



## Phytochemical and toxicity analysis of *Leucas zeylanica* crude extracts

Faizuan Abdullah<sup>1\*</sup>, Noor Aida Fazira Mohd Salleh<sup>1</sup>, Muhammad Luqman Selahuddeen<sup>1</sup>

<sup>1</sup>Department of Chemistry, Faculty of Science, Universiti Teknologi Malaysia, 81310 Johor Bahru, Malaysia.

### Abstract

*Leucas zeylanica* (L.) R. Br. (*L. zeylanica*) originated from Lamiaceae family which is also known as “*Pokok ketumbit*” by local Malaysian is famous for therapeutic treatment uses especially in India and Sri Lanka. Throughout this study, four different solvents which were n-hexane, chloroform, methanol, and distilled water used in the extraction method using cold maceration technique. Optimization was done and methanol became the best solvent that produced highest percentage yield compared to the other solvents and also been chose to carry out a few other analysis. The extracts were subjected to qualitative and quantitative analyses to determine the phytochemical constituents present in aerial parts of *L. zeylanica*. The Fourier Transform Infrared spectroscopy – Attenuated Total Reflection (FTIR-ATR) results showed few significance peaks according to extracted solvents. Qualitative analysis on the methanolic extract showed that *L. zeylanica* contains phenol, flavonoid and tannin through phytochemical screening tests using colorimetric method. The Gas Chromatography – Mass Spectrometry (GC-MS) results demonstrated few fatty acids been extracted in both n-hexane and chloroform extracts with high peak area, while Liquid Chromatography Tandem with Mass Spectrometry (LC-MS-MS) results identified that chloroform extract showed fragment spectrum of tricetin [M+H]<sup>+</sup> ion at m/z 328.1 with retention time of 19.49 min, while methanol extract had two fragment spectrum of tricetin and apigenin at m/z 282.3 and 270.4 in positive ion mode at 19.53 min and 16.70 min respectively. Quantitative analysis on methanolic extract was done via Ultra Violet-visible spectrophotometric assay for estimation of total phenolic content (TPC), total flavonoid content (TFC) and total tannin content (TTC). TPC showed a gallic acid standard calibration curve,  $y = 0.0063x + 0.02$  and estimated amount was  $151.54 \pm 0.04$  mg of gallic acid equivalent/ 1 g of extract, meanwhile TFC displayed a quercetin standard calibration curve of  $y = 0.0050x + 0.037$  and estimated amount of flavonoids was  $71.76 \pm 0.2$  mg of quercetin equivalent/ 1 g of extract. Additionally, The toxicity test for the *L. zeylanica* extract showing that this compound is safe and non-toxic in term of skin irritation, cytotoxicity and also genotoxicity.

Keywords :

*Leucas zeylanica*, phytochemical constituents, tricetin, apigenin, toxicity, skin irritation, cytotoxicity.

## 1 Introduction

*Leucas zeylanica*, from a Lamiaceae family is a wild plant that grows in various habitats and widely distributed throughout many countries such as Southeast Asia, but rarely in East Asia (Napagoda et al., 2018). Noteworthy, the local name for *L. zeylanica* in Malaysia is known as “*Pokok Ketumbit*” and broadly distributed in bushes especially in Kelantan state near to the seaside with maximum height of 30 cm (Abdullah et al., 2019). *L. zeylanica* is widely used in traditional medical treatment for various diseases which been reported as anti-inflammatory, antimicrobial, analgesic, antioxidant, anti-diarrheal, anthelmintic, and insecticidal activities (Hung et al., 2019). The morphology of *L. zeylanica* plant was shown in Figure 1 below. Phytochemical screening study done on methanolic extract of *L. zeylanica* and confirmed that the presence of alkaloids, steroids, flavonoids, tannins, and glycosides (Choudhary, Sunojkumar, Mishra, 2017).

\* Corresponding Author.

Email Address : [faizuan@utm.my](mailto:faizuan@utm.my)

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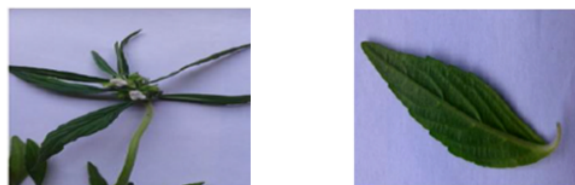
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The recent study done by (Nidhal et al., 2020) found that thirty compounds been isolated from *L. zeylanica* which were two norditerpenoid, three flavonoid glycosides, six flavonoids, two phytosterols, two phenylpropanoids, two phthalate esters, two phenolic compounds, five terpenoids, one aliphatic glycoside, one nucleobase, one amino acid, two alkaloids, and one cytochalasin.

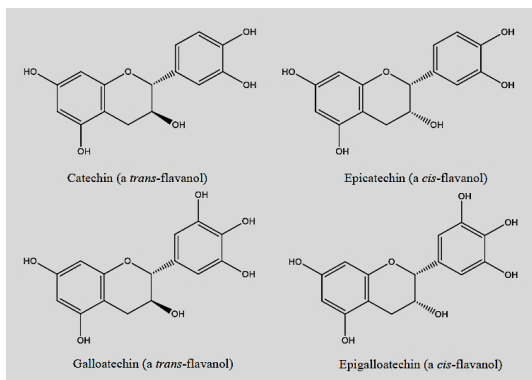


**Figure 1** Morphology features of *L. zeylanica*: (a) leaves, flowers, and stem, (b) appearance of the abaxial side of the leaves.

Previous study had reported that *L. zeylanica* has several usage traditionally including as a sunscreen for skin, anti-inflammatory, antiseptic and also anthelmintic (Abdullah et al., 2019).

Condensed tannins (CT) had been broadly analysed for their anthelmintic consequence and nutritional benefits and the nature of this monomeric can be either catechin (C) and its *cis* isomer epicatechin (EC) that make up procyanidins or gallicocatechin (GC) and epigallocatechin (EGC) as shown in Figure 2. A study reported that condensed tannins can give a direct antiparasitic ef-

fect or an indirect host effect due its capability to bind to dietary protein and therefore, the rumen degradation can be protected and the protein availability in the small intestine of the host increased. [9] said that CT had direct anthelmintic effects against *Ascaris suum* with evidence shown reduced migratory potentiality of newly hatched third-stage larvae and reduced motility and survival of fourth-stage larvae recuperated from pigs due to a complex set of factors including the degree of polymerization and the distinctiveness of the monomeric flavanol units in the CT molecules.



