

ISOLATION AND TENTATIVE IDENTIFICATION OF ANTIOXIDATIVE CONSTITUENTS FROM DICHLOROMETHANE EXTRACT OF *MURAYA KOENIGII* LEAVES USING CHROMATOGRAPHIC TECHNIQUE

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Abstract

This investigation aimed to identify antioxidative constituents from *M. koenigii* leaves using thin layer chromatographic (TLC) technique. The screening of phytochemicals was conducted using methanol extracts as well as the developed TLC of extracts. Isolation of antioxidative constituents was held using preparative TLC. Secondary metabolites that had been screened from methanol extract were recognized as tannins, alkaloids, flavanoid and terpenoids. Screening for phytochemical using developed TLC of extracts revealed the presence of antioxidative constituents from dichloromethane extract after spraying with DPPH reagent. The antioxidative constituents are proposed as (A) Cyclic Terpene ester, (B) 4-benzyloxy-4-[2,2,-dimethyl-4-dioxolanyl] butylaldehyde, (C) Oleic acid, (D) Piperidione (alkaloid derivatives) and (E) 9-oxo-methyl ester after analyzing with GCMS aided with Wiley Database matching individually. Based on its potent antioxidant properties, the use of *M. koenigii* leaves in the development of natural medicine for the treatment of various diseases cannot be neglected.

Keywords: *Murraya koenigii*, secondary metabolites, antioxidant activity

Introduction

Murraya koenigii (L.) Spreng, commonly known as curry leaf in Malaysia, belongs to the family of Rutaceae, which is native to India and now distributed in most of Southern and Southeast Asia (Satyavati et al 1987). It has a slightly pungent, bitter, and feebly acidulous taste and is frequently used for cooking in Malaysia and other Asian countries. It is believed to function as traditional a folk remedy that contains several potent bioactive compounds with health-promoting properties. Different parts of *M. koenigii* have been used for the treatment of cough, hypertension, hysteria, hepatitis, rheumatism, poisonous bites, and skin eruptions. Besides, curry leaf has been reported to have antitumor (Mohd Nor et al., 2009), antioxidant (Muthumani et al., 2009), anti-inflammatory (Dineshkumar et al., 2010), antihyperglycemic (Tembhurne and Sakarkar, 2009) and hypoglycemic effect. (Crozier et al., 1997). However, the searching of secondary metabolite sources which responsible for its antioxidant behaviour of Malaysian curry leaf is still scarce. The present investigation was conducted to isolate and determine the bioactive compounds as well as its antioxidant profile from dichloromethane extract of leaf extracts of *M. koenigii* from Malaysia.

Materials and Methods

Extraction

The fresh leaves of *M. koenigii* was washed and cut into small pieces and was air-dried at room temperature (26°C) for 2 weeks. The dried leaves were grinded to a uniform powder. Then 200 g of coarse powder the fresh leaves of *M. koenigii* was soaked in 500 ml of each petroleum ether, dichloromethane and methanol solvents in the cold for 3 times in one week with

occasional shaking. The solvent to sample ratio of 10 : 1 (v / w) solvent to dry weight ratio was used as ideal (Das et al., 2010). The solvent from the total extract was filtered through a Whatmann filter paper No. 42 (125 mm) and then through cotton wool. Next, the mixture was extract about 30 minutes by using rotary evaporator with the water bath set at 40°C. The percentage yield of extracts ranged from 7–19 % w / w (Ayoola et al., 2008).

Thin Layer Chromatography of Plant Extracts

The differences ratio of solvent system was carried out on the TLC plate for the differences solvent polarity. The preparative TLC silica gel 60F254, 7X6 cm (Merck) plate was developed baseline with a pencil. Then glass capillary tube was used to spot extracts at distance of 1 cm from baseline. After the spot reached at distance 1 cm from upper line, the plates was dried and observed under Ultraviolet light to detect the bands on the TLC plates.

Fourier Transform Infra-Red (FTIR) Analysis of Crude Extract

The FTIR model of Perkin Elmer Spectrum 100 FT-IR. ATR- FTIR was used to identify functional groups in *M. koenigii* leaves. The expected functional groups were alkane, alkene, alcohol, carboxylic acid, ester, benzene aromatic ring, amine and carbonyl group.

