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# High performance thin layer chromatography fingerprint profile, isolation, its antioxidant activity and spectroscopic characterization of marker compound of *Murraya koenigii*

**Ladli kumari, Papiya Mitra Mazumder, Manik, UR Lal and R Dubey**

**Abstract**
**Objectives**
The present study was aimed at identifying, isolation, antioxidant activity and quantification of a marker compound obtained from *M. koenigii* leaves.
**Methods**
HPTLC fingerprinting and *in-vitro* antioxidant activity was carried out for four isolated fractions given the trivial name fraction 1 (MF1), fraction 2 (MF2), fraction 3 (MF3) and fraction 4 (MF4) of methanolic leaf extract of *M. koenigii* (MEMK). From the HPTLC fingerprint the florescent band (under 350 nm) at $R_f$: 0.93 (mobile phase n-butanol: acetic acid: water (2:2:4, v/v/v) was found in the fractions of MEMK. So, the florescent band (under 350 nm) at $R_f$: 0.93 was isolated as marker compound Mk-F4 from MEMK. The marker compound Mk-F4 was quantified by using HPTLC technique. The quantification protocol was followed as per the ICH Q2-R1 guidelines.
**Results**
MF4 showed more effective antioxidant activity as compared to other fractions MF1-MF3 w.r.t. the standard substance used being ascorbic acid in all three models viz DPPH radical scavenging, nitric oxide radical scavenging and hydrogen peroxide scavenging assay may be due to the presence of more percentage of Mk-F4 in MF4. The percentage (W/W) yield of Mk-F4 was found to be 90.0, 95.1 and 16.1% in MF3, MF4 and MEMK, respectively.
**Conclusion**
The present study indicated that *M. koenigii* and its isolated bioactive compounds be a very useful antioxidant and may form the basis of development of various therapeutic agents with better activity.

**Keywords:** Column chromatography, HPTLC fingerprinting, Gas chromatography-Mass spectroscopy, Nuclear magnetic resonance spectroscopy

## 1. Introduction
The chemical constituents of the medicinal plants, the secondary metabolites have more pronounced pharmacological actions [1]. Marker compound means chemical constituents that can be used to verify its potency and identity [2]. The discovery of a novel chemical component from a medicinal plant form the basis of development of various therapeutic agents with better activity [3]. It is very difficult to identify marker compounds for all traditional medicines, because some medicines have unknown active constituents and others have multiple active constituents [4]. The extraction methods used to process the herbal plants can affect the quantities of biologically active compounds in the extract. Thus the quality control of the active constituents or marker compounds in the herbal extract is of great importance in medicinals [5].
Several natural products such as alkaloids, flavonoids, terpenoids, saponins and glycosides are isolated from medicinal plants and are being reported to possess anti-diabetic activities [6]. Herbal drugs are used to treat different diseases because of effectiveness, lesser side effects and relatively low cost [7]. Therefore, it is important to isolate the active constituent from traditional used antidiabetic plants. *M. koenigii* is used as a spice and condiment in India and other tropical countries. *M. koenigii* have been used as folk medicine for the treatment of rheumatism, traumatic injury and snake bite and it has been reported to have antioxidant, antidysenteric activities, antimicrobial, anti-inflammatory, hepatoprotective and

anti- hypercholesterolemic efficacy [8-9]. It is reported that it contains amino acids [10]. But there is no identified marker reported so far. Hence the present study is aimed to identify, isolate and quantify the marker compound in *M. Koenigii* along with evaluation of the antioxidant activity of the fractions

## 2. Material and method

### 2.1 Apparatus

NMR spectra were recorded on Bruker DRX 400 NMR spectrometers with methanol (MeOD) as internal standard. GC-MS spectra were recorded on a model GC-MS-QP-2010-Plus1, Shimdzu and elemental analysis (CHNO) on (Elementar, Vario EL III). Column chromatography was carried out silica gel (60-120 mesh, Merck, Darmstadt, Germany). Analytical thin-layer chromatography (TLC) was performed on Kieselgel 60F $_{254}$ (Merck, Darmstadt, Germany) glass plates.