**Figure 2** Structure of flavanol monomeric subunits.

This research will emphasize on the preparation of four different crude extracts from aerial parts of *L. zeylanica* using successive cold maceration technique and characterize them spectroscopically, phytochemical screening of bioactive compounds in the crude extracts and analyse qualitatively their chemical compositions using GC-MS and LC-MS-MS, , investigate the potential antibacterial and anthelmintic compounds by estimation of total phenolic, flavonoid and tannin contents of the crude methanolic extract using UV-Vis spectrophotometric assays, and lastly the toxicity analysis including eye irritation, skin irritation and genotoxicity. The chemical compositions contained in *L. zeylanica* will be an important parameter to study the pharmacological efficacy of the plant (Napagoda et al., 2018).

## 2 Experimental

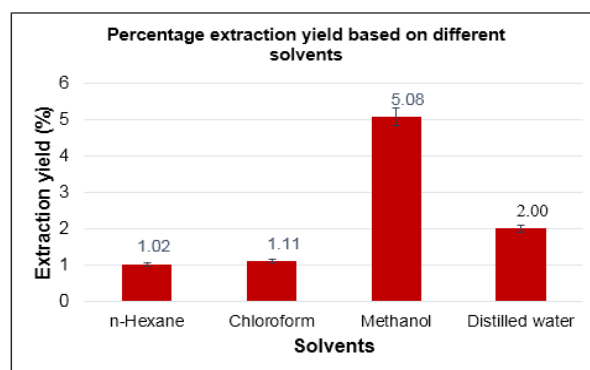
The experiment was divided into three main stages. The first stage was focused on the extraction of aerial part *L. zeylanica* using cold maceration technique in four different solvents, n-hexane, chloroform, methanol with sequential extraction and distilled water as aqueous extraction. As starting, the aerial part of plants was ground to a fine powder texture form using the mechanical grinder and stored at 4°C for further use. This extraction technique was done for an average of 4 days per solvents for optimum efficiency. The n-hexane, chloroform and methanol were concentrated using a rotary evaporator meanwhile, the aqueous extract was concentrated using the freeze-drying instrument. The second stage was qualitative analysis including thin-layer chromatography (TLC) analysis using the different solvent ratio of n-Hexane : Ethyl acetate and phytochemical screening tests by colorimetric method for confirmation of phenol, flavonoid and tannin in the methanolic extract. The crude extracts were then characterized spectroscopically using FTIR-ATR and qualitatively through GC-MS and LC-MS-MS for screening analysis. Then, the quantitative estimation of total phenolic content (TPC), total flavonoid content (TFC) and total tannin content (TTC) were carried out on methanolic extract of *L. zeylanica* using UV-Vis spectrophotometric assay by Folin-Ciocalteu, aluminium chloride in colorimetric, and Folin-denis method respectively. A series of standards for TPC, TFC and TTC which gallic acid, quercetin and tannic acid respectively were prepared for each estimation test and calibration curve,  $y = mx + c$  was made to identify the concentration of phenols, flavonoids, and tannins in the extract. Additionally, skin toxicity was evaluated by interleukin18 production in the HaCaT keratinocyte, Lo-

cal Lymph Node Assay (LLNA:BrdU-ELISA) method, 3T3 Neutral red uptake (NRU) assay and in vivo phototoxicity test according to ISO 10933-10 Protocol (using rabbit). Genotoxic potential and cytotoxicity test of *L. zeylanica* was also analyzed by cytokinesis-block micronucleus assay (MNvit test—cytoB) in HepG2 cells according to ISO 10993-5 guidelines. In vitro cytotoxicity was evaluated in by a yellow tetrazolium salt (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) or MTT assay. Cell morphological changes were observed by using light microscope (Mohd Bakhori, Mahmud, Mohamad, Masudi, Seeni, 2021).

## 3 Results and discussion

### 3.1 Extraction method on aerial parts of *L. zeylanica*

This study proposed an extraction method called cold maceration in four different solvents which were sequential extraction done with increasing polarity solvents, n-hexane, chloroform and methanol meanwhile, aqueous extraction is done using distilled water. The extraction yield percentage had been calculated to prove the different solvents affected different extraction efficiency due to solvent polarity. The results of the extraction yield percentage of four different crude extracts were illustrated in Figure 3. The highest extraction yield was methanol extracts which gave 5.08% and the lowest yield was n-hexane extracts with 1.02% from sequential extraction. The extraction yield for aqueous extracts was 2.00%. The highest yields obtained in the methanolic extract of *L. zeylanica* could be ascribed due to its high polarity. The formation of complexes by phenolic constituents with other biomolecules such as carbohydrates, proteins, terpenes, and inorganic compounds might be another possible reason. The methanol can be more easily to retrieve from this establishment compared to other solvents. Correspondingly, the use of methanol to reveal as the best solvent for extraction is aptly documented in the literature (Priya, Nirmala, Shankar, Malarvizhi, 2018; Saritha, Rajesh, Manjulatha, Setty, Yenugu, 2015). In this research, n-hexane had might extracted non-polar compounds, chloroform extracted semi-polar compounds and meanwhile, methanol and water had might extracted polar compounds from the plant-based on solvent polarity. This can be confirmed with further qualitative and quantitative analyses done below.