Phytochemical Screening Test

This experiment was conducted as stated similarly by Pavitra et al. 2009 by means to screen phytochemicals such as alkaloids, tannin, saponin, triterpenoid, steroid, flavonoids and cardiac glycoside. Generally, a measured methanol extract of *M. koenigii* leaves was mixed with chemical reagents to screen the listed phytochemicals.

Qualitative Antioxidant Analysis Using TLC

The TLC plate (1.5 cm x 6 cm) of each crude extract was developed in a suitable developing solvent. After drying process, the TLC plate was sprayed with antioxidant spraying reagent of 0.2% solution of diphenyl picrylhazine (DPPH) in methanol. The yellow-white spots against purple background indicated the presence of antioxidant constituents. The TLC plate of extracts which demonstrated greater intensity of yellow white spot on TLC plate will be considered for isolation of antioxidative constituents.

Isolation of antioxidative constituents using preparative TLC

The TLC plate of extracts which demonstrated greater intensity of yellow white spot was redeveloped using 10 cm x 10 cm TLC plate. After developing process and drying process was completed, the desired band was fully scraped off and was dissolved in its dissolving solvent prior to filtration. A simple filtration process was conducted through cotton wool packed into tiny glass dropper to remove silica gel. The filtrate was left for several hours for drying process at room temperature. The dried compound was kept in refrigerator prior to use.

Gas Chromatography (GC-MS)

The structure identification of antioxidative constituents was performed using Gas Chromatography Mass Spectrometer (GCMS) with Wiley database matching individually. The isolated compound were introduced by using splitless injection of 2.0 µL in methanol fitted with cross linked of 5% phenyl ethyl siloxane capillary column. Mass detector has been used and the injector temperature was 220°C, with an oven temperature of 60 – 250°C with rate of 5°C min⁻¹. Carrier gas that has been used was helium gas at flow rate 1.5 mL min⁻¹.

Result and Discussion

Phytochemical Screening Analysis

Table 1 demonstrates the types of phytochemicals present in *M. koenigii* leaves.

Table 1 Phytochemicals in *M. koenigii*

Phytochemicals	Observation
Terpenoid	+
Flavonoid	+
Saponin	-
Alkaloid	+
Tannin	+
Cardiac glycoside	-

(+) = present; (-) = absent

The screening was conducted by means to examine the presence of tannins, cardiac glycoside, alkaloids, saponin, flavonoids and terpenoids in *M. koenigii* leaves as depicted in Table 1. Previous investigation revealed phytochemicals from *M. koenigii* leaf were belong to alkaloids, tannins and flavonoids (Handral et al., 2012) as well as terpenoid (Vijayvargia and Vijayvergia, 2016) and our current results totally agreed with this previous study. The absence of saponin in *M. koenigii* leaf was consistent with the finding by (Salna et al., 2011). However there was no appearance of cardiac glycoside although it was previously found to have positive result of cardiac glycoside (Mathur et al., 2011).

Generally phytochemicals in plants are substances that play important roles in their growth and development. They protect plants from harmful agents such as insects and microbes as well as stressful events such as ultraviolet (UV) irradiation and extreme temperatures. Each phytochemicals possess their own advantages and has unique properties. Interestingly, they also provide health benefits when consumed since certain plants are richer in phytochemicals such as phenolic compounds like phenolic acids, flavonoids, and other phenolics as well as terpenoid which is antioxidant. Hence, the presence of terpenoid, flavonoid and alkaloid may responsible for antioxidant properties in *M. koenigii* leaves.

Determination of functional groups from FTIR analysis

Table 2 depicted various kinds of functional groups presents in *M. koenigii* leaves after analysed with FTIR spectrometer. Conceptually, organic molecules absorbed energy from infrared radiation when the frequency of infrared radiation matched with the frequency vibration of covalent bond in molecules and as a result molecules vibrate at higher amplitude. Since different functional groups of organic molecules absorbed energy of infrared radiation at different frequency, so any functional group present in any organic molecules can be determined easily by referring to its frequency of vibration or wavenumber (cm^{-1}) solely. By recognizing functional groups, one can have the general idea of what type of group of the compound is, however the complete structure of the compound still cannot be determined with FTIR data alone. According to **Table 2**, the absorption peak of 1643.65 cm^{-1} which was corresponded to the functional group of carbon double bond gave earlier information about possible aromatic chemical structure. The presence of C-N stretching functional group at 1010.80 cm^{-1} revealed the existence of alkaloidal structure in which it was consistence with phytochemical screening data. The C=O stretching also was observed at 1643.65 cm^{-1} but it was little bit lower than its normal value of 1715 cm^{-1} . This phenomenon is normally occurred

as a result of conjugation effect in the structure. Conjugation effect weakened the C=O bond and shifted absorption frequency to lower value. The hydroxyl group OH stretching also appeared but seemed to overlap with C-H stretching but this is normal for carboxylic acid FTIR data. Therefore we could possibly proposed several structures from *M. koenigii* leaves generally as the groups of aromatic compound, terpenoid, carboxylic acid, carbonyl compound and alkaloid.