### 2.2. Chemicals

1,1-diphenyl-2-picryl hydrazyl (DPPH), ascorbic acid from Sigma Aldrich, US and Merck, US. All other reagents and solvents were analytical grade, purchased from Merck (Darmstadt, Germany).

### 2.3. Plant Materials

The leaves of *M. koenigii* L. Spreng (Family: Rutaceae) was collected from the campus of Birla Institute of Technology, Mesra, Ranchi during the month of March-April 2011**.** The plant material was taxonomically identified and authenticated by K. Karthikeyan, Scientist 'C', Botanical Survey of India (BSI), Central National Herbarium, Howrah, with ref. no. CNH/103/2011/Tech-II/620. The voucher specimen is stored in the herbarium section of Birla Institute of Technology, Mesra, Ranchi, Department of Pharmaceutical Sciences and Technology for future reference.

### 2.4. Preparation of extract and fractions

Powdered air dried leaves of *M. koenigii*, weighing about 50 g, was extracted successively in soxhlet apparatus with the series of solvents of increasing polarity as follows: petroleum ether, chloroform and methanol. Each time before extracting with the next solvent, the material was dried. Methanolic extracts (MEMK) were filtered through Whatman filter paper and concentrated. TLC was used for isolation of different compounds present in MEMK [11]. The MEMK (10 g) was repeatedly separated by column using silica gel (60-120 µ size) as adsorbent and n-butanol: acetic acid: water in different ratio as the mobile phase [12]. It led to the isolation of four fractions namely MF1, MF2, MF3 and MF4. Then the fractions were concentrated under reduced pressure on rotavapor (Buchi Labortechnik AG, CH-9230 Flawil 1/Switzerland) to obtain a residue (MF1: 0.6%, MF2: 2.1%, MF3: 2.4% and MF4: 1.2% w/w).

### 2.5 HPTLC finger print profiles for extracts and fractions

**HPTLC Conditions**

Post-Chromatographic characterization of MEMK, MF1, MF2, MF3 and MF4 was performed on precoated silica gel aluminium plate 60 GF254 (10 cm×10 cm with 250 µm thickness, E. Merck, Darmstadt, Germany supplied by Anchrome Technologies, Mumbai, India) using a Camag Linomat IV sample applicator (Camag, Muttenz, Switzerland) and a 100-µl syringe (Hamilton, Rena, Nevada, USA). The sample was spotted in the form of bands of 6 mm length and 10 mm higher from the bottom, 10 mm from the left margin, and 10 mm apart, at a constant application rate of 15 µL/s using nitrogen aspirator. Plates were developed using a mobile phase consisting of n-butanol: acetic acid: water in the ratio of 4:4:2. Linear ascending development was carried out in 10 cm×10 cm twin trough glass chamber (Camag Muttenz, Switzerland) equilibrated with the mobile phase. The optimized chamber saturation time for mobile phase was 30 min at room temperature. After development, the HPTLC plates were dried with a hot air blower. The slit dimension settings of length 5 mm and width 0.45 mm and a scanning rate of 20 mm per second was employed. Densitometric scanning was performed on Camag TLC scanner II in the reflectance mode at $\lambda_{max}$ at 350 nm and operated by win CATS software version (1.1.3.0.). The source of radiation utilized was deuterium lamp. Evaluation was done via peak areas [13].

**Procedure**

Extracts and fractions of *M. koenigii* were applied on HPTLC plate and the plate was developed in n-butanol: acetic acid: water (4:4:2, v/v/v) solvent system. The plates were dried at room temperature. The plate was scanned at 350 nm after spraying with detection reagent (Ninhydrin reagent) and plate was heated at 105 ◦C for 5 minutes. The $R_f$ values and color of the resolved bands were noted.

