MA stem


Track 17, ID: MA callus
Peak Start Rf Start Height Max Rf Max Height Max % End Rf End Height Area Area % Assigned substance
1 0.90 0.4 0.93 10.1 100.00 0.96 1.9 264.4 100.00 stigmasterol



lupeol through HPTLC has been performed (Singh *et al.* 2009).

Quantification of kaempferol and quercetin in medicinal plants such as *Podophyllum hexandrum* and *Cassia angustifolia* using HPTLC and in *Thespesia populnea* have been done (Bhandare and Laddha 2016, Panchal *et al.* 2017).

HPTLC analysis of *Merremia aegyptia* and *Merremia dissecta* for Stigmasterol and Kaempferol reported that seed, stem and callus parts of *M. aegyptia* showed significant amount of stigmasterol (Rf=0.92) which was recorded to be 59ng/µl, 9.8 ng/ µl and 9.0 ng/ µl respectively. The amount of compound in calli was lowest as compared to that found in *in vivo* plant parts whereas, Kaempferol, (Rf=0.71) an anticancer compound was best quantified from *M.aegyptia* callus extract which was 4.9 ng/ µl whereas *M. dissecta* seed extract reported it to be 3.6 ng/ µl.

Conclusion

Genus *Merremia* is an ornamental vine genus belonging to family Convolvulaceae. Stigmasterol and Kaempferol have various therapeutic uses such as stigmasterol is reported to have antioxidant, thyroid inhibitory, antiperoxidative, hypoglycaemic (Panda *et al.* 2009) hypocholesterolemic (Battaab *et al.* 2006) and anti-inflammatory activity (Gabay *et al.* 2010) whereas Kaempferol has antioxidant, anticancerous and antibacterial properties. (Singh *et al.* 2009)

From the results obtained, it can be concluded that *M. aegyptia* and *M. dissecta* are great source of these two potential antioxidant compounds, stigmasterol and kaempferol. One is a plant sterol and other belongs to flavone group of plant secondary metabolites. These were found in adequate amount in the *in vivo* plant parts in both the plant species.

As per the Literature survey and our knowledge, identification and quantification of such useful secondary metabolites from *M. aegyptia* and *M. dissecta* has not been reported ever and is the first report from both these species and therefore, opens large dimensions to the future field of research

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