SIMULTANEOUS ANALYTICAL DETERMINATION OF METHYL SALICYLATE AND THYMOL IN SELECTED MALAYSIAN TRADITIONAL MEDICINES

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SIMULTANEOUS ANALYTICAL DETERMINATION OF METHYL SALICYLATE AND THYMOL IN SELECTED MALAYSIAN TRADITIONAL MEDICINES

ABSTRACT

Various topical formulations comprise of methyl salicylate and thymol due to their analgesic and anti-inflammatory properties. The huge demand has led traditional medicines being susceptible to adulteration with synthetic drugs or their analogues to enhance their efficacy and to minimise the cost of obtaining the limited natural substance. The objectives of this study are to analyse a suitable analytical method for simultaneous determination of methyl salicylate and thymol in selected Malaysian traditional medicines using High Performance Liquid Chromatography (HPLC) and to screen the selected Malaysian traditional medicines for possible methyl salicylate and thymol adulteration using the analytical method. Most literature search showed the determination of methyl salicylate and thymol as an individual compound or in combination with other compounds instead of both being detected simultaneously using a single method. Methyl salicylate and thymol were separated at about 3.8 and 6.2 min, respectively at a flow rate of 1 mL/min on C8 column with methanol and water (65:35) as the mobile phase, column temperature of 35°C and detector wavelength of 230 nm within 9 minutes of run time. Method validation was conducted and this method was rapid, sensitive, linear, specific, precise and accurate. The validated method was applied for screening of methyl salicylate and thymol in 10 samples of liniment and ointment. Half of the samples were detected with methyl salicylate and none with thymol. Majority of the positive samples were unregistered traditional medicines. Hence, the method can be adopted for routine quality control analysis of methyl salicylate and thymol in traditional medicines.

Keywords :methyl salicylate, thymol, HPLC, simultaneous, traditional medicines

PENENTUAN ANALITIKAL SERENTAK METIL SALISILAT DAN TIMOL DALAM UBAT-UBATAN TRADISIONAL MALAYSIA YANG TERPILIH

ABSTRAK

Pelbagai formulasi topikal mengandungi metil salisilat dan timol berikutan sifat analgesik dan antiinflamasi mereka. Permintaan yang tinggi telah menyebabkan ubat tradisional terdedah kepada campur palsu oleh ubat-ubatan sintetik atau analog mereka untuk meningkatkan efikasi dan meminimumkan kos. Objektif kajian ini ialah untuk menganalisis kaedah analitikal yang sesuai bagi penentuan serentak metil salisilat dan timol di dalam ubat-ubatan tradisional Malaysia yang terpilih menggunakan kromatografi cecair prestasi tinggi dan menyaring ubat-ubatan tradisional Malaysia yang terpilih untuk kemungkinan campur palsu oleh metil salisilat dan timol menggunakan kaedah analitikal ini. Kebanyakan penilitian maklumat menunjukkan penentuan metil salisilat dan timol sebagai sebatian individu atau bergabung dengan sebatian-sebatian lain dan bukannya dikesan serentak dengan menggunakan satu kaedah. Metil salisilat dan timol masing-masing telah diasingkan kira-kira pada 3.8 dan 6.2 minit, pada kadar alir air 1 mL/min menggunakan turus C8 dengan metanol dan air (65:35) sebagai fasa gerak, suhu turus 35°C dan jarak gelombang pengesan 230 nm dalam masa 9 minit. Tatacara pengesahsahihan telah dijalankan dan kaedah ini telah terbukti cepat, sensitif, linear, spesifik, persis dan jitu. Kaedah ini telah digunakan untuk penyaringan metil salisilat dan timol di dalam 10 sampel minyak dan salap. Separuh daripada sampel tersebut telah dikesan dengan metil salisilat manakala tiada sampel dikesan dengan timol. Majoriti sampel positif merupakan ubat tradisional yang tidak berdaftar. Justeru, kaedah ini boleh digunakan untuk analisis rutin kawalan kualiti metil salisilat dan timol di dalam ubat-ubatan tradisional.

Kata kunci : metil salisilat, timol, HPLC, serentak, ubat-ubatan tradisional

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LIST OF SYMBOLS AND ABBREVIATIONS

F_{cal}	:	Calculated F value
t_{cal}	:	Calculated *t* value
R^2	:	Coefficient of determination
F_{crit}	:	Critical F value
t_{crit}	:	Critical *t* value
F_{lof}	:	F value (lack-of-fit)
F_{reg}	:	F value (regression)
LD_{50}	:	Median lethal dose
r	:	Recovered concentration
S_{res}	:	Residual standard deviation
S^2_{res}	:	Residual variance
t_r	:	Retention time
b	:	Slope
v	:	Spiked concentration
S_a	:	Standard deviation at *y*-intercept
S_b	:	Standard deviation of slope
SS_{lof}	:	Sum of squares lack-of-fit
SS_{pe}	:	Sum of squares pure error
SS_{reg}	:	Sum of squares regression
SS_{tot}	:	Sum of squares total
a	:	*y*-intercept
APDI	:	Absolute percent difference against initial response
DAD	:	Diode-array detector
DCA	:	Drug Control Authority
DRGD	:	Drug Registration Guidance Document
GMP	:	Good Manufacturing Practice
HPLC	:	High performance liquid chromatography
ICH	:	International Council for Harmonisation of Technical Requirements for Pharmaceuticals for Human Use
LOD	:	Limit of detection
LOQ	:	Limit of quantitation
BERNAMA	:	Malaysian National News Agency
NSAID	:	Nonsteroidal anti-inflammatory drug
OTC	:	Over-the-counter
RSD	:	Relative standard deviation
SST	:	System suitability testing
UV	:	Ultraviolet

LIST OF APPENDICES

CHAPTER 1: INTRODUCTION

1.1 Introduction

Musculoskeletal injuries and rheumatic disorders have seen patients seeking solutions in the form of over-the-counter (OTC) medicines for pain relief. This is mainly attributed to the inability of common treatment modalities such as opioids and surgical intervention to offer long term benefit to the patients. These OTC medicines consist of either single or multiple compounds that provide pain relief owing to their local analgesic action and anti-inflammatory property. Compounds can be derived from naturally-occurring sources or chemically synthesized to be formulated into various dosage forms (Anderson et al., 2017).