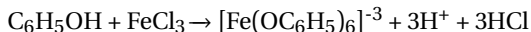


**Figure 3** Extraction yield percentages of crude extracts based on different solvents.

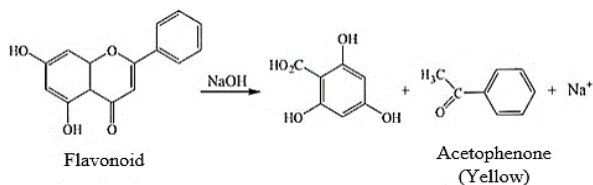
### 3.2 Phytochemical analysis of methanolic *L. zeylanica* crude extract

Phytochemical analysis including phenol test, flavonoid test and tannin test carried out in this study for the evaluation of secondary metabolites in *L. zeylanica* revealed the presence of phenols, flavonoids and tannins with colour changes observed in methanolic crude extract. Firstly, the positive results showed by

phenol test when the original green colour of crude methanol extracts changed to dark bluish green colour immediately as the 5% of  $\text{FeCl}_3$  was added. The colour change is because of phenols form a coloured complex with ferric ions when the -OH groups attached directly on aromatic ring is detected by  $\text{FeCl}_3$  and that complex has an intense colour that may differ from blue, green or even red depending on the nature of the phenol. The complex is ferric phenoxide,  $[\text{Fe}(\text{OC}_6\text{H}_5)_6]^{-3}$  and formed due to a ligand-exchange reaction. The reaction below between phenol and  $\text{FeCl}_3$  produced dark bluish green complex.



Besides, on the flavonoid test, the crude extract was turned to yellow colour as the addition of NaOH due to the formation of acetophenone compound as shown in Figure 4. The purpose of addition 10% NaOH in this test is to deprotonate the polyphenolic molecules contained in flavonoids. Also, the alkaline condition may have been necessary to disrupt the bond between the flavonoids and other components of the brain. Furthermore, tannin test is based on the principle of phenolics react with Fe salts and forming a blue or green black product. Additionally, condensed tannin and hydrolysable tannin can be differentiating throughout this test when the study reported that the products of condensed tannins are having green black colour meanwhile, the hydrolysable tannins will become blue black products. Thus, the methanolic extract of *L. zeylanica* can be predicted to consist of condensed tannin due to the green black colour observed from the test (Nidhal et al., 2020; Priya et al., 2018).



**Figure 5** Structure of flavanol monomeric subunits.

### 3.3 Qualitative analysis of *L. zeylanica* crude extracts

#### 3.3.1 Thin-layer chromatography (TLC)

The TLC analysis was done on three different crude extracts of *L. zeylanica*, n-hexane, chloroform and methanol in five solvent ratios of Hexane:Ethyl acetate. Since the TLC plate used in this study is non-polar, therefore non-polar compounds travelled faster than polar compound because polar compound will stick together on the plate. The solvent used also one of the factors contributing to the moving of the analyte and the solvent in this study was non-polar. Based on the results, the solvent ratio Hexane: Ethyl acetate (4:1) showed better compound separations compared to the other four ratios in each crude extract. Additionally, there were few observed colours formed on the plate due to the reaction between the compound with the spraying agent, which was vanillin and the colours were purple, pink, green, yellow, and blue. From those colours, a prediction of the class of compound can be made. The purple colour could indicate the presence of alkaloids, meanwhile, the green colour referred to chlorophyll. The yellow colour formed indicated the presence of flavonoid and the pink colour showed the presence of terpenes compound.

#### 3.3.2 Fourier transform infrared spectroscopy-attenuated total reflection (FTIR-ATR)

The FTIR-ATR analysis was done on four different crude extracts and few significance peaks were shown in Figure 5 below that ex-

plaining the possible compounds that may exist in the *L. zeylanica*. Firstly, the spectra of n-hexane crude extract showed the presence of both  $\text{sp}^3$  C-H and O-H stretching bonds at strong bands 2923.63  $\text{cm}^{-1}$  and 2853.65  $\text{cm}^{-1}$ , two medium peaks with strong intensity at the value of 1736.76  $\text{cm}^{-1}$  and 1710.75  $\text{cm}^{-1}$  referred to the presence of C=O stretching bond, a band of 1664.48  $\text{cm}^{-1}$  is assigned to (C=C) stretching, two high intense bands at 1455.05  $\text{cm}^{-1}$  and 1376.09  $\text{cm}^{-1}$  indicated the presence of C-H bending vibrations having a different mode of the scissoring vibration and symmetric bend of methyl group respectively, 1160.33  $\text{cm}^{-1}$  band identified the presence of C-O stretching of alkyl carbon and oxygen, and 837  $\text{cm}^{-1}$  and 721.45  $\text{cm}^{-1}$  were identified for presence of out-of-plane C-H stretching and CH<sub>2</sub> bending respectively.

The result obtained for chloroform dried crude extract was as expected and incomparable with n-hexane since there are not many polarities different between these two solvents. A few bands were detected including a weak and broad band at 3399.77  $\text{cm}^{-1}$ , strong bands at 2922.02  $\text{cm}^{-1}$  and 2852.24  $\text{cm}^{-1}$ , high intense bands of 1735.49  $\text{cm}^{-1}$  and 1710.18  $\text{cm}^{-1}$ , sharp bands at 1462.78  $\text{cm}^{-1}$  and 1376.05  $\text{cm}^{-1}$ , a range between 1250  $\text{cm}^{-1}$  and 1160  $\text{cm}^{-1}$  and strong band at 755.80  $\text{cm}^{-1}$  referred to the presence of O-H stretching, C-H stretching of saturated carbon-carbon bonds in aliphatic -CH<sub>3</sub> groups, C=O stretching C-H bending vibration, C-H asymmetrical and symmetrical stretching, C-O stretching bands, and C-Cl stretching absorption band

