

Bio-efficacy of acetonic leaf extract of *Murraya koenigii* with reference to its antibacterial spectrum against food-borne bacteria

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ABSTRACT

The food-borne antibacterial activity of *Murraya koenigii*, acetone leaves extract (ALE) was investigated against standard strains of *Escherichia coli*, *Listeria monocytogenes* and *Staphylococcus aureus* causing various food-borne diseases in human. The ALE showed a significant inhibition of the *L.monocytogenes*, *S. aureus* but the low inhibition of *E.coli*. The bioactive metabolites analysis of ALE by TLC, HPLC, UV-Vis spectroscopy, FTIR and NMR exhibited the presence of diverse types of bioactive phyto-constituents such as flavonoids, saponins, phenolic bioactive compounds etc. which might be responsible for bacterial inhibition in-vitro. FTIR studies of ALE, revealed the presence of functional groups peaks such as phenol, alkanes, alkenes, aromatic, aliphatic and amine plant bioactive compounds. NMR showed the presence of aliphatic groups and -OH groups compounds which were structurally and functionally similar due to the chemical arrangement of functional groups. Thus, *M. koenigii* provided a natural remedy for the control of food-borne pathogens.

KEY WORDS: ANTIMICROBIAL ACTIVITY; FTIR; HPLC;NMR; PHYTO-CHEMICAL ANALYSIS

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INTRODUCTION

Food-borne bacterial diseases in worldwide are proliferate day-by-day and becomes a serious concerns for both consumers and the food fabrication. Majority of food-borne diseases are caused by food spoilage bacteria and other microorganism (Sousa, 2008). Hence, the problem of food contamination arising through bacterial infections a matter of major concern for the public health in both developed and developing nations (Shi and Zhu, 2009). Food spoilage is a sundry process involving food-borne microorganisms causing loss of 25% world's food supply and a vast degree of infection illness. Fresh products such as fruits, vegetables, dairy products etc. are most likely to be contaminated by food-borne pathogens such as *Campylobacter*, *Escherichia coli*, *Listeria monocytogenes*, *Staphylococcus aureus*, etc. (Dhama et al., 2015). The majorly pathogenic bacteria (66%) have played main part in food spoilage or food borne illness (Khare and Rawat, 2018).

A good number of such toxicogenic bacteria have been discovered and few of them such as *L. monocytogenes* among, causing their survival at even extremely low temperatures where the other bacteria do not grow. The economic burden and food-borne diseases by contamination of food is continuing in the contemporary technical advancement (Ray and Bhunia, 2013). It has been estimated that in developing countries like India, 30% of people suffer from outbreaks of food-borne pathogens (Scallan et al. 2011). Traditional remedy, especially the medicinal plants and their extracts, still play a major role in the developing countries to combat bacterial infections (Pirzada et al., 2009). According to the WHO, the majority of worldwide population depends on traditional remedial system as a resource of natural drugs for human healthcare (Agyare et al. 2018).

The folk medicine makes valuable remedial for several diseases due to very low side effects (Zhang et al. 2016). The active phytochemical constituents of medicinal plants have bio-efficacy such as antioxidant, insecticidal and antibacterial activities. The folk medicine and its different parts extract exhibit significant anti-bacterial activity without any adverse serious side effects to host. The leaves of *M. koenigii* contain good sources of antioxidant activity, revamp the food protection and diminish food-borne infections, and can be used as preservatives (Genena et al. 2008). *M. koenigii* is a medicinal plant belongs to the family Rutaceae, is native to India and Sri Lanka. Its leaves are used in recipes in India and neighboring countries as spices due to aromatic nature. The leaves of *M. koenigii* work in relief from the food-borne pathogenic infection, vomiting and dysentery. The present work is therefore, aimed to study the bio-efficacy of ALE against both Gram-positive and

Gram-negative food-borne bacteria because of their significance its combat human food-borne pathogens.

MATERIALS AND METHODS

Plant material: The healthy leaves of *M. koenigii* were collected from their natural habitat growing at different locations of district Haridwar, Uttarakhand (29.945°N North 78.163° East) during September to October 2015 and 2016. The plant was identified following of authentic literature based on its characteristic features and a herbarium was kept in the Department of Botany and Microbiology, Gurukula Kangri Vishwavidyalaya, Haridwar, India.