Various topical formulations such as ointments, gels and liniments comprise of methyl salicylate and thymol due to the aforementioned properties (Bachute & Shanbhag, 2016). Examples of on-shelf products are Axe Brand Medicated Oil (methyl salicylate), Tiger Balm Plus Ointment (methyl salicylate) and Kein Tau Medicated Oil (methyl salicylate and thymol). Methyl salicylate is a naturally occurring compound in wintergreen (*Gaultheria procumbens*) and sweet birch (*Betula lenta*). Following topical application, methyl salicylate will be metabolised to salicylic acid and produces its effects by the resulting vasodilation, enhancing the blood flow and increasing the temperature of the tissues (Anderson et al., 2017). Thymol can be found in abundance in any plants of the genus *Thymus* such as common thyme (*Thymus vulgaris)*. Besides exhibiting analgesic and anti-inflammatory activity, thymol also demonstrates antioxidant, antimicrobial, wound healing and immunomodulatory properties (Nagoor Meeran et al., 2017).

Traditional medicine refers to any product used in the practice of indigenous medicine, in which the drug consists solely of one or more naturally occurring substances of a plant, animal or mineral, of parts thereof, in the unextracted or crude extract form, and a homoeopathic medicine. It shall not include any sterile preparation, vaccines, any substance derived human parts, any isolated and characterized chemical substances. (National Pharmaceutical Regulatory Division, 2016 p.302)

Globally, the usage of traditional medicines has seen remarkable growth due to their alleged health benefits regarding efficacy and safety as well as its good availability. Approximately 80% of the global population depended on traditional medicines for their healthcare. By 2020, it is projected that the global market will be valued at US$115 billion due to the surge in demand for traditional medicines. Locally, the demand for traditional medicines is also on the rise as Malaysia has a rich floral biodiversity. It was reported by the Malaysian National News Agency (BERNAMA) that the annual sales of traditional medicines from 2000 to 2005 experienced almost a 5-fold increase to reach RM 4.5 billion. It was also found that majority of Malaysians utilised traditional medicine throughout their lives (Abubakar et al., 2018).

In contrast to pharmaceuticals, the huge demand of traditional medicines has led them being susceptible to adulteration and contamination with synthetic drugs or their analogues to enhance their efficacy and to minimise the cost of obtaining the limited natural substance. Adulteration is mainly described as the artificial addition of impurities or substandard components or the elimination of a critical component that will lead to reduction in quality. Various reports found traditional medicines to be adulterated with anti-obesity agents, phosphodiesterase-5 inhibitors and non-steroidal anti-inflammatory

drugs (NSAIDs), consequently producing numerous adverse events upon chronic use (Haneef et al., 2013).

Hence, there is a need to detect for the presence of adulterants specifically concerning both methyl salicylate and thymol as they can cause detrimental health impacts to consumers since majority of consumers may be medically illiterate. This study is conducted to determine both compound simultaneously in traditional medicines through a more rapid and energy-efficient approach in comparison to existing methods. Furthermore, analytical method from this study can facilitate the relevant stakeholders such as regulatory bodies and manufacturers in ensuring the safety and quality of traditional medicine in the market.

1.2 Objective

The objectives of this study are :

i. to analyse a suitable analytical method for simultaneous determination of methyl salicylate and thymol in selected Malaysian traditional medicines using High Performance Liquid Chromatography (HPLC); and

ii. to screen the selected Malaysian traditional medicines for possible methyl salicylate and thymol adulteration using the suitable analytical method.

1.3 Significance of the Study

Most literature search showed the determination of methyl salicylate and thymol as an individual compound or in combination with other compounds in traditional medicines instead of both being detected simultaneously using a single method. Hence, the proposed method can be adopted for routine quality control analysis of methyl salicylate and thymol in traditional medicines and further utilised in regulatory settings.

1.4 Problem Statement

In the context of adulteration in Malaysia, methyl salicylate and thymol were among the commonly reported adulterants in traditional medicines. In the past, the presence of both compounds in a single traditional preparation was never reported but recent findings described otherwise (National Pharmaceutical Regulatory Agency, 2019). In view of this recent adulteration trend, it is imperative to have a validated method in place to effectively determine methyl salicylate and thymol simultaneously as most existing methods only focused on either compound and are not energy efficient. Analytical method from this study will further enhance the capacity of regulatory bodies in facing this challenge.

1.5 Research Scope

This study focuses on the validation of a method to simultaneously determine methyl salicylate and thymol in traditional medicines using HPLC and to apply the validated method for methyl salicylate and thymol screening in selected Malaysian traditional medicines. The dosage forms of traditional medicines that will be evaluated in this study are liniment and ointment as they formed the majority of adulteration cases involving these compounds (National Pharmaceutical Regulatory Agency, 2019). The screening results of traditional medicines in the market will provide valuable insight on the adulteration state concerning these compounds in Malaysia.

CHAPTER 2: LITERATURE REVIEW

2.1 Methyl Salicylate

Methyl salicylate (methyl 2-hydroxybenzoate) is a compound comprises of alcohol and ester functional groups (Figure 2.1) that normally appears as a colourless yellowish or reddish liquid with distinctive wintergreen scent. Methyl salicylate is slightly soluble in water, soluble in alcohol, glacial acetic acid and most organic solvents such as chloroform and ether. It is also sensitive to light and heat. The boiling point of this compound is in the range of 220 to 224°C and it absorbs maximum ultraviolet (UV) radiation >290 nm (National Center for Biotechnology Information, 2019a).

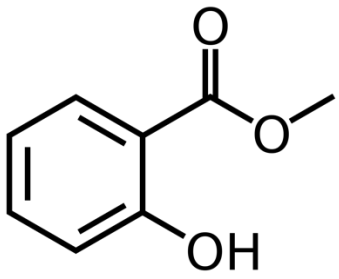

Figure 2.1: Methyl salicylate (Bachute & Shanbhag, 2016)

In plants, it functions as a signalling molecule to alert neighbouring plants to activate their defence in events of herbivorous insect attack. It also enlists other insects to prey on the attacker. Due to its abundance in species of the genus Gaultheria, methyl salicylate was initially extracted from wintergreen (*Gaultheria procumbens*), which is illustrated in Figure 2.2. Traditionally, indigenous North Americans utilised the leaves and barks from wintergreen to treat rheumatic disorders, fever, stomach complaints and skin injuries. Synthetic production of methyl salicylate has led to its application as a fragrance

ingredient and flavouring agent in food and cosmetics as well as an active ingredient in OTC formulations (Anderson et al., 2017).