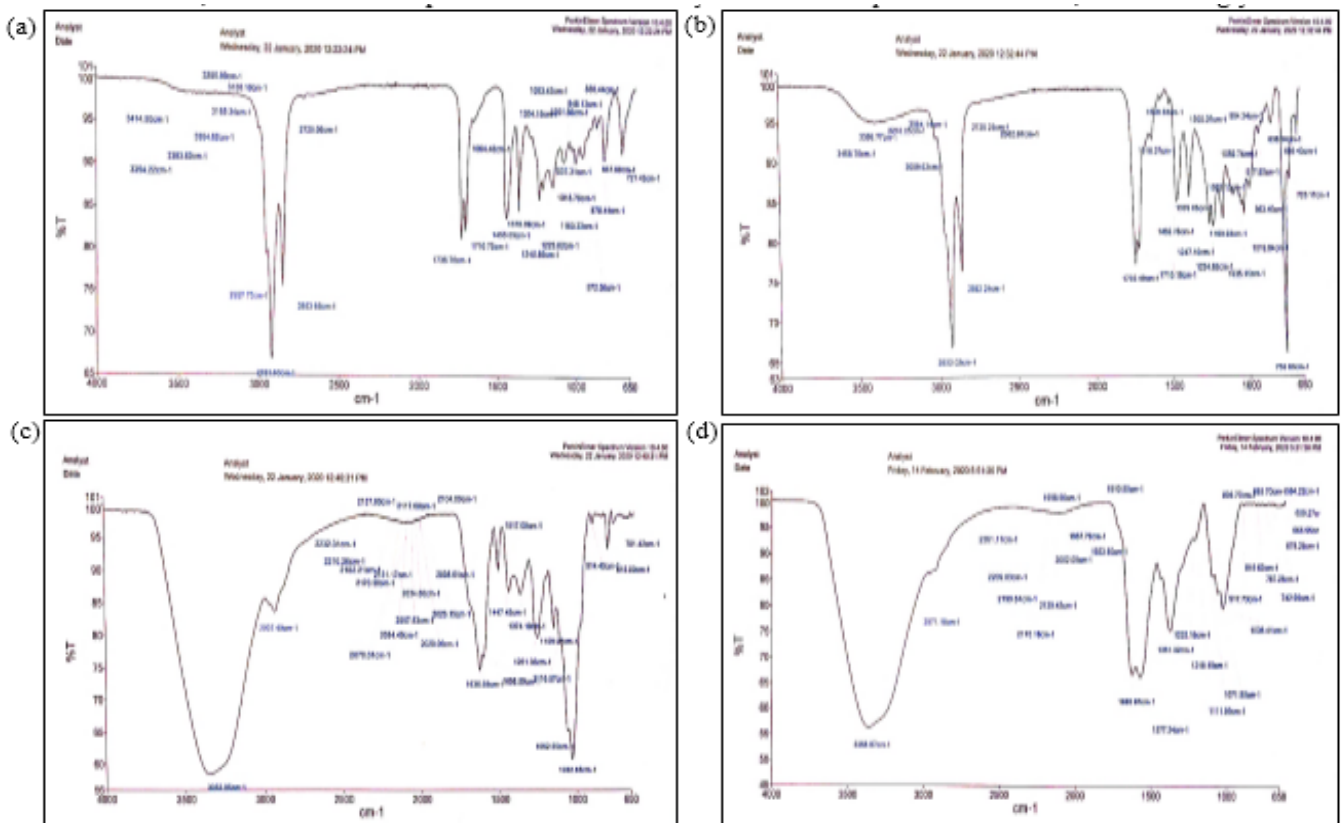
Besides, the spectra of methanolic extracts showed the presence of phenolic compounds. A strong and broad absorption peak at 3353.85  $\text{cm}^{-1}$  showed evidence stretching of hydroxyl groups (OH) in aliphatic and phenolic structures, the band present in a range between 3500  $\text{cm}^{-1}$  and 3000  $\text{cm}^{-1}$  referred to the vibrations of OH groups, a weak absorption band at 2937.40  $\text{cm}^{-1}$  is referred to the presence of axial deformation of the methylene CH bond, two moderately intense absorption bands at 1630.68  $\text{cm}^{-1}$  and 1606.59  $\text{cm}^{-1}$  where the C=C stretching of aromatic rings identified, a moderately sharp band at 1517.00  $\text{cm}^{-1}$  assigned to the stretching of C-C and C-O bonds in aromatic rings, a band at 1374.19  $\text{cm}^{-1}$  shows evidence bending of aromatic groups, and two sharply broad peaks at 1062.55  $\text{cm}^{-1}$  and 1032.85  $\text{cm}^{-1}$  referred to in-plane C-H bending and symmetrical C-O stretch in aromatic rings. In addition, the result obtained for distilled water dried crude extract is quite similar to methanol in term of absorption peaks. The absorption peaks at 3368.07  $\text{cm}^{-1}$ , 2971.16  $\text{cm}^{-1}$ , 1630.87  $\text{cm}^{-1}$  and 1577.24  $\text{cm}^{-1}$ , 1381.32  $\text{cm}^{-1}$ , and 1100  $\text{cm}^{-1}$  referred to the presence of O-H stretching groups, asymmetrical C-H stretching, C=C stretching, bending of C-H and O-H, and in-plane C-H bending in aromatic rings.

Thus, all the FTIR spectra from every four crude extracts indicated the presence of many functional groups such as alkanes, alkene, carboxylic acids, unsaturated ester, aldehyde, ketone, and phenolic compounds. So, suggested classes of compounds are n-hexane and chloroform crude extracts may have compounds of terpenes, fatty acids, steroids and long chains of alkanes meanwhile, the methanol and aqueous crude extracts may consist of compounds flavonoids, tannins and glycosides.

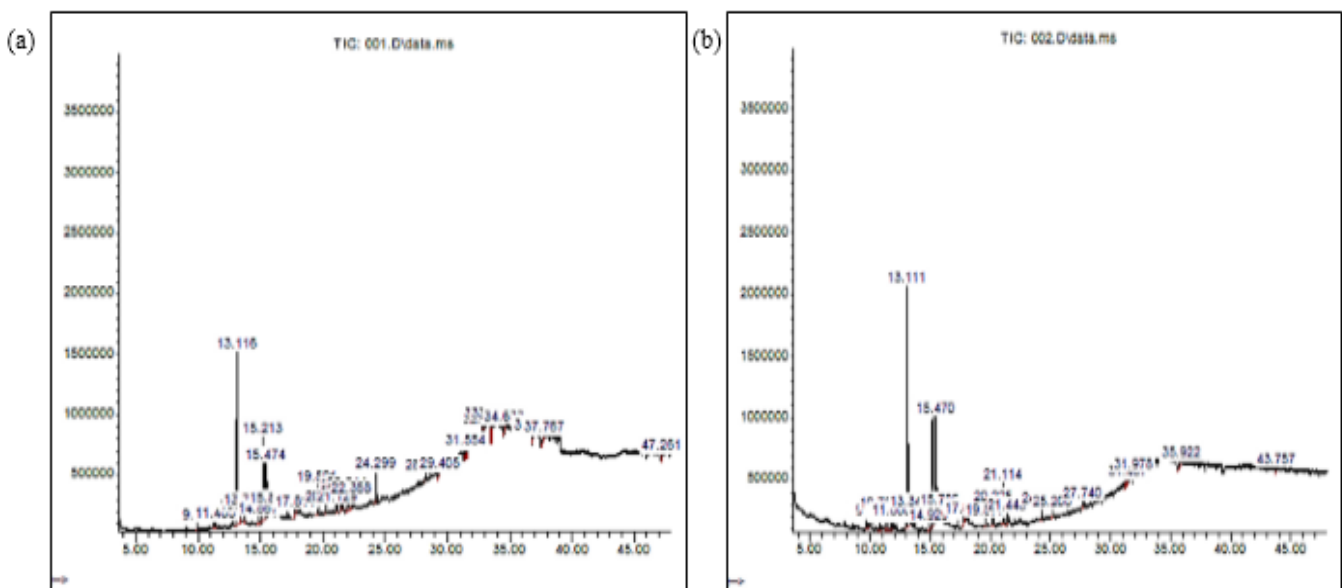
#### 3.3.3 Gas chromatography – mass spectrometry (GC-MS)