### 2.6. *In vitro* antioxidant activity of fractions of MEMK

The fractions MF1, MF2, MF3 and MF4 of MEMK were dissolved in ethanol at the concentration 2 mg/ml to make a test solution of 25, 50, 75, 100 and 125 µg/mL concentration. Standard solutions of 25, 50, 75, 100 and 125 µg/mL 1-ascorbic acid were also prepared.

**A. DPPH radical scavenging assay**

The DPPH free radical scavenging activity of fractions MF1, MF2, MF3 and MF4 of MEMK was carried out according to the method of Deore *et al* [14]. 0.2 ml of test solution at different concentrations (25-125 µg $ml^{-1}$) were mixed with 0.8 ml of Tris- HCL buffer (100 mM,7.4). 1 ml of DPPH (500 mM in ethanol) solution was added to the above mixture and this mixture was shaken vigorously and incubated for 20 min in the dark. The absorbance of the resulting solution was measured at 517 nm by UV-Visible spectrophotometer (Shimadzu UV-Visible spectrophotometer 1700, India). All the assays were carried out in triplicates against ascorbic acid (AA) as a positive control. Blank was prepared without the addition of DPPH and for control, 0.2 ml of ethanol (without test sample) was added. Decreased absorbance values indicated a higher free radical scavenging activity. The percentage of DPPH scavenging effect was calculated.

**B. Nitric oxide radical scavenging assay**

Nitric oxide radical scavenging capacity of fractions MF1, MF2, MF3 and MF4 of MEMK was determined according to the method of Chaurasia *et al* [15]. 3 ml of the ethanolic solution of the test samples at different concentrations (25-125 µg/ml) were mixed with 1 ml of sodium nitroprusside (10 mM) in standard phosphate buffer solution (0.02 m, pH 7.4). The mixture was shaken vigorously and incubated at 25

$^0$C for 150 min. Control experiments without the test compounds, but with an equivalent amount of buffer were prepared in the same manner as done for the test. Thereafter, 0.5 ml of the incubation solution was removed and diluted with 0.5 ml of griess reagent (1% sulphanilamide, 2% o-phosphoric acid and 0.1% naphthyl ethylenediamine dihydrochloride) and allowed to stand at room temperature for 30 min. The absorbance of the chromophore formed during the diazotization of nitrite with sulphanilamide and subsequent coupling with naphthyl ethylene diamine dihydrochloride was read spectrophotometrically by (Shimadzu UV-Visible spectrophotometer 1700, India) at 546 nm. The increased absorbance of the reaction mixture indicated higher scavenging effect. The percentage inhibition of nitric oxide radical generation was calculated.

**C. Hydrogen peroxide scavenging assay**

Measurement of hydrogen peroxide scavenging activity of fractions MF1, MF2, MF3 and MF4 of MEMK was based on the method described by Deschner *et al* [16]. Hydrogen peroxide solution (2 mM) was prepared with standard buffer (pH 7.4). Test samples (25-125 µg/ml) in distilled water were added to hydrogen peroxide solution (0.6 ml). Then the absorbance was measured spectrophotometrically by (UV-Visible spectrophotometer 1700, Shimadzu, India) at 230 nm after 10 min in triplicates against blank solution containing phosphate buffer without hydrogen peroxide solution and compared with ascorbic acid as positive control. Increased absorbance of the reaction mixture indicated strong scavenging effect. The percentage scavenging of hydrogen peroxide was calculated.

**Statistical analysis**

The percentage of inhibition was calculated as:

% of inhibition = {(absorbance of control-absorbance of sample)/absorbance of control} × 100

Results were expressed as mean by triplicates measurement and $IC_{50}$ was graphically determined by a linear regression method using MS Windows based graph pad prism (Trial Version 5.01) software. Results were expressed as Mean ± Standard deviation.