Figure 2.2: Wintergreen, also known as checkerberry or teaberry (*Gaultheria procumbens*), with red berries (Photo sourced from https://www.britannica.com/plant/Gaultheria/imagesvideos#/media/1/227145/233894).

Methyl salicylate is a common active component in OTC preparations such as liniments, ointments and creams to take advantage of its therapeutic values involving analgesic, anti-inflammatory and counterirritant effects. Being lipophilic, methyl salicylate can promptly penetrate the skin layer and undergo metabolism to form salicylic acid. Previous human studies projected 12 to 20% of topical methyl salicylate to be immediately absorbed within the initial 10 hours. However, methyl salicylate absorption is also dependent on other factors such as product composition and skin condition. Upon absorption, salicylic acid exhibited a wide distribution mainly through passive pH-dependent mechanisms. Salicylic acid is metabolised extensively in the liver by conjugating with glycine to produce salicyl acyl and salicylic acid glucuronide. Furthermore, oxidation of salicylic acid to gentisic acid, gentisuric acid and 2,3-dihydroxybenzoic acid can also occur to a lesser extent in comparison to other pathways. At normal dose, the salicylates undergo renal elimination with free unchanged

salicylic acid form 10% of the urine salicylate composition. Studies also found that at low doses, the half-life of salicylate is approximately 2-3 hours with further increase in dose lead to longer half-life (Anderson et al., 2017; Valussi, 2015).

2.2 Thymol

Thymol (2-isopropyl-5-methylphenol) is a colourless phenolic monoterpene (Figure 2.3) derived from terpinene in a two-step biosynthesis: γ-terpinene aromatization followed by *p*-cymene hydroxylation. Thymol has a strong flavour and aromatic scent. This phenolic compound is highly soluble in alcohols, basic solutions and other organic solvents but is slightly soluble in water at neutral pH. The boiling point is 233°C and it absorbs maximum UV radiation at 274 nm (Nagoor Meeran et al., 2017; National Center for Biotechnology Information, 2019b).

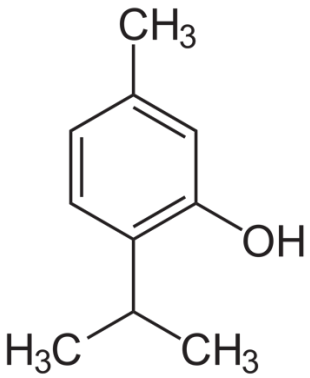

Figure 2.3: Thymol (BG Chemie, 2000)

Thymol is commonly present in extracted essential oils of plants from the Lamiaceae family; for instance, plants of the genus *Thymus*, *Ocimum* and *Satureja* (Marchese et al., 2016). An example is the common thyme (*Thymus vulgaris*) which is illustrated in Figure 2.4. Previously a vermifuge, this phenolic compound has been utilised as pharmaceutical stabiliser and antiseptic (National Center for Biotechnology Information, 2019b). This

phenol is also an active component in food flavourings, cosmetics, gargles and topical preparations. Its pharmacological attributes, as stated in the previous chapter, are due to the phenolic hydroxyl group's pharmacophore (Nagoor Meeran et al., 2017).

Figure 2.4: Thyme (*Thymus vulgaris*) (Photo sourced from https://www.britannica.com/plant/thyme/images-videos#/media/1/594537/7770).

Thymol's therapeutic properties like analgesia, anti-inflammation, wound healing and antiseptic has seen thymol become an integral ingredient in numerous topical formulations in the market. Studies demonstrated that the analgesic properties of thymol are potentially attributed to the inhibition of voltage-gated sodium channels. In terms of anti-inflammation, thymol exhibited significant oedema and leukocytes influx reduction in the wounded area. Besides, its wound-healing effects are described by the significant higher wound retraction rates and improve granulation reaction (Marchese et al., 2016). Thymol vapour undergoes rapid absorption after dermal application. Upon absorption, peak blood thymol concentration is achieved in 1 hour. This phenol is mainly metabolised to water-soluble sulphates and glucuronides. Thymol undergoes renal elimination with small fractions are excreted unchanged (U.S. National Library of Medicine, 2019).

2.3 High Performance Liquid Chromatography (HPLC)

HPLC is a type of column chromatography which is extensively utilised in the analysis of traditional medicines. Main advantage of HPLC is its versatility to analyse nearly all compounds and is not restricted to a compound's stability or volatility (Kamboj, 2012). This feature is contributed by the development of different column chemistry that can be used for optimal separation of compounds (Joshi, 2012).

This chromatographic technique comprises of small particle size (3 to 50 μm) packed in a column with an internal diameter between 2 to 5 mm connected to a pressurised solvent source at one end (Kamboj, 2012). The solvent which acts as the mobile phase will flow through the column under high pressure, causing each component in a mixture to elute out at different times based on their respective polarity (Joshi, 2012). The eluted components are subsequently detected by a detector, which is connected to the other end of the column. When combined with UV detection such as diode-array, HPLC can acquire UV spectra of eluting peaks from 190 to 800 nm (Heinrich et al., 2012).

Although gas chromatography is generally preferred in the analysis of volatile compounds, HPLC was the technique of choice for analysis of methyl salicylate and thymol due to its short run time (9 minutes). In contrast with gas chromatography methods utilised by Adib et al. (2017), Bachute & Shanbhag (2016), Krzek et al. (2003) and Subhash K et al. (2016) which reported a run time of 11, 20, 20 and 16 minutes, respectively, the run time of this HPLC method was the shortest. In addition, only the study conducted by Bachute & Shanbhag (2016) analysed both compounds simultaneously while the rest analysed methyl salicylate in combination with other compounds.