Bacterial strains: The standard cultures of *Escherichia coli* MTCC 25922, *Staphylococcus aureus* MTCC 25923 and *Listeria monocytogenes* MTCC 657 were procured from the Microbial Type Culture Collection, (MTCC), Chandigarh, India. The test bacteria were sub-cultured onto nutrient agar medium in order to determine their viability. Stock cultures were maintained on nutrient agar slants at 4°C and inoculated in nutrient broth at 37°C prior to further use.

Extraction of plant material: The *M. koenigii* leaves were washed with distilled water and the dried leaves were pulverized to get powder in form. 200 g leaf powder was used for bioactive chemical extraction using acetone as solvent. Acetonic leaf extract (ALE) was concentrated by vacuum evaporator under the control temperature and pressure to obtain a gummy/semi-solid mass, which was preserved in a refrigerator at 4°C for further uses (Oniszczyk and Podgórski, 2015).

Antibacterial activity: The selected food-borne bacterial strains were prepared by transferring microbial inocula from stock cultures to test tubes containing Mueller-Hinton Broth (MHB) and incubated at 37°C for 24 h. Antibacterial activity was tested by agar well-diffusion method (Correa et al., 2017). 100 µL of diluted inoculum of 10^5 cfu mL⁻¹ of 24 h old cultures of *E. coli*, *L. monocytogenes* and *S. aureus* were separately mixed in Mueller Hinton Agar (MHA) medium, with thorough shaking. Medium was poured in sterilized Petri plates and were allowed to solidify. A sterile cork borer of (6 mm diam.) was punch wells in medium. The stock extract of ALE (100 %) was diluted using acetone solvent to get 25, 50 and 75% concentrations for measuring antibacterial activity. DMSO (dimethyl sulphoxide) was used as control. The plates were incubated at 37°C for 24 h for the antibacterial activity (Balouiri et al., 2016). The data was interpreted on the basis of the size of the diameter of zone of inhibition (mm).

Phytochemical screening: The acetonic leaf extract (ALE) was subjected for qualitative analysis of phytoconstituents viz., alkaloids, amino acids, flavonoids,

saponins and tannins following Trease and Evans (1983). Dragendorff's test for alkaloid was carried out in which diluted hydrochloric acid (0.1 mL) and Dragendorff's reagent (0.1 mL) were added separately in 2 mL of ALE in test tubes. After proper mixing, formation of orange brown colored precipitate indicated the presence of alkaloids. For saponins test ALE (1 mL) was separately diluted with distilled water up to 20 mL and vigorously shaken in a graduated cylinder for 15 min. Development of stable foam indicated the presence of saponins; this test is also known as foam formation test. ALE (5 mL) was separately treated with 1 mL of 10% aqueous lead acetate solution. Formation of yellow colored precipitate indicates the presence of flavonoids. For tannins analysis, ALE (5 mL) were separately allowed to react with 1 mL of 5% ferric chloride solution. Appearance of greenish black color indicated the presence of tannins. ALE (2 mL) and 0.25% ninhydrin reagent were added in fresh test tube and boiled for few minutes. Formation of blue color indicated the presence of total amino acids.

Thin layer chromatography (TLC): For quantitative analysis of different bioactive marker, ALE was applied on prepared TLC plate and developed in a TLC chamber saturated with different suitable mobile phase such as ethyl acetate: methanol (3:1), hexane : chloroform : methanol (5:1:1) and chloroform : methanol (5:1). Based on clear bands and proper R_f values, the chloroform: methanol (5:1) was found the most suitable solvent system for the separation of bioactive metabolites. (Ramalho *et al.*, 2006).

Fourier transform infrared spectroscopy (FTIR): FTIR was conducted following Kumar *et al.* (2014). The Infra-red spectra of acetone extract was analysis for the determination of functional groups responsible for biological activities. ALE was mixed with KBr (spectroscopic grade) and pressed to form 1-mm pellet. Perfectly dried powder of the ALE was placed on the sample chamber of Nicolet Avatar 330 FTIR spectrometer (Thermo Electron Co., Madison, WI, USA) for the record of spectra and in the range of 600–3600 cm^{-1} . The absorption frequencies appeared in functional group region as well as fingerprint region of the spectra was observed to record FT-IR spectral (Liu *et al.*, 2006).