### 2.7. Isolation and characterization of marker compound

**Isolation of compound Mk-F4 from MEMK**

The dried powder of the leaves (200 g) of *M. koenigii* was extracted with petroleum ether, chloroform and methanol (500 mL) separately in soxhlet apparatus. Then the extracts were concentrated by distilling the solvent. Concentrated methanolic extracts (MEMK) were subjected to column fractionation in different ratio as mobile phase (n-butanol: acetic acid in 10, 20, 25, 50, and 100% v/v) (into four fractions MF1, MF2, MF3 and MF4. Concentrated MF4 were subjected to repetitive preparative thin layer chromatography using silica gel Gas stationary phase (20×20 cm glass plates) and n-butanol: acetic acid: water 4:4:2 v/v/v as mobile phase. It led to the isolation of a single pure compound (Mk-F4). The isolated compound was concentrated under reduced pressure to dryness and then the structure was established on the basis of elemental analysis and spectroscopic evidences (UV, $^1$H NMR, $^{13}$C NMR, GC-MS).

### 2.8. Quantification of isolated marker compound Mk-F4 in MEMK and its fractions (MF1-MF4) using HPTLC Method

**Sample Preparation**

Standard stock solution. A solution of compound Mk-F4 (500 µg/mL) was prepared in HPLC grade methanol.

Stock solution of sample 2 mg/mL of MEMK and 1mg/mL of fractions (MF-MF4) were prepared in methanol.

Calibration curve- From the standard stock solution 2.0-10.0 µL solutions were applied on precoated plate of Silica Gel G, to produce the range of 1.88-10.19 µg of Mk-F4 per spot, respectively (Table 1 & 2). Calibration curve is given in (Fig. 1).

**Sample-** 5 µL of each of the extract and fractions were applied. Mobile Phase- The mobile phase of the system n-butanol: acetic acid: water (4:4:2).

Stationary Phase- The stationary phase was precoated plate, Silica Gel G 60 F 254.

**Applicator-** The applicator phase was CAMAG LINOMAT 5.

**Development-** Plate was developed in a twin trough chamber. Detection- Spray with ninhydrin reagent and heat at 105 $^0$C for 5 minutes. The plate was scanned at 350 nm after spraying. The Chromatograph of MEMK [18], its fractions MF1, MF2, MF3 and MF4 and isolated marker compound Mk-F4 (Fig. 2) were reported.

## 3. Results and discussion

### 3.1. HPTLC Fingerprint Profile

HPTLC fingerprint showed that violet purple colored band (after derivatization) at $R_f$: 0.93 was in MEMK. The florescent band (under 350 nm) at $R_f$: 0.93 was selected as marker compound and identified as Mk-F4.

**Table 1:** Calibration curve data for compound Mk-F4 by HPTLC method

| Track | Concentration (µL) | $R_f$ | Area of peak | Concentration of Mk-F4 (µg) | Calculated Mk-F4 (µg) |
|---|---|---|---|---|---|
| **1** | 2 | 0.93 | 3910.1 | 1.889 | |
| **2** | 4 | 0.93 | 7833.0 | 3.870 | |
| **3** | 5 | 0.93 | 7818.69 | Unknown[a] | 3.890 |
| **4** | 5 | 0.92 | 7407.18 | Unknown[b] | 4.111 |
| **5** | 6 | 0.93 | 10023.60 | 5.773 | |
| **6** | 8 | 0.92 | 13168.0 | 7.771 | |
| **7** | 10 | 0.92 | 16990.0 | 10.199 | |
| **8** | 10 | 0.95 | 1324.0 | Unknown[c] | 0.246 |
| a= MF3, b= MF4 and c = methanolic extract (MEMK) | | | | | |

**Table 2:** Quantification of compound Mk-F4

| Stationary phase | Precoated Silica Gel 60 GF254 |
|---|---|
| Mobile phase | n-butanol: acetic acid: water (4:4:2) |
| Calibration range of Mk-F4 | 1.88 - 10.19 µg/spot |
| Detection | Ninhydrin reagent heated at 105 ◦C for 5min and detected at 350 nm |
| Regression equation | Y=1574.X + 936.4 (area wise) |
| R value | 0.998 (area wise) |

### 3.2 Antioxidant activity

The antioxidant properties of the chromatographically separated fractions (MF1-MF4) of MEMK were carried out by standard following methods.