2.4 Adulteration of Traditional Medicine

Adulteration of traditional medicines has been a major problem in the quality control of these products. It can occur either deliberately or accidentally. Besides enhancing the therapeutic effect of the traditional medicines, deliberate adulteration is mainly performed to reduce their production cost especially in procuring premium herbs (Kamboj, 2012). In terms of accidental adulteration, traditional medicines may be unintentionally adulterated with pharmacologically-active chemicals that are naturally occurring in a plant species. Various studies revealed that undeclared synthetic drugs or pharmacologically-active agents are the perpetrators. Among the common adulterants are sildenafil analogues (sex stimulant), sibutramine (slimming agent) and dexamethasone (anti-inflammatory agent) (MyHEALTH, 2017). When consumed, these undeclared drugs possess the potential to instigate serious adverse effects on health (Lin et al., 2018). In Malaysia, Drug Control Authority (DCA) prohibited the registration of any traditional medicines that contain adulterants to ensure their quality and safety (Jayaraj, 2010).

From 2014 to December 2019, 36 registered products were adulterated with various chemicals including scheduled poisons. During the same period, there have been 15 cases of methyl salicylate adulteration detected while thymol recorded 8 cases. In 2018, it was important to point out that 2 registered products namely *Minyak Ibu Gamat Asli Plus* and *Bam Serai Plus* were detected with undeclared methyl salicylate and thymol. As a result, the registration of both traditional medicines was cancelled and withdrawn (National Pharmaceutical Regulatory Agency, 2019). *Minyak Ibu Gamat Asli Plus* and *Bam Serai Plus* were traditionally used for relieving joint and muscle pains. Besides, *Ibu Gamat Asli Plus* was also used to treat minor cuts and wounds. These adulterations were meant to enhance the effectiveness and the therapeutic claims of these traditional medicines by capitalising on the aforementioned therapeutic properties of both adulterants.

2.5 Adverse Effects of Adulteration

As adulterated traditional medicines are taken continuously, they pose a major health risk to the consumers. In terms of methyl salicylate and thymol adulteration, consumers unknowingly are exposed to their long term effects. The fact that these adulterated traditional medicines capable of producing the desired therapeutic effects in a short period will further encourage their use (MyHEALTH, 2017).

Repeated application of adulterated traditional medicines can lead to adverse effects related to methyl salicylate and thymol. In methyl salicylate metabolism, the earlier described glycine and glucuronide pathways are saturated easily at supratherapeutic doses, thus generating higher levels of free salicylic acid. As a consequence, the half-life of salicylate can be prolonged up to 15 to 20 hours and chronic salicylate toxicity can arise (Anderson et al., 2017). Salicylate toxicity can be severe and fatal after topical application or accidental oral ingestion. Its toxicity effects are derived from the uncoupling of oxidative phosphorylation, inhibition of Krebs cycle enzymes and inhibition of amino acid formation. These will impair various organ systems such as central nervous system (tinnitus, confusion, drowsiness), cardiovascular system (tachycardia, hypotension, arrhythmia), respiratory system (tachypnoea, hyperventilation) and digestive system (nausea, vomiting). Other symptoms include acid-base abnormalities (respiratory alkalosis, metabolic acidosis), dehydration, electrolyte disturbances, impaired platelet aggregation and muscle necrosis (Valussi, 2015). Acute salicylate toxicity will manifest as mild gastrointestinal symptoms, diaphoresis, fever and tinnitus. Multi-organ dysfunction involving seizures, coma, non-cardiogenic pulmonary oedema, and cardiovascular failure may follow as toxicity increases in severity (Davis, 2007). From the literature review, there was a report regarding chronic salicylate toxicity in an individual who applied a methyl salicylate-based cream for his psoriasis. Upon

application, he started to exhibit symptoms of acute toxicity such as tinnitus, tachypnoea and acid-base abnormalities. In this case, methyl salicylate absorption was enhanced by the presence of lesions on the individual's skin. Furthermore, studies also showed that methyl salicylate can cross the placenta, which is potentially detrimental to the foetus. Although the risks are limited due to the minute amount of methyl salicylate absorbed after topical application, chronic use may lead to low birth weight, anaemia, antepartum and postpartum haemorrhage and perinatal mortality (Valussi, 2015).

On the other hand, thymol is considered non-toxic after acute dermal application (LD_{50} rat dermal > 2000 mg/kg body weight) when compared with toxicity profile of its acute oral administration (LD_{50} rat oral 980 mg/kg body weight; LD_{50} mouse oral 640-1800 mg/kg body weight). However, it was found to be corrosive when applied to the skin of rabbits. Studies involving humans showed that thymol as a single component or in combination with others may lead to skin complications such as primary skin irritation and skin sensitisation in isolated cases (BG Chemie, 2000). Although thymol is a mild irritant when applied locally, chronic dermal application may result in systemic toxicity symptoms such as nausea, vomiting, diarrhoea, tachypnoea, hypotension, agitation and seizures (U.S. National Library of Medicine, 2019).

Besides adopting this developed method for routine quality control analysis of methyl salicylate and thymol in traditional medicines, this method can also serve as a basis for potential application in toxicity and safety studies of both compounds in animals and humans.

2.6 Method Validation

Analytical method validation is the initiation of documented evidence that guarantees the consistency of the analytical method together with the accompanying instruments in generating the intended results. These results accurately signify the quality attributes of the tested product (Shabir, 2005). Furthermore, this documented evidence also reflects the analytical method's performance as well as approximates the uncertainty of results at a specified confidence level (Chandran & Singh, 2007).

This developed method was validated in accordance to International Council for Harmonisation of Technical Requirements for Pharmaceuticals for Human Use (ICH) Harmonised Tripartite Guideline. The parameters or performance characteristics that were validated include linearity, range, detection limit, quantitation limit, specificity, accuracy and precision. In addition to the aforementioned parameters, solution stability and system suitability testing were also evaluated (ICH, 2005).

CHAPTER 3: METHODOLOGY

3.1 Introduction

The methodology of the study can be divided into two main components which are the analytical method validation as well as methyl salicylate and thymol screening. Summary of the overall methodology is illustrated in Figure 3.1.