Table 2 The type of functional groups in *M. koenigii*

Wave number, cm ⁻¹	Functional group
2923.58	sp ³ C-H stretching/OH stretching
1643.65	C=O stretching/C=C aromatic stretching
1181.17	C-O stretching
1010.80	C-N stretching

Screening of antioxidative constituents from *M. koenigii* crude extracts

The presence of antioxidative constituents in any plant extract can be easily determined by spraying the developed TLC with DPPH spraying reagent solution. The formation of yellow spot against purple background gave the sign of antioxidative behavior of the extract. The duration of the formation of yellow spot in each extract was closed related to the content of antioxidants in each extract. The faster the formation of yellow spots the greater the antioxidant content. **Figure 1** illustrated TLC of each extract after spraying with DPPH reagent.

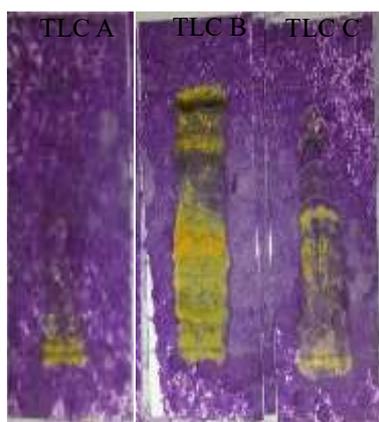


Figure 1 The emerging of yellow spot against purple background in *M. koenigii* leaves.

TLC A: TLC of petroleum ether extract after spraying with DPPH

TLC B: TLC of dichloromethane extract after spraying with DPPH

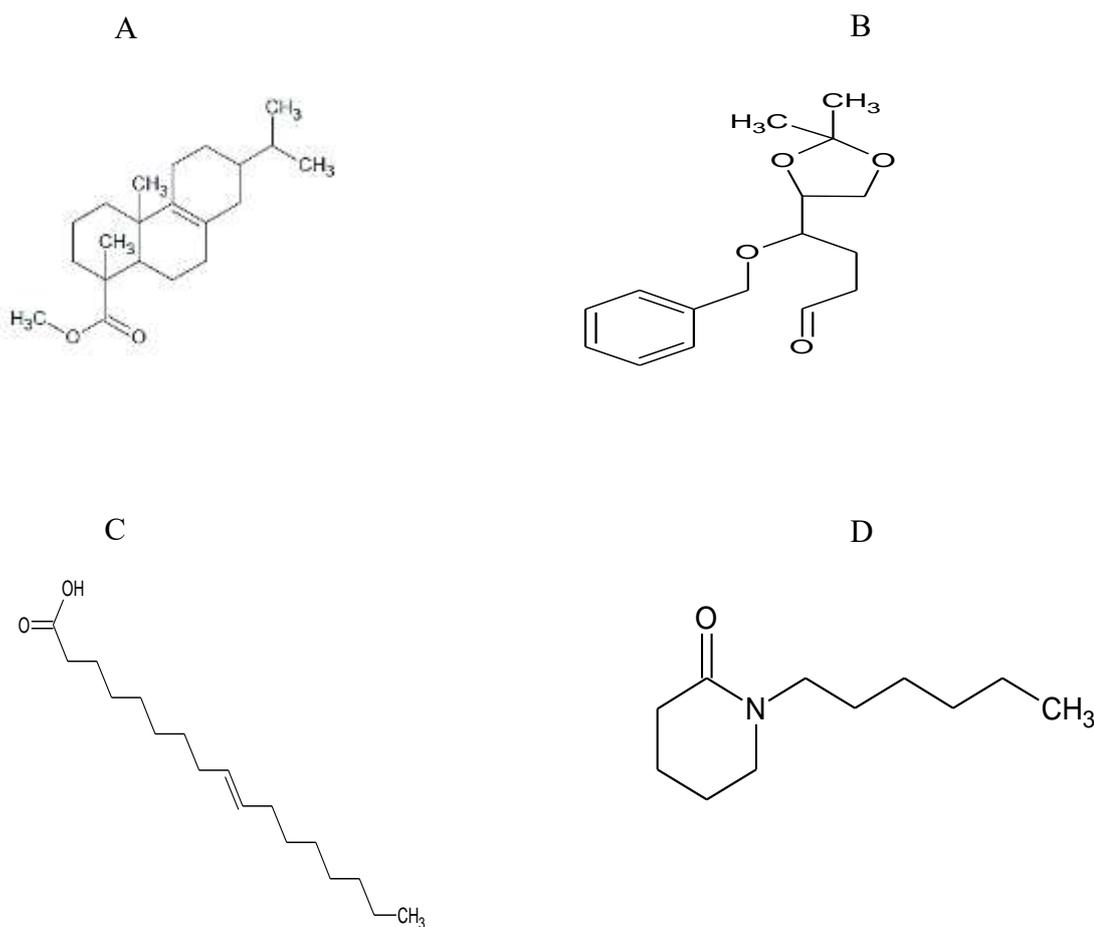
TLC C: TLC of methanol extract after spraying with DPPH