### DPPH radical scavenging assay

DPPH radical contains an odd electron, which is responsible for the absorbance at 517 nm and also for the deep purple colour [17]. When DPPH accept an electron donated by an antioxidant compound in test sample, it is decolorized which can be quantitatively measured from the changes in absorbance. The reduction in the concentration of DPPH radical due to the scavenging activity by MF4 showed the potent free radical scavenging activity 67.87 ± 3.2 than MF1: 25.26 ± 2.0, MF2: 25.35 ± 1.8 and MF3: 55.89 ± 6.2 percentage of inhibition, respectively, at 125µg/ml as compared by standard ascorbic acid (AA): 70.86 ± 8.1 (Table 3). The $IC_{50}$ (the inhibitory concentration at which there is 50% decrease of free radical) of MEMK fraction (MF1-MF4) was found to be 252.32 µg $mL^{-1}$, 251.32 µg $mL^{-1}$, 114.76 µg $mL^{-1}$ and 121.42 µg $mL^{-1}$, respectively. Free radical scavenging activity of the extract is concentration dependent, as the concentration of the test sample increases, the free radical scavenging activity increases and lower $IC_{50}$ value reflects better protective action.

**Table 3:** Percentage inhibition of fractions of MEMK by DPPH method

| Concentration (µg/ml) | Ascorbic acid | MF1 | MF2 | MF3 | MF4 |
|---|---|---|---|---|---|
| **25** | 15.02 ± 4.5 | 6.80 ± 1.4 | 6.34 ± 2.1 | 10.01 ± 2.4 | 14.66 ± 3.7 |
| **50** | 24.34 ± 3.8 | 10.90 ± 1.7 | 9.70 ± 1.5 | 15.08 ± 2.1 | 23.97 ± 2.4 |
| **75** | 45.94 ± 6.3 | 14.92 ± 2.3 | 13.13 ± 2.0 | 27.90 ± 1.8 | 43.59 ± 7.5 |
| **100** | 65.03 ± 4.9 | 23.24 ± 4.3 | 21.33 ± 2.1 | 38.19 ± 1.4 | 63.49 ± 6.9 |
| **125** | 70.83 ± 8.1 | 25.26 ± 2.0 | 25.35 ± 1.8 | 55.89 ± 6.2 | 67.87 ± 3.2 |

Data expressed as Mean ± Standard deviation

### Nitric oxide radical scavenging assay

The MF4 showed effectively the generation of nitric oxide scavenging activity at 125 µg/ml: 68.74 ± 8.6 than MF1, MF2 and MF3: 24.90 ± 1.2, 21.12 ± 1.3 and 30.04 ± 1.2. The radical scavenging of standard ascorbic acid at the same concentration was 71.487 ± 3.2 respectively (Table 4). The $IC_{50}$ of MF1-MF4 was found to be 309.41 µg $mL^{-1}$, 317.32 µg $mL^{-1}$, 243.98 µg $mL^{-1}$ and 89.36 µg $mL^{-1}$, respectively. From results of nitric oxide assay, it proved that MF4 was an effective scavenger of NO and competes with oxygen leading to a reduced production of NO than MF1-MF3 and compared with standard ascorbic acid with low $IC_{50}$ value [18].