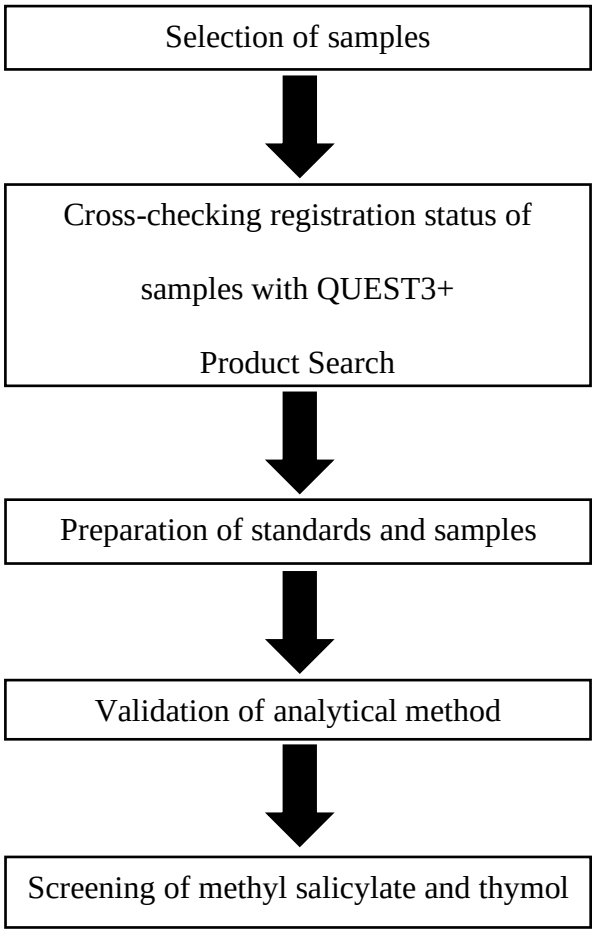

Figure 3.1: Flowchart of methodology

3.2 Reagents and Materials

The reference standards of methyl salicylate (purity: 97%) and thymol (purity: 98%) were obtained from Toronto Research Chemicals (TRC) Inc., Canada. HPLC grade methanol (Fisher Scientific Korea Ltd., Methanol HPLC Grade, Seoul, Republic of Korea) and ultrapure water was utilised throughout the study.

3.3 Analytical Procedure

Determination of methyl salicylate and thymol were performed using an HPLC unit (Agilent, Agilent 1260 Infinity Quaternary Pump, California, United States) that consisted of a thermostatted autosampler; Model:1260 ALS, quaternary pump; Model:1260 Quat Pump VL, thermostatted column compartment; Model: 1260 TCC and diode-array detector(DAD); Model: 1260 DAD VL.

3.4 Instrumental Conditions

For samples and standards, the injection volume was 5 μL. The mobile phase composition was 65% methanol: 35% water in isocratic mode. The separation was conducted using a Zorbax Eclipse Plus C8, 4.6 mm × 150 mm, 5 μm column at a flow rate of 1 mL/min. The column oven temperature was set at 35°C and the detector wavelength setting was 230 nm. The run time for both methyl salicylate and thymol analysis was 9 minutes.

Isocratic elution was preferred over gradient elution as both compounds of concern does not largely differ in terms of polarity and retention as well as do not require complex separation (Hansen et al., 2012). C-8 column is a type of reversed-phase column consists of octylsilane which is bound to porous silica particles. In contrast to the common C-18 column, C-8 column was chosen due to its weaker retention for relatively hydrophobic

compounds (methyl salicylate and thymol), which in turn produces shorter retention times (Joshi, 2012).

3.5 Preparation of Standard

100 mg of weighed methyl salicylate and thymol reference standards were each transferred into a 25 mL volumetric flask. The flask content was dissolved and made up to volume by methanol. Then, 1 mL of the resulting solution from both volumetric flasks were pipetted into a 10 mL volumetric flask and made up to volume using methanol. The resulting concentration of methyl salicylate and thymol standard solutions was 0.4 mg/mL. Both standard solutions were kept in an amber vial and stored in a chiller at 4°C.

3.6 Selection of Sample

Ten different brands of traditional medicines were purchased from various sources such as traditional medicine stores, pharmacies, night markets and online shopping platforms. The chosen dosage forms were liniment and ointment. The samples were selected based on market availability at the time of the study and the absence of methyl salicylate and thymol as active ingredients on their labels. In addition, the samples must be locally manufactured in Malaysia. Then, their product registration status was cross-checked with QUEST3+ Product Search, which can be accessed via the National Pharmaceutical Regulatory Agency (NPRA), Ministry of Health Malaysia official website. From the ten selected samples, five samples were liniment and the remaining samples were in the form of ointment. In terms of their registration status, five were found to be registered with NPRA while the rest were unregistered. These samples were stored in its original container at room temperature and shielded from light. The list of samples utilised during this study is listed in Table 3.1.

Table 3.1: List of samples

No.	Sample Code	Dosage Form	Registration Status
1.	LR1	Liniment	Registered
2.	LR2	Liniment	Registered
3.	LU1	Liniment	Unregistered
4.	LU2	Liniment	Unregistered
5.	LU3	Liniment	Unregistered
6.	OR1	Ointment	Registered
7.	OR2	Ointment	Registered
8.	OR3	Ointment	Registered
9.	OU1	Ointment	Unregistered
10.	OU2	Ointment	Unregistered

3.7 Preparation of Sample

About 1.0 g of sample was weighed and transferred into 50 mL volumetric flask. 30 mL of methanol was then added and the mixture was heated on sonicator bath at 55°C until fully liquified. The solution was cooled to room temperature and made up to 50 mL with methanol. 1 mL of the above sample solution was diluted to 50 mL with methanol. Then, 5 mL of the resulting solution was further diluted to 25 mL with methanol. Finally, the sample was filtered through a 0.45 μm syringe filter.

3.8 Method Validation Procedure

3.8.1 System Suitability

For system suitability tests, standard solutions from Section 3.5 were diluted together with methanol to produce methyl salicylate and thymol concentration of 0.1 mg/mL. The resulting mixture of methyl salicylate and thymol standard solution was further filtered through a 0.45 μm syringe filter and six replicates were injected into the HPLC system for system suitability determination. Table 3.2 shows the system suitability requirements of the analytical procedure.