UV-Vis spectrophotometry: For the UV-VIS spectrophotometer (Perkin Elmer, USA Model: Lambda 950) analysis, the sample was prepared by diluting to 1:10 within the same solvent used in extraction of material (Do *et al.*, 2014). The extract was examined under visible and UV light in the wavelength range 200–800 nm. The UV-visible spectra were performed to identify the compounds containing σ -bonds, π -bonds, lone pair of electrons and aromatic rings. The UV-VIS spectrum was observed both in visible and UV-VIS light lambda 200–800 nm as given by Maji *et al.* (2016).

HPLC analysis: HPLC analysis was performed by Perkin Elmer Series 200 system in isocratic conditions using a C-18 (250mm x 4.6 mm, 5 μ m) at 25°C. Running conditions included injection volume, 20 μ l; mobile phase, methanol: acetic acid (0.4%) (800: 200 v/v); flow rate (1 ml/min). *M. koenigii* ALE (2.5 mg) was dissolved with 5 ml acetone. Bioactive compounds present in test sample were identified by chromatographic peaks with the retention time (RT) at 220 nm and 254 nm by UV detector. HPLC analysis was performed according to the method of Altun *et al.* (2002).

^1H and ^{13}C NMR analysis: Sample was dissolved in respective deuterated solvents (CDCl_3), 600 μ l was poured in NMR tube and observed on the applied magnetic field (Tachibana *et al.*, 2001), to obtain the Nuclear Magnetic Resonance (DRX-300Mega Hz Bruker, Switzerland).

RESULTS AND DISCUSSION

Phytochemical screening: Preliminary phytochemical analysis of ALE of *M. koenigii* revealed the presence of saponins, alkaloids, amino acids and flavonoids. However, terpenes was not detected in ALE of *M. koenigii* (Table 1).

Antibacterial activity: ALE caused broad zone of inhibition. Significantly, wider zone of inhibition of actively growing bacteria on surface of Petri plates indicated the antibacterial potentials of ALE. ALE had the maximum activity against *L. monocytogenes* (12 mm), while the minimum inhibition was recorded against *E. coli* (7 mm). The zone of inhibition at 100% concentrations of ALE was most prominent followed by 75, 50, and 25 %. Which was corresponding to concentration the order of effectiveness of ALE was *L. monocytogenes* > *S. aureus* > *E. coli* on the basis of sensitivity (Table 1).

Thin layer chromatography: Different active metabolites such as the alkaloids, flavonoids, glycosides, terpenoids and saponins with many high resolution bands appeared with different R_f values (Figure 1).

Table 1. Antibacterial activity of ALE of *M. Koenigii* against food-borne bacteria.

ALE	Zone of inhibition		
	<i>L. monocytogenes</i>	<i>S. aureus</i>	<i>S. aureus</i>
%			
100	11 \pm 2.5	9 \pm 1.02	9 \pm 1.02
75	10 \pm 1.01	8 \pm 0.87	8 \pm 0.87
50	8 \pm 0.75	5 \pm 0.72	5 \pm 0.72
25	4 \pm 0.45	3 \pm 0.25	3 \pm 0.25
Control	NI	NI	NI
Erythromycin	25	23	23

Values are mean of three independent observations \pm SD; NI= No inhibition

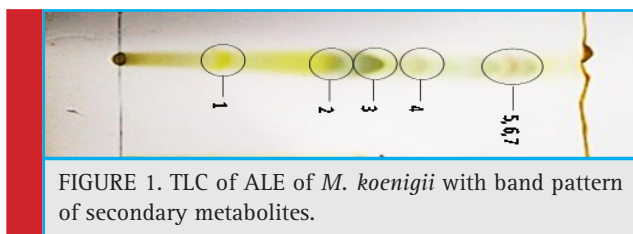


FIGURE 1. TLC of ALE of *M. koenigii* with band pattern of secondary metabolites.

Fourier transform infrared spectroscopy (FTIR): The functional groups of bioactive chemicals of ALE both known and unknown bands appeared at $3959.59 - 553.53\text{cm}^{-1}$. The intense broad absorbance at 3515.99cm^{-1} was the characteristic of the hydroxyl functional group in alcohols and phenolic compounds. The absorbance was relatively intense and broad at 3515cm^{-1} characterized for hydroxyl functional groups. Two bands at $3444.63, 3259.47$ and a weaker band at 1600.81cm^{-1} exhibited amine group. Further, intense and weaker absorptions were observed in the C-H aromatic bands at $3130.25, 2960.53\text{cm}^{-1}$, respectively. On the other hand, low intensity of the absorption was observed in the two aldehyde bands at 2812.02 and 2704.01cm^{-1} similar to another two alkyne bands at 2329.85 and 2189.06cm^{-1} . Single intense absorption peak appeared that determines the hydroxyl band at 3515.99cm^{-1} , while alkene band at 1361.65cm^{-1} has low absorption spectra. The appearance of ester band at 995.20cm^{-1} along with others having 4 small alkyl halide bands at $862.12, 754.12, 678.90$ and 553.53cm^{-1} were significantly diverse but showed majority of alkaloids with distinct secondary metabolites of unknown functional groups referred to the absorption spectra. On the other hand, spectral data of most of the extract confirmed the presence of bioactive groups