**Table 4:** Percentage inhibition of fractions of MEMK by nitric oxide radical scavenging method

| Concentration (µg/ml) | Ascorbic acid | MF1 | MF2 | MF3 | MF4 |
|---|---|---|---|---|---|
| **25** | 18.15 ± 1.7 | 5.82 ± 0.9 | 4.70 ± 1.5 | 12.80 ± 2.3 | 17.78 ± 2.3 |
| **50** | 25.44 ± 1.5 | 10.24 ± 1.2 | 7.59 ±.0.9 | 15.94 ± 1.5 | 21.96 ± 3.6 |
| **75** | 46.90 ± 5.8 | 16.83 ± 0.7 | 10.53± 1.2 | 23.60 ± 2.5 | 42.80 ± 6.7 |
| **100** | 65.53 ± 7.3 | 20.81 ± 1.4 | 16.60± 1.6 | 26.47 ± 1.7 | 59.43 ± 4.2 |
| **125** | 71.48 ± 3.2 | 24.90 ± 1.2 | 21.12 ±1.3 | 30.04 ± 1.2 | 68.74 ± 8.6 |

Data expressed as Mean ± Standard deviation

### Hydrogen peroxide scavenging assay

Hydrogen peroxide itself is not very reactive, it can generate the highly reactive hydroxyl radical (OH) through the fenton reaction. Thus, the scavenging of hydrogen peroxide is an important antioxidant defense mechanism [19]. The scavenging ability of hydrogen peroxide by MF4 showed the higher scavenging activity at 125 µg/ml: 49.38 ± 7.2 percentage of inhibition than MF1-MF3: 19.45 ± 2.5, 20.95 ± 3.1 and 31.29 ± 2.1. The radical scavenging of standard ascorbic acid at the same concentration was 72.02 ± 8.3 respectively (Table 3). The $IC_{50}$ of MF1-MF4 was found to be 394.17 µg $mL^{-1}$, 374.92 µg $mL^{-1}$, 347.16 µg $mL^{-1}$ and 132.16 µg $mL^{-1}$, respectively.

**Table 5:** Percentage inhibition of fractions of MEMK by $H_2O_2$ radical scavenging method

| Concentration (µg/ml) | Ascorbic acid | MF1 | MF2 | MF3 | MF4 |
|---|---|---|---|---|---|
| **25** | 17.96 ± 3.8 | 7.23 ± 1.7 | 8.67 ± 0.9 | 10.34 ± 1.4 | 14.09 ± 2.7 |
| **50** | 24.78 ± 2.7 | 9.20 ± 1.2 | 9.60 ± 2.1 | 12.38 ± 2.0 | 19.39 ± 2.1 |
| **75** | 41.94 ± 5.1 | 12.48 ± 2.0 | 13.74 ± 1.3 | 16.34 ± 1.2 | 25.67 ± 1.8 |
| **100** | 62.76 ± 4.3 | 16.02 ± 1.8 | 17.82 ± 1.5 | 24.28 ± 2.4 | 38.34 ± 3.8 |
| **125** | 72.02 ± 8.3 | 19.45 ± 2.5 | 20.95 ± 3.1 | 31.29 ± 2.1 | 49.38 ± 7.2 |

Data expressed as Mean ± Standard deviation

### 3.3. Isolation and characterization of marker MK-F4 compound

On the basis of phytochemical data it was found that the isolated compound was an amino acid. Data from finger printing results provide information about presence of the major amino acids in MF3 and MF4 and showed the potent antioxidant activity. Hence, these amino acids were targeted for isolation.

**Compound Mk-F4 -** Isolated compound Mk-F4 has sticky type of nature. It gives violet purple color with ninhydrin reagent.

**Analysis**

**TLC-** $R_f$: 0.92, Solvent system- n-butanol: acetic acid: water (4:4:2).

**Detection-** Ninhydrin (heat at 105 ˚C for 5 minutes).

**Elemental analysis -** The elemental analysis (Elementar, Vario EL III) revealed that the compound contains 55.69% of C, 8.52% of H, 10.65% of N and 24.61% of O.

**GCMS-** RT: 9.818, MW: 129 (Fig. 3); NMR- $^1$H NMR (400 M HZ, MeOD): δ 1.6 (m, 2H, H-4), 1.9 (m, 2H, H-3), 2.2 (s, 3H, H-7), 2.3 (m, 2H, H-5), 3.1 (s, 1H, H-2, J = 7.0). $^{13}$C NMR (100 M HZ, MeoD): δ 19.46 (C-4), 22.75 (C-3), 39.97 (C-2), 55.85 (C-5), 70.77 (C-7), 171.76 (C-6) (Fig. 4).