Table 3.2: System suitability requirements (Shabir, 2005)

Parameter	**Acceptance Criteria**
System precision	i) % RSD of standard retention time ≤ 1%
	ii) % RSD of standard peak area ≤ 1%
	iii) % RSD of standard peak height ≤ 1%
Theoretical plate count	≥ 2000
Tailing factor	≤ 2
Resolution	≥ 2

3.8.2 Limit of Detection and Limit of Quantitation

In this study, both limit of detection (LOD) and limit of quantitation (LOQ) were calculated based on the standard deviation of residual. To achieve this, at least six levels of mixed standard solutions from Section 3.5 with regular distribution between each level were prepared. The mixed standard solutions were then analysed in three different batches under reproducibility conditions

(standard solutions are freshly prepared and analysed on different days). Parameters for the calibration curve were then calculated, followed by the determination of LOD and LOQ using the equations below:

$$\text{LOD} = \frac{(3 \times S_{res})}{b} \tag{3.1}$$

$$\text{LOQ} = \frac{(10 \times S_{res})}{b} \tag{3.2}$$

where S_{res} is the residual standard deviation and b is the slope of the regression line.

3.8.3 Linearity and Range

Linearity and range were established by preparing a series of calibration solution of methyl salicylate and thymol, usually beginning from the LOQ level up to the highest expected concentration. Six concentration levels from mixed standard solutions (Section 3.5) were prepared. These calibration solutions were then analysed in three different batches under reproducibility conditions (standard solutions are freshly prepared and analysed on different days).

The concentration of the calibration solutions together with their response values were later subjected to regression analysis to obtain the linear equation, coefficient of determination (R^2), *y*-intercept of the regression line (a), slope of the regression line (b), residual standard deviation (S_{res}), standard deviation at y-intercept (S_a), standard deviation of the slope (S_b), sum of squares regression (SS_{Reg}), sum of squares lack-of-fit (SS_{lof}), sum of squares pure error (SS_{pe}) and

sum of squares total (SS_{tot}). Then, the respective mean squares (regression, lack-of-fit and pure error) and subsequently the Fisher ratio for regression (F_{reg}) and lack-of-fit (F_{lof}) could be computed.

To confirm the linearity of the analytical method, both the regression and linearity models need to be accepted. If F_{reg} was higher than the critical value $F_{(0.95,1,n(p-1))}$, the hypothesis was that the variation of *y* was explained by a regression model. $F_{(0.95,1,n(p-1))}$ was the value of Fisher distribution for 1 and $n(p-1)$ degrees of freedom at 95% confidence level. If the first hypothesis was valid, the second hypothesis will be tested which concerns the validity of the linear model. The linearity model was acceptable if F_{lof} was lower or equal to the critical value $F_{(0.95,n-2,n(p-1))}$. Thus, if both the regression and linearity models were valid, a further Student's t-test was carried out to determine whether *a* passed through the origin (zero). Calculated *t* value (t_{cal}) was obtained from the division of absolute value of *a* with S_a. If t_{cal} was lower or equal to the critical *t* value for *n-2* degree of freedom at 95% confidence level (t_{crit}), it can be established that *a* passed through the origin (zero). On the other hand, the coefficient of determination (R^2) computes the proportion of variance that is explained by the regression model. Generally, the coefficient of determination should be more or equal to 0.997 for the regression line (Shabir, 2005).

In terms of the working range, it is the upper and lower concentration intervals of the analyte in the sample for which the analytical method has demonstrated an acceptable level of linearity. For this study, the working range was determined based on the established linear range and a minimum of five

concentration levels was needed to demonstrate linearity as stipulated by ICH guidelines (Shabir et al., 2007).

3.8.4 Specificity

Specificity analysis was performed by the standard addition method. Four different samples (two each for liniments and ointments) were spiked with one selected concentration within the working range. Analysis of the spiked and unspiked samples was conducted.

The specificity was confirmed by adjusting a straight line between spiked concentration (v) and recovered (r) concentrations. The relationship between the spiked concentration (v) and recovered (r) concentration was expressed in the form of regression line ($r = a + bv$). If the specificity was valid, the slope and intercept were statistically not different from 1 and 0, respectively, which indicated that the overlap line ($r = a + bv$) was the equivalent of line $y = x$. By using least-square regression analysis, the slope (b), standard deviation of the slope (S_b), intercept (a) and standard deviation at intercept (S_a) were computed. Then, two hypothesis testing was carried out to confirm specificity. Firstly, t-test (t_1) was utilised to test the hypothesis that the slope was significantly equal to 1 while the second t-test (t_2) was utilised to test the hypothesis that the intercept was significantly equal to 0. Calculated t value (t_1) was obtained from the division of absolute value of (b-1) with S_b. Likewise, calculated t value (t_2) was obtained from the division of the absolute value of a with S_a. Thus, if both conditions were accepted and valid, it indicated that the overlap line was the equivalent to $y = x$, consequently deeming the analytical method to be specific.

The specificity of the analytical method was also investigated by injecting blank solution (methanol), mixed standard solution, blank sample solution and spiked sample solution to demonstrate the absence of any interferences from other excipients and sample components. The excipients and other sample components should not interfere with the elution of methyl salicylate and thymol (Shabir, 2005).

Also, a test for general matrix effect was also performed to study any significant difference between the calibration solution in solvent and calibration solution in the sample matrix. A set of calibration solution in methanol and another set each in both sample types (liniment and ointment) were prepared as established in the linearity studies. All sets were analysed on the same day. Least squares regression, F-test and t-test parameters were calculated. An F-test to obtain F_{cal} and F_{crit} at the critical value of $F_{(0.95,\, n-1,\, n-1)}$ was conducted to examine the differences between all matrices' residual variances, S^2_{res}. Then, two hypothesis testing were carried out to compare the slope of the calibration lines using Student's t-test. Calculated *t* value (t_{cal}) was compared to the critical *t* value. Thus, if t_{cal} was less or equal to t_{crit}, it indicated that both of the slopes (methanol-liniment matrix or methanol-ointment matrix) were not different and the calibration for routine analysis can be performed in the solvent. Conversely, if the t_{cal} was more than t_{crit}, the calibration for routine analysis should be conducted in sample matrix solution.