The GC-MS analysis of two different extracts from *L. zeylanica* aerial parts revealed the identification of 6 compounds in n-hexane extract, while 10 compounds in chloroform extract in Table 1 belonging to different chemical families which having boiling point up to 300°C. The GC-MS chromatogram of both crude extracts (Figure 6) was not much different to each other same goes to the identified compounds.





**Figure 5** FTIR spectra of dried (a) n-hexane, (b) chloroform, (c) methanol, and (d) distilled water crude extracts.



**Figure 6** GC-MS chromatogram of (a) n-hexane and (b) chloroform crude extracts.

The compounds consist in n-hexane extract having a probability range between 91% – 99% which were 8-heptadecene, octadecane, n-hexadecanoic acid, oleic acid, 9,12-octadecadienoic acid, and squalene meanwhile, chloroform extracts consist of octadecane, neophytadiene, *n*-hexadecanoic acid, tetradecanoic acid, oleic acid, octadecanoic acid, *cis*-13-eicosenoic acid, cyclo-eicosane, squalene, and eicosane with probability range of 99% - 81%. Henceforth, those compounds are shown in the analysis verified that most of the non-polar compounds were extracted in *n*-hexane and chloroform solvents which mostly fatty acids. The major constituents were *n*-hexadecanoic acid and oleic acid due to among the highest peak areas for both crude extracts. The fatty acids such as *n*-hexadecanoic acid and oleic acid are non-polar due to long hydrocarbon chains and show hydrophobicity characters.

Based on the results, each compound eluted with increasing

boiling points accordingly which the highest boiling point will elute later. The compound that having a lower boiling point will take a shorter time to completely vaporize and eluted out. This is also related to the polarity of the compounds. As the polarity of a molecule increases, the boiling point will also increase and cause higher retention time as the compound need to take a longer time to be pushed along the column by the gas phase. Additionally, the instrument was set for temperature programming due to the wide range of volatility or boiling point of compounds in the extracts.

Most of the compound's presence in *L. zeylanica* is important from pharmacological and botanical viewpoints. There are researches declared that the secondary metabolites and bioactive phytoconstituents identified by GC-MS in hexane extracts from plants have antimicrobial, anti-inflammatory, antioxidant and antidiabetic activities. (Choudhary et al., 2017) stated that most of the fatty acids are known to exhibit good antifungal, antibacte-

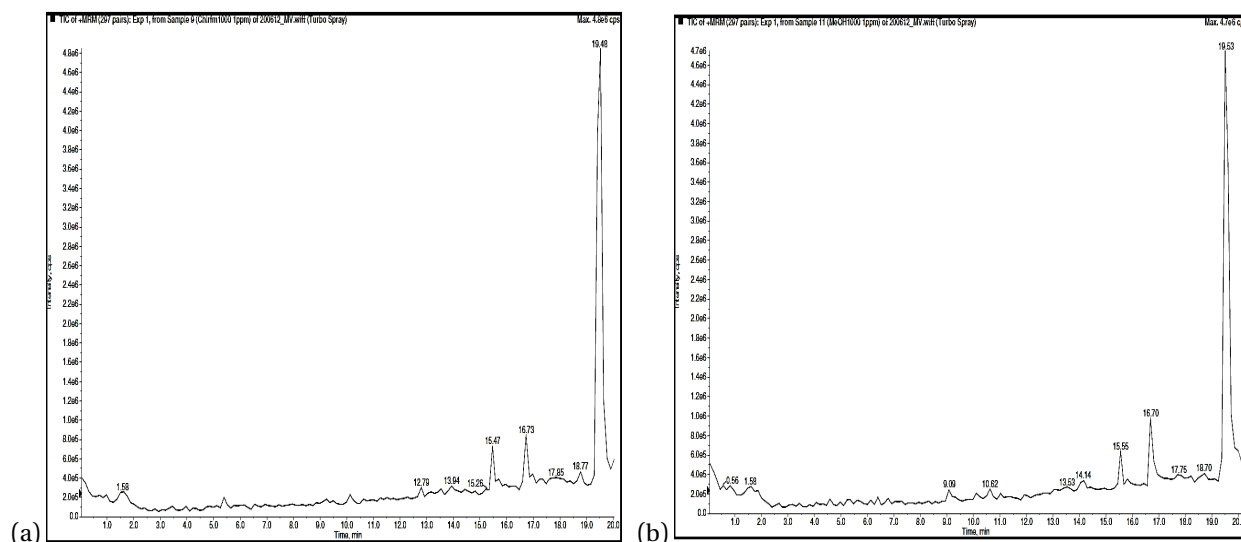
rial and anti-inflammatory properties. Thus, the most important is the FTIR analysis above was well supported by GC-MS analysis to prove the presence of some chemical constituents in the crude extracts.