### 3.4 Quantification of Mk-F4 in MEMK and its fractions using HPTLC

The quantification protocol was followed as per the ICH Q2-R1 guidelines [20]. Table 4-5 and Figure 1 depicts the calibration curve data of isolated marker compound Mk-F4 by HPTLC method and HPTLC chromatogram of MEMK and its fractions MF1, MF2, MF3 and MF4. The percentage (W/W) amount of Mk-F4 was found to be 90.0, 95.1 and 16.1% in MF3, MF4 and MEMK respectively, and was absent in other fractions MF1 and MF2 (Table 4).

**Substance Mk-F4@350 nm regression mode: Linear sdv= 2.9%**

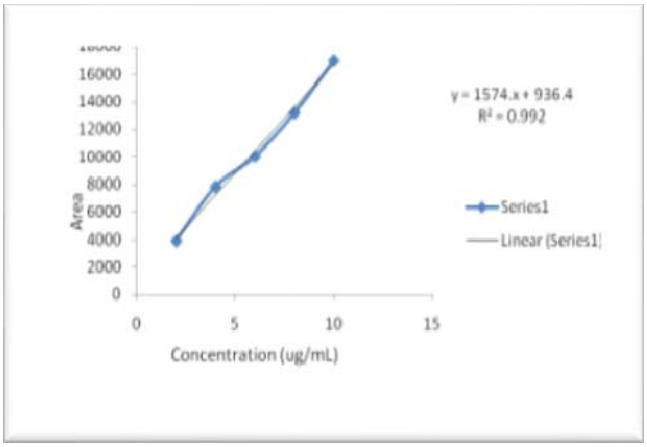



**Fig 1:** Calibration curve of compound Mk-F4

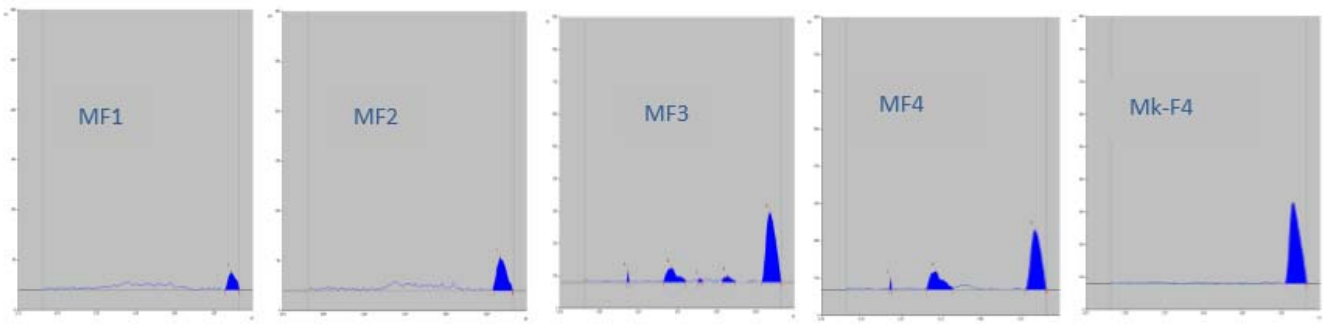
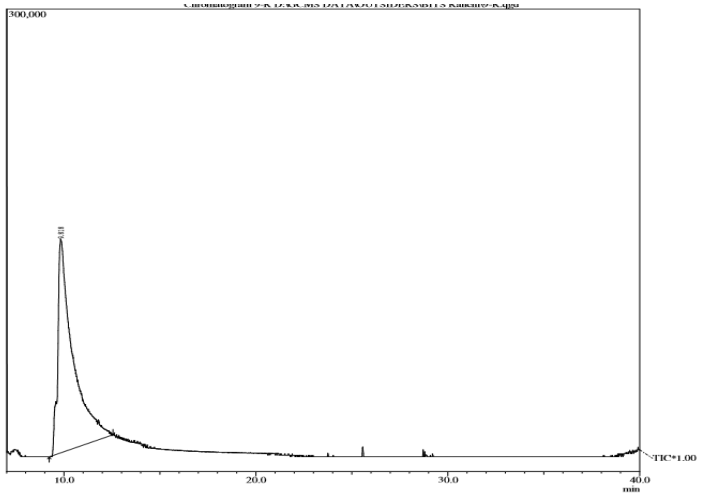