3.8.5 Precision

3.8.5.1 Repeatability

Repeatability testing was conducted by spiking three batches of sample blanks in duplicates at three different concentrations covering the working range. Calibration solution based on the established linear range was also prepared. Then, the mean assay values for each batch were applied for determining mean value of the respective concentration levels. Repeatability was expressed in percentage of relative standard deviation (RSD). For repeatability, the percentage RSD of assay results should be ≤ 2% (Shabir, 2005).

3.8.5.2 Intermediate Precision

Intermediate precision was performed by a different analyst on different days utilising a different HPLC instrument with different standards and samples preparation. Similar methods as stated in Section 3.8.5.1 were performed for this testing. The acceptance criteria for intermediate precision requires the percentage RSD of both analysts' assay results to be ≤ 2% (Shabir, 2005).

3.8.6 Accuracy

The accuracy of the analytical method was determined by the standard addition method. Similar to Section 3.8.5.1, three batches of sample blanks in duplicates were spiked at three different concentrations covering the working range. This was in line with ICH recommendation by which a minimum of nine determinations over a minimum of three concentration levels covering the working range was required for accuracy determination (Shabir et al., 2007). The percent recovery was calculated from the mean assay value of each batch.

The mean recovery should be within the recovery limits depending on the concentration level. At the concentration of 10 μg/mL, the acceptable recovery limit should be within 80 to 115% while at the concentration of 100 μg/mL, the limit should be within the range of 85 to 110% (AOAC International, 2013).

3.8.7 Solution Stability

The stability of analytical solutions was assessed in two approaches. Firstly, the mixed standard solution from Section 3.8.1 and sample solutions (spiked with 0.1 mg/mL of methyl salicylate and thymol) were prepared and injected as per method. The mixed standard and sample solutions were retained in the HPLC autosampler and reinject after 24 hours to assess their stability at room temperature.

Secondly, the stability of methyl salicylate and thymol standard solutions stored in the chiller were examined at day 0 and day 6, respectively. Mixed standard solution of methyl salicylate and thymol with concentration of 0.15 mg/mL was freshly prepared and injected at the aforementioned time intervals. The change in the peak area of methyl salicylate and thymol in all the solutions with respect to time was expressed as absolute percent difference against initial response (APDI). APDI values within a range of ±5% are deemed acceptable (Shailajan et al., 2015).

3.9 Screening of Methyl salicylate and Thymol

Samples listed in Table 3.1 were screened for methyl salicylate and thymol as per the proposed method. The screening was conducted by analysing the samples concurrently

with a mixed standard solution of methyl salicylate and thymol prepared at LOD level. Following HPLC analysis, the samples were screened for the presence of methyl salicylate and thymol peaks by comparing their respective chromatograms to that of the LOD standard solution. If methyl salicylate and/or thymol peaks are present, the particular peak area will be compared to its corresponding peak area from the LOD standard solution. A sample is detected with methyl salicylate and/or thymol with the condition that the sample's peak area higher than that of the LOD standard solution.

CHAPTER 4: RESULTS AND DISCUSSION

4.1 HPLC Analysis of Methyl Salicylate and Thymol Standard

A mixture of methyl salicylate and thymol standard solution were injected into the HPLC system according to the aforementioned chromatographic conditions. Chromatogram of standard solutions exhibited peaks corresponding to methyl salicylate and thymol, which eluted at 3.861 minutes and 6.211 minutes, respectively as illustrated in Table 4.1 and Figure 4.1. Figure 4.1 clearly showed that both compounds were adequately resolved and free from any interferences.

In terms of elution order for both compounds, it was found to be comparable with a previous study conducted by Bachute & Shanbhag (2016), although the run time for this proposed method is relatively shorter at 9 minutes. The UV spectrums of methyl salicylate and thymol in the mixed standard solution are shown in Figure 4.2 and Figure 4.3, respectively.

Table 4.1: Retention time of methyl salicylate and thymol

Compound	**Retention Time, t_r (minutes)**
Methyl salicylate	3.861
Thymol	6.211

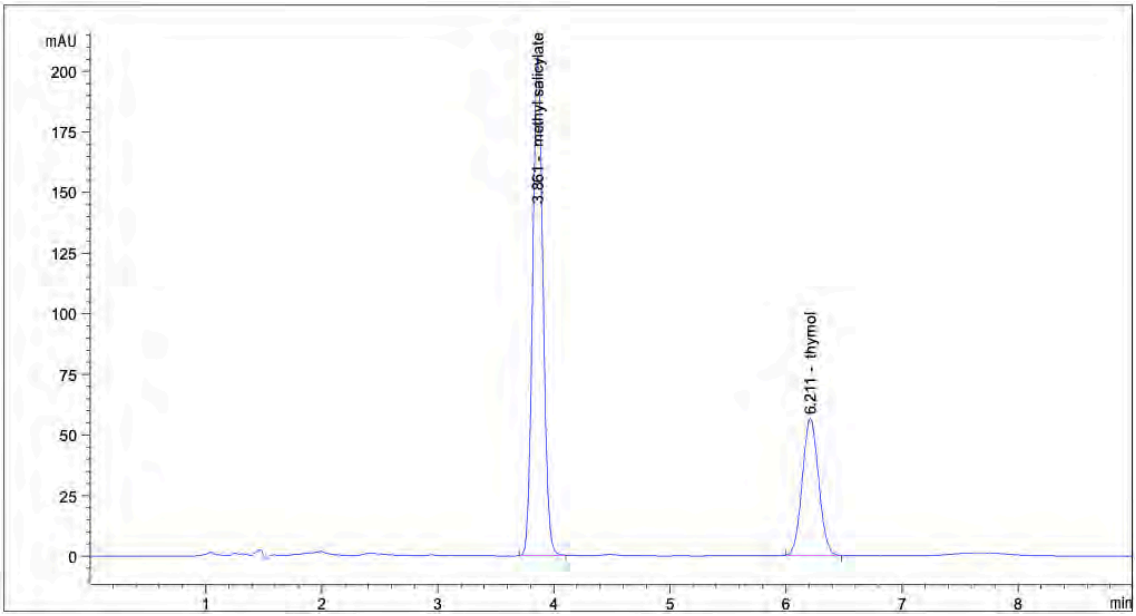

Figure 4.1: HPLC chromatogram of a mixture of methyl salicylate and thymol standard solution at t_r = 3.861 minutes for methyl salicylate and t_r = 6.211 minutes for thymol