**Table 1** Chemical compositions of n-hexane extract from *L. zeylanica* by GC-MS analysis.

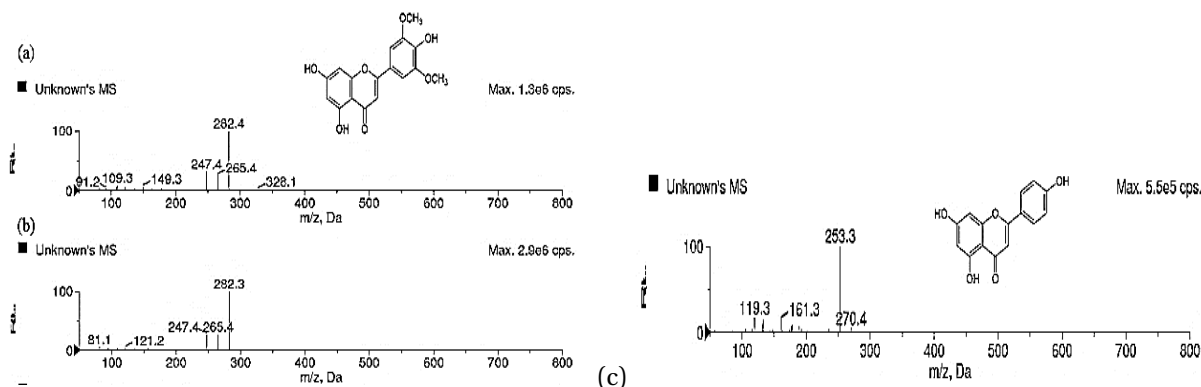
Peak	Retention time (min)	Peak (%)	area	Name of compound	Molecular formula	Molecular weight (g/mol)	Probability
1	9.9455	0.3193		8-heptadecene	C <sub>17</sub> H <sub>34</sub>	238.50	97
2	11.2628	0.4073		otadecane	C <sub>18</sub> H <sub>38</sub>	254.50	96
3	13.1165	22.7504		<i>n</i> -hexadecanoic acid	C <sub>16</sub> H <sub>32</sub> O <sub>2</sub>	256.42	99
4	15.2132	21.4491		oleic acid	C <sub>18</sub> H <sub>34</sub> O <sub>2</sub>	282.50	99
5	17.78035	0.1619		9,2-octadecadienoic acid	C <sub>17</sub> H <sub>32</sub> O <sub>2</sub>	280.40	91
6	24.2994	3.1749		squalene	C <sub>30</sub> H <sub>50</sub>	410.70	99

**Table 2** Chemical compositions of chloroform extract from *L. zeylanica* by GC-MS analysis.

Peak	Retention time (min)	Peak (%)	area	Name of compound	Molecular formula	Molecular weight (g/mol)	Probability
1	11.2620	0.6198		octadecane	C <sub>18</sub> H <sub>38</sub>	254.500	90
2	11.6860	0.5568		neophytadiene	C <sub>20</sub> H <sub>38</sub>	278.50	97
3	13.1110	28.3460		<i>n</i> -hexadecanoic acid	C <sub>16</sub> H <sub>32</sub> O <sub>2</sub>	256.42	99
4	13.3480	1.7530		tetradecanoic acid	C <sub>14</sub> H <sub>28</sub> O <sub>2</sub>	228.37	83
5	15.2020	28.1630		oleic acid	C <sub>18</sub> H <sub>34</sub> O <sub>2</sub>	282.50	99
6	15.4700	15.6500		octadecadienoic acid	C <sub>18</sub> H <sub>36</sub> O <sub>2</sub>	284.50	98
7	17.7780	0.7871		9, <i>cis</i> -13-eicosenoic acid	C <sub>20</sub> H <sub>38</sub> O <sub>2</sub>	310.50	96
8	17.8600	0.3093		cycloeicosane	C <sub>20</sub> H <sub>40</sub>	280.50	81
9	24.2820	1.2080		squalene	C <sub>30</sub> H <sub>50</sub>	410.70	99
10	25.2060	0.6294		eicosane	C <sub>20</sub> H <sub>42</sub>	282.50	90



**Figure 7** LC-MS-MS chromatogram of dried (a) chloroform and (b) methanol crude extracts



**Figure 8** Mass spectrum of: tricin compound in (a) chloroform and (b) methanol crude extracts, and (c) apigenin compound

### 3.3.4 Liquid chromatography tandem with mass spectrometry (LC-MS-MS)

LC-MS-MS has been applied to both chloroform and methanol crude extracts of *L. zeylanica* at the same concentration of 1000 ppm and tandem mass spectrometry opened a palette of operational procedures useful to find structurally related compounds. Firstly, chloroform crude extract had detected 4 peaks with retention time 12.79 min, 15.47 min, 16.73 min, and 19.49 min, meanwhile, methanol crude extract has detected 3 peaks with retention time 15.55 min, 16.70 min and 19.53 min (Figure 7). Then, each peak was fragmented, resulting in few fragmentation spectrum where the retention time of chloroform extract at 19.49 min had almost the same fragments and possible structure with the retention time of methanol extract at 19.53 min. Also, the methanol extract showed another fragment at 16.70 min with possible structure. Moreover, the chromatogram pattern for both extracts is almost the same.

Based on few fragmentation spectrum, there were two compounds identified, tricin and apigenin which also been isolated before from *L. zeylanica*. The spectrum fragmentation mass of tricin was observed by  $[M+H]^+$  ion at  $m/z$  328.1 of  $C_{17}H_{14}O_7$  in chloroform and  $m/z$  282.3 in methanol extract at the retention time of 19.49 min and 19.53 min respectively. Moreover, the base peak for both fragments of chloroform and methanol extracts were  $m/z$  282.4 and 282.3 respectively. Then, apigenin compound with the retention time of 16.70 min in methanol extract identified where the spectrum fragmentation yielded molecular ion at  $m/z$  270.4 ( $[M+H]^+$ ) in positive ion mode and highest intense of a base peak at  $m/z$  253.3 as shown in Figure 8. Both compounds had their importance in biological activities such as tricin revealed as antioxidant, antiaging, anticancer, and cardioprotective potentials, while apigenin as diabetes treatment, Alzheimer's disease, anticancer, antioxidant, anti-inflammation, and depression and insomnia.