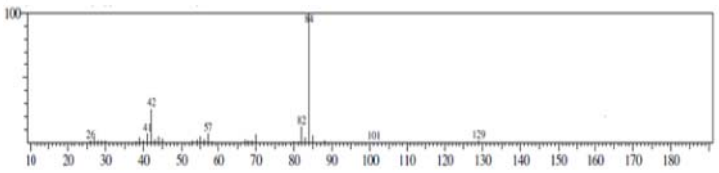

**Fig 2:** HPTLC chromatogram of fractions (MF1, MF2, MF3 and MF4) of MEMK and compound Mk-F4 showing peaks of phytoconstituents

### 4. Conclusion

The proposed method is a simple and fast alternative for quantitative determination of Mk-F4 in methanolic extract of *M. koenigii* that is responsible for having antioxidant activity which was quantified by the presence of more percentage of Mk-F4 in MF4 which showed higher antioxidant activity than the other fractions. Moreover MF1 and MF2 showed least antioxidant activity which also proves the point. Thus, the HPTLC finger printing profile has been used as a diagnostic tool to identify and to determine the quality and purity of the *M. koenigii* in present studies.

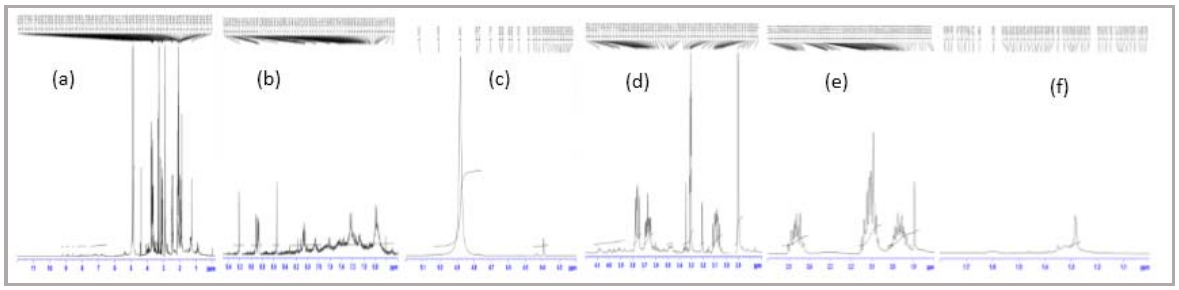


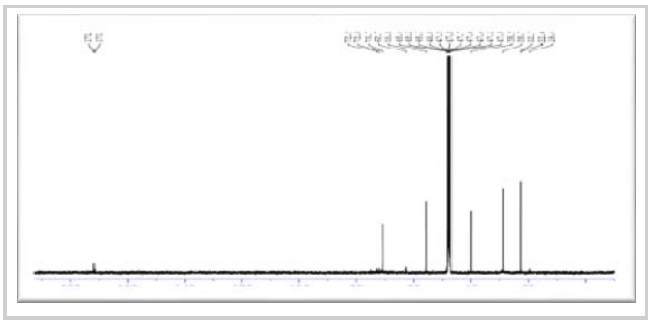

(a)




(b)

**Fig 3:** GC-MS (a-b) of compound Mk-F4



(g)

**Fig 4:** $^{1}H$ NMR (a-f) and $^{13}C$ NMR (g) of compound Mk-F4

## 5. Acknowledgment

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