such as $-O-H, -C-H, =C-H, -C\equiv C, N-H, -C-O, -COOH, C-Cl, C-Br$ and alkene. Important IR absorption frequencies displayed the presence of C, H, Br and alkane string bioactive compounds (Figure 2).

UV spectroscopy: The ALE was examined by UV spectroscopy for proximate analysis. The UV-VIS profile in the range of $200 - 800\text{nm}$ wavelength exhibited the sharpness of the peaks and proper baseline. The flavonoids spectra typically consisted of two absorption maxima in the ranges $200-290\text{nm}$ and $300-530\text{nm}$. The precise position and relative intensities of these maxima gave valuable information in the nature of flavonoids. Occurrence of peaks at $207-557\text{nm}$ reveals the presents of flavonoids in the *M. koenigii* (Figure 3).

HPLC analysis: HPLC profiling of ALE showed the presence of bioactive metabolites in different peaks observed at 220nm and 254nm in UV. The eight compounds were separated at different retention times at 220nm and at 254nm . These spots showed different bioactive constituents (Table 2).

Nuclear magnetic resonance (NMR) spectrophotometry: ^1H NMR and ^{13}C NMR spectrum of isolated compound reveals a one strong solvent (CDCl_3) peak at 7.264ppm . NMR spectrum of ALE extract resulted 11 peaks due to the presence of $-OH$ group, C-H and C=O group. In ^1H NMR spectrum a less intensive peak appeared at $1.6, 1.7\text{ppm}$ due to $\text{R-CH}_2\text{-R}$ group and 4.0ppm for $=\text{CH}$ group. The four broad spectrum peaks constitute $2.1, 2.2\text{ppm}$ for C=O and $2.3, 2.6\text{ppm}$ spectrum due to the presence of C-N group and one 1.25ppm for $-OH$ in ALE of *M. koenigii* (Table 3).

Foods-borne infectious diseases are serious concern of worldwide. About 250 different food-borne diseases

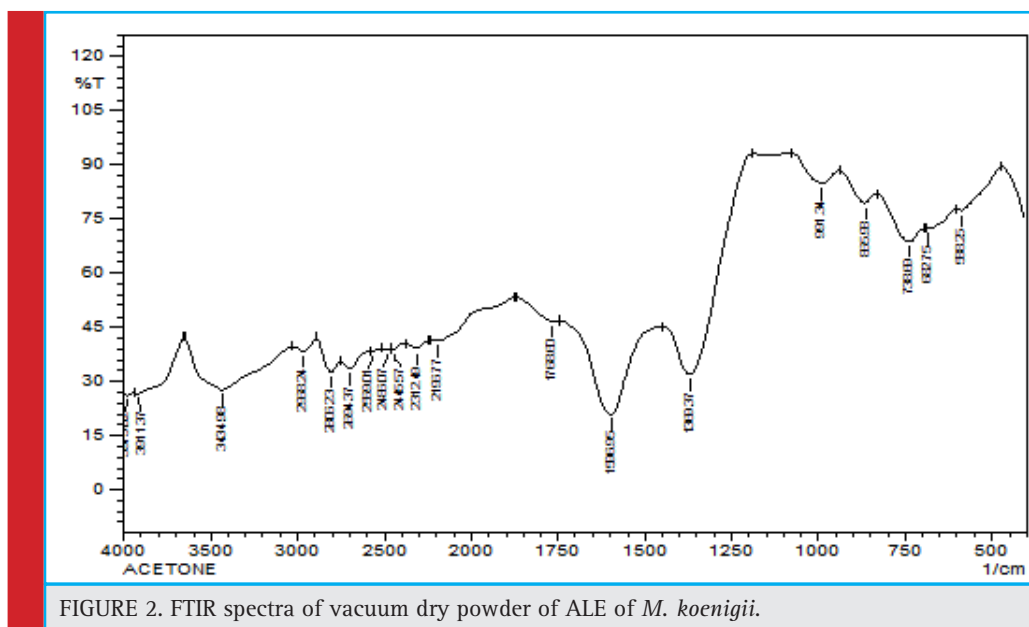


FIGURE 2. FTIR spectra of vacuum dry powder of ALE of *M. koenigii*.