## 3.4 Quantitative analysis on methanolic *L. zeylanica* crude extract

### 3.4.1 Total phenolic content (TPC)

The total phenolic content in methanolic extract of the aerial part of *L. zeylanica* was calculated from calibration curve (Figure 9) using Folin-Ciocalteu assay method. TPC was estimated using gallic acid as a standard in the different series concentration of 40 ppm, 80 ppm, 120 ppm, 160 ppm, and 200 ppm. The F-C method is an electron transfer based assay which gives reducing capacity that expressed as phenolic content of an antioxidant. Furthermore, the basic mechanism for this reaction is an oxidation or reduction reaction in alkaline medium with the phenolic group oxidized while the metal ion reduced. Under basic condition at a pH of  $\sim 10$  due to  $Na_2CO_3$ , phenolate anion formed that leading by dissociation of phenolic proton which can reduce the F-C reagent. Then, a

redox reaction takes place between the phenolate anion and F-C reagent (yellow) when the molybdenum undergoes reduction and yellow solution change to blue after a period of approximately at 60 min at room temperature in the dark condition. The colour shifts due to the production of a blue metallic complex,  $[(PMoW_{11}O_4)^4]$  (phosphotungstic-phosphomolybdenum complex) when the oxidation states for both molybdenum and tungsten is between 5 and 6 (Ferraz, Thomaz, Antunes, Lopes, 2021; Schaumlöffel, Fontoura, Santos, Pontes, Gutterres, 2021).

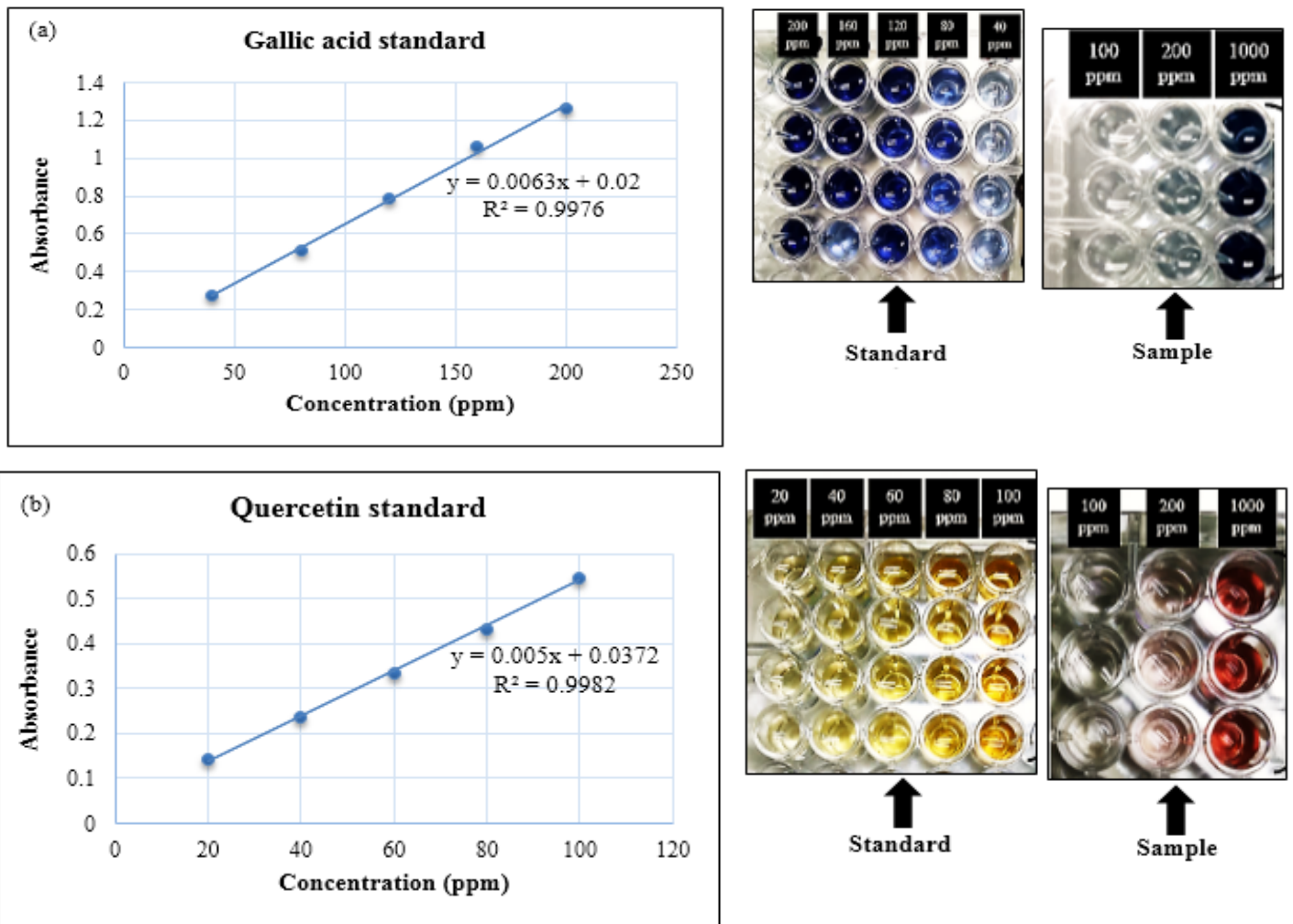
The result was derived by constructing a calibration curve ( $y = 0.0063x + 0.02$ ,  $R^2 = 0.9976$ ) with gallic acid (GA) taken into consideration the relationship between absorbance and concentration. From the results obtained, the maximum phenol concentration in 1000 ppm of methanolic dried crude extract was observed at  $151.54 \pm 0.04$  mg of gallic acid equivalent/ 1 g of extract showing that high content of equivalent phenolic structures in methanolic *L. zeylanica* extract.

### 3.4.2 Total flavonoid content (TFC)

The TFC analysis was analysed using aluminium chloride in a colorimetric method and was calculated from calibration curve (Figure 9) of 20 ppm, 40 ppm, 60 ppm, 80 ppm, and 100 ppm. Throughout the reaction, aluminium chloride,  $AlCl_3$  will form acid-stable complexes with carbonyl group at C4 and hydroxyls at C3 (flavonols) and C5 in flavonols and flavones. This complexation reaction is done in the presence of  $NaNO_2$  in alkaline medium due to the addition of  $NaOH$  based on the nitration of any aromatic ring bearing a catechol group with its three or four positions unsubstituted or not sterically blocked.