As can be seen in **Figure 1**, the dichloromethane extract seemed to demonstrate higher antioxidants content as depicted in TLC B compared to petroleum ether extract and methanol extract. Theoretically, any antioxidant donates electrons to free radicals, neutralize and prevent them to cause harm. In our case the DPPH which act as free radical supplier was neutralized and reduced by the donation of electron from antioxidant in extracts and immediately reduces

its colour from purple to yellow as it appeared on TLC. Since TLC B contains greater number of antioxidant qualitatively, it was chosen for isolation of antioxidative compounds using preparative TLC method.

Proposed antioxidative constituents from dichloromethane extract of *M. koenigii*

Five antioxidative compounds were successfully isolated through preparative TLC, whereby compound A was isolated at retention factor 0.622, compound B and D were isolated at retention factor of 0.917 and compound C and E at retention factor of 0.561. All isolated compounds were subjected to GCMS by means of structure determination. **Figure 2** illustrates the proposed structures of all isolated compounds after analyzing using GCMS with Wiley Database matching individually. According to **Figure 2**, the C=O and C-O functional groups can be seen in all structures except in compound D with no C-O and they were consistent with the FTIR data at 1643.65 cm^{-1} and 1181.17 cm^{-1} respectively. Compound D is an alkaloid which was identified by the presence of nitrogen atom linkage. Conjugation effect was also observed in this compound since the C=O and nitrogen atom was arranged alternately. The resonance effect makes the C=O behaved as single bond character and weaken the bond and lead to lower absorption frequency of C=O bond as showed in FTIR data compared to its normal absorption. After analyzing using GCMS with Wiley database matching individually, compound A,B,C,D and E were proposed as terpene ester, 4-benzyloxy-4-[2,2,-dimethyl-4-dioxolanyl] butylaldehyde, Oleic acid, Piperidione and 9-oxo-methyl ester respectively.



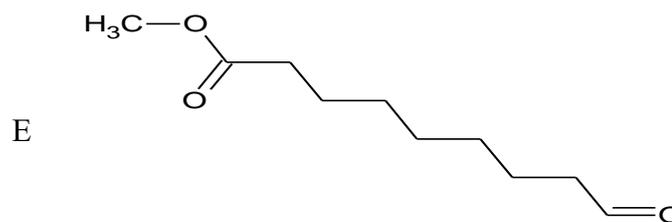


Figure 2 Antioxidative constituents from dichloromethane extract of *M. koenigii* leaves

Conclusion

All extracts from *M.koenigii* leaves have shown its potent antioxidative behavior in which dichloromethane extract is the most antioxidative. Isolation and identification of antioxidative constituents from preparative TLC and GCMS have proposed 5 antioxidative constituents which namely as (A) terpene ester (B) 4-benzyloxy-4-[2,2,-dimethyl-4-dioxolanyl] butylaldehyde, (C)Oleic acid, (D) Piperidione and (E) 9-oxo-methyl ester. Further research work using Nuclear Magnetic Resonance (NMR) need to be conducted for structure confirmation.

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Conflict of interests

Author declares no conflict of interest.

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