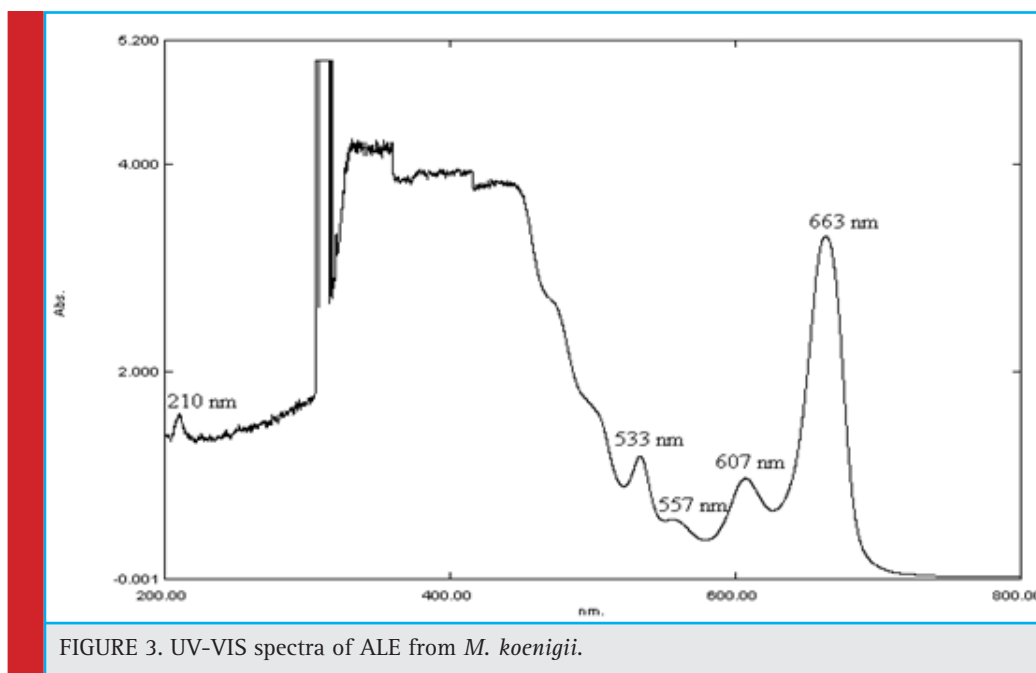


FIGURE 3. UV-VIS spectra of ALE from *M. koenigii*.

Table 2. Peak list and Rf value of the chromatogram of the ALE of *M. koenigii*.

RT	Area	Height	Purity Angle	UV range (nm)
4.364	3102730	195562	6.639	220
9.628	266428	41190	3.636	220
18.936	24814	178512	2.54	220
20.110	364394	64676	0.375	220
21.309	762224	135192	0.222	220
22.782	1052445	192003	0.232	220
4.365	47873872	2730580	6.430	254
7.550	126028	25924	3.784	254

have been reported, and bacteria are the major and common causative agents of two thirds of food borne infectious disease outbreaks (Le et al., 2003). Medicinal plants are widely used against dysentery and food-borne infec-

Table 3. ¹H-NMR and ¹³C-NMR spectral data of bioactive constituents of ALE.

Type of proton	Chemical shift (δ) ppm	Description of proton
ROH	1.25	Alcohol
C-H	1.4, 1.5	Alkane
R-CH ₂ -R	1.6, 1.7	Alkyl
C=O	2.1, 2.2	Carbonyl
C-N	2.3, 2.6	C attached to N and Halogens (Cl, Br, and I)
C=O	3.9	C attached to O
=CH	4.0	Alkene

tion throughout the world but only selected plants have been validated by scientific community. *M. koenigii* leaves are used mainly for flavor and medicinal purpose worldwide shown its potential to serve as food ingredients in India and other Asian countries (Perera and Li., 2012; Shanthala and Jamuna., 2005). We have observed that the leaves of *M. koenigii* have highly anti-bacterial activity against *L. monocytogenes*, *S. aureus* and *E. coli*.