In observation, the quercetin standard solution turns to yellow solution as the addition of  $Al(III)$  with different tone of colour according to increasing concentration order meanwhile, for the sample methanolic extract solution, after the addition of  $Al(III)$ , a yellow solution of the complex was formed due to formation of flavonoid- $Al^{3+}$  complex and then immediately turned to red after the addition of  $NaOH$  due to nitration of catechol with  $NaNO_2$  [14]. The result was attained from a calibration curve ( $y = 0.0050x + 0.037$ ,  $R^2 = 0.9982$ ) and the maximum phenol concentration in 1000 ppm of methanolic dried crude extract was identified at  $71.76 \pm 0.2$  mg of quercetin equivalent/ 1 g of extract. The result obtained show that a high content of equivalent flavonoid structures in methanolic *L. zeylanica* extract.

Since the flavonoids are included in the phenolic compound as secondary metabolites, it also exhibits antioxidant activity and the potency are depends on the number and position of free OH groups. Both phenolic and flavonoids are important components in antioxidant due to the responsibility for deactivating free radicals based on their capability to donate hydrogen atoms to free radicals. Additionally, some present studies revealed the responsibility of flavonoids for the observed anthelmintic activity. A study suggested that for further studies need to be done for the treatment of helminthiasis to prove the highly benefits of chrysin as a novel putative anthelmintic drug [15].



**Figure 9** Standard calibration curve for the estimation of (a) TPC and (b) TFC with proven images

Since the gallic acid as the representative compound for TPC to find the similar skeleton structure presence in the plant, based on isolated compounds from *L. zeylanica*, two phenolic compounds having similar structure as gallic acid which are tyrosol and catechol. TFC was carried out using quercetin standard to act as representative compound to identify all the compounds that having similar skeleton structure as quercetin and confirm the presence of different classes of compounds. Therefore, all the six flavonoids isolated by Nidhal et al., (2020) are having similar skeleton as quercetin. The high estimation amount due to the presence of these compounds are such as daidzein, luteolin 3',4'-dimethyl ether, apigenin, tricetin, chrysoeriol, and linarinigenin.

### 3.4.3 Limit of detection (LOD) and limit of quantification (LOQ)

The LOD and LOQ are two common parameters to assess the sensitivity of analytical methods. The LOD and LOQ of standard compounds of gallic acid and quercetin were calculated using the formula. The results demonstrated in Table 3 is sufficient for the estimation of TPC and TFC in the methanolic extract of *L. zeylanica* using UV-vis spectrophotometric assays.

**Table 3** Limit of detection and quantification of standard compounds.

Compound	LOD (mg/L)	LOG(mg/L)
Gallic acid	11.832	35.856
Quercetin	0.023	0.077

## 3.5 Toxicity test

For skin irritation or toxicity test, HaCaT keratinocytes-associated IL-18 assay *L. zeylanica* (2–200mg/mL) did not show significant cytotoxic effects in HaCaT keratinocytes, contrasting with PPD (40mg/mL), a contact sensitizer that significantly reduced cell viability ( $p < 0.001$ ). In addition, PPD showed a SI = 4.10 associated with a significant increase of IL-18 levels ( $p < 0.05$ ). On the other hand, the high concentration of *L. zeylanica* (200mg/mL) showed a low SI = 1.14 with IL-18 levels similar to control and vehicle groups, suggesting a non-skin sensitization effect. (De Jong, Carraway, Geertsma, 2020).

In the LLNA:BrDU-ELISA test, a substance is considered potentially sensitizing if SI 1.6. Base on the result, that rabbit exposure to skin sensitizers HCA and eugenol, both at 25%, for three days, promoted a stimulation index of 2.4 ( $p < 0.01$ ) and 1.9 ( $p < 0.05$ ), respectively. In contrast, animals exposed to *L. zeylanica* at 5% showed a value of 0.7, similar to the control group. The *L. zeylanica* extract exhibited non-significant cytotoxic effects Thus, *L. zeylanica* was classified as a potential pharmaceutical and cosmetics substance in the LLNA:BrDU-ELISA. Additionally, no changes in the clinical signs were observed in animals exposed to *L. zeylanica*. (Kiranda, Mahmud, Abubakar, Zakaria, 2018; Vijayarathna Sasidharan, 2012)

## 4 Conclusion

This study is mainly focused on the efficiency of the extraction technique and the analysis of the various compounds consist

in aerial part of *L. zeylanica*. The investigation on the efficiency of various extraction solvents used for an extraction technique called cold maceration was successfully developed through phytochemical screening tests, qualitative analyses via FTIR-ATR, GC-MS, and LC-MS-MS, and quantitative estimation of TPC, TFC and TTC using UV-vis spectrophotometric assay. Herein, methanolic extract of *L. zeylanica* yielded the highest percentage of crude with 5.08% followed by aqueous extract in 200 g of dried powder yielded 2.00% and then chloroform and n-hexane with 1.11% and 1.02% respectively. Moreover, the methanolic extract confirmed the presence of phenols, flavonoids and tannins based on the phytochemical screening done. GC-MS analysis can be concluded that chloroform signified to have higher efficiency compared to n-hexane to extract more chemical constituents since it demonstrated 10 compounds extracted in chloroform and 6 compounds in n-hexane. Besides, LC-MS-MS results identified that chloroform extract showed fragment spectrum of tricetin  $[M+H]^+$  ion at  $m/z$  328.1 with the retention time of 19.49 min. Meanwhile, methanol extract had two fragment spectrum of tricetin and apigenin at  $m/z$  282.3 and 270.4 in positive ion mode at 19.53 min and 16.70 min respectively. In short, *L. zeylanica* proved to have a lot of phytoconstituents which having various beneficial use in medicinal purpose and also been used for traditional treatments for a long time ago. The toxicity test for the *L. zeylanica* extract showing that this compound is safe and non-toxic in term of skin irritation, cytotoxicity and also genotoxicity. The most important is all the discussed results above revealed that *L. zeylanica* crude extracts are having a high potential for pharmaceuticals and cosmetics ingredients. Therefore, laboratory research should be done more to explore the mechanism reactions of the potential cosmetics and pharmaceutical beneficial compounds especially the flavonoids

## Declaration of competing interest

The authors declare no known competing interests that could have influenced the work reported in this paper.

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