Recently, Sablania et al. (2019) found that the *M. koenigii* leaves have antibacterial activity against *Bacillus subtilis*, *Staphylococcus aureus*, *Pseudomonas aeruginosa*, *E. coli* and other microorganism. The most significantly inhibitory effect has been observed against *L. monocytogenes*. ALE resulted in the maximum zone of inhibition against *L. monocytogenes* followed by *S. aureus* and quite less against *E. coli*. Due to the presence of OH (hydroxyl group) at 1.25 ppm in ALE, it may have similar mode of action against bacteria like erythromycin as stated by (Kohanski et al., 2007). Some of the bioactive chemicals may have similar mode of action such as DNA damaging, protein synthesis inhibition and bacteriostatic activity against food-borne bacteria. The anti-bacterial effects against Gram-positive bacteria and Gram-negative may be attributed due to the presence of several bioactive chemicals in ALE (Table 2). The natural bioactive chemicals which contain -OH groups, that were causing of protein damage and membrane lipid inhibition of bacteria (Brogden., 2005).

Similarly, Gupta et al. (2018) reported strong antibacterial properties by *M. koenigii* leaves extract mainly due to the saponin and active protein. But the action of additional anti-food-borne bioactive compounds of *M. koenigii*

cannot be ruled out. The microbicidal activity of ALE was higher against the Gram-positive bacteria in comparison to the Gram-negative bacteria. Majority of Gram-positive bacteria have been drastic food-borne pathogens in comparison to that of Gram-negative bacteria. The data of UV-VIS spectral absorption showed the presence of flavonoids at absorbance 207-280 nm also reported by Kavitha and Uduman (2017) whereas phenolic acid derivatives at 317-340 nm (Zavoi et al., 2011). In the present study, ALE spectral absorption sharp baseline and peaks were clearly shown in spectral graph. The Preliminary plant bioactive metabolites screening and TLC Rf values ALE of *M. koenigii* revealed to the presence of diverse type of bioactive metabolites such as alkaloids, amino acid, saponins, flavonoids. On comparison of the leaves shows that the ALE has similar flavonoids and glycosides compounds reported. A similar finding has also been reported as also by Kumar et al., (2013).

Since, the functional groups of bioactive metabolites describe the nature and behavioural characteristic therefore, it is valuable for the preliminary separation and determination of bioactive metabolites constituents, Meepagala *et al.* (2013) have reported that HPLC chromatograms of ethyl acetate extract of leaves of *M. koenigii* relative with the peaks of extract retention time 8.2, 9.1 and 10.3 as isomahanine and mahanine according to their chemical name. Shah et al. (2018) found the prominent peak of tannic acid at a retention time of 3.21 min. During our study, the H1 and C-13-NMR spectroscopy analysis of ALE of *M. Koenigii* showed the presence of the aliphatic group, such as alkane, alkyl and alkene of bioactive chemicals which may be responsible for the anti-bacterial nature of ALE. The NMR spectrum of proton and carbon also predicts the anti-food-borne bioactive metabolites structure on the basis of FTIR and UV-VIS data interpretation as evidenced by previous workers (Pretsch et al., 2013; Sarker et al., 2006)

The results of ALE are useful for the existing information for identification and validation of *M. koenigii* leaves for the future prospective against food-borne bacteria. The bioactivity of ALE and the therapeutic use of *M. koenigii* for various ailments and look promising for using the bioactive phyto-chemicals in pure form as an effective natural drug agent in future. Therefore, the leaves of *M. koenigii* can be used for developing commercial management in food industry; chemical preventative such as benzoic acid and sodium benzoate are also work as antimicrobial which are normally used to expand shelf-life of food products (Nair, 2001).

CONCLUSION

It may be concluded that the ALE of *M. koenigii* anti-bacterial efficacy against some food-borne bacterial

pathogens due to the presence of several phytochemical compounds such as alkaloid, phenol, flavonoids and tannin were observed. The UV-VIS profile showed the absorbance of flavonoids in the ALE. FTIR analysis confirmed the presence of functional groups of bioactive metabolites such as phenol, alkanes, alcohol and amines, while HPLC outline of *M. koenigii* showed the presence active metabolites in ALE and the NMR spectrum predicted the structure of bioactive compounds based on data of FTIR and UV-VIS. The results of this study offer a platform for using *M. koenigii* leaves as herbal alternative for various food-borne disease and infections caused by bacterial pathogens after animal trials. Further work is in progress.

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