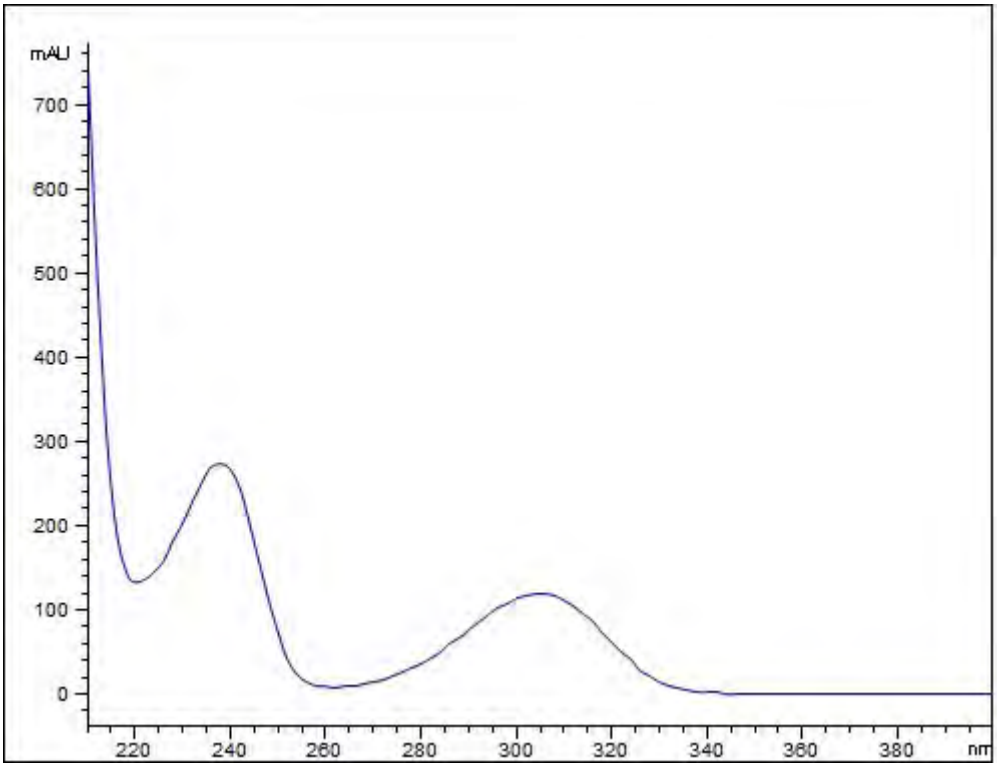

Figure 4.2: UV spectrum of methyl salicylate

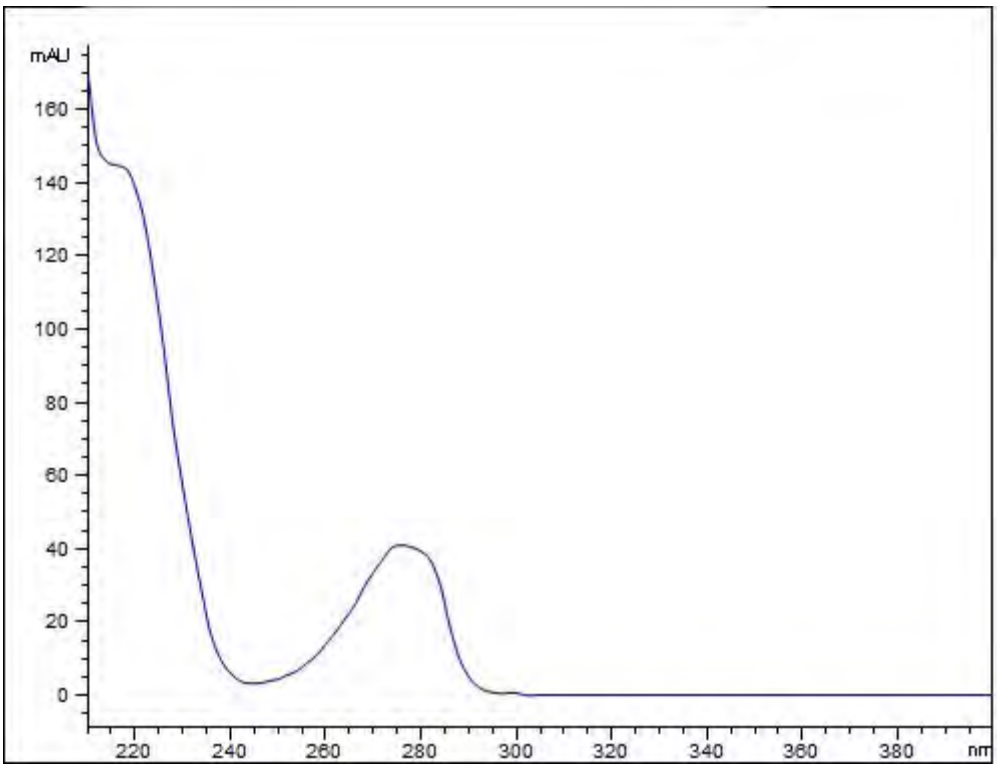

Figure 4.3: UV spectrum of thymol

4.2 Method Validation

Validation of this method has been conducted utilising standards and samples that represented the actual routine analysis involving the determination of methyl salicylate and thymol. As earlier stated, the analytical method was validated with regards to system suitability, solution stability, linearity, range, detection limit, quantitation limit, specificity, accuracy and precision.

4.2.1 System Suitability

System suitability testing or system precision is a crucial aspect of any analytical method. It is based on the concept that all related aspects of the method such as instruments and samples form a vital system that can be assessed (ICH, 2005). From this study, both peaks were adequately resolved and the precision for both peaks of interest was within acceptable limits. The

percent relative standard deviation of both peaks were computed in terms of their peak area, retention time and peak height. In addition, the tailing factor, theoretical plate number and resolution were also calculated. The summarised results of system suitability tests and chromatogram of mixed standard solution are shown in Table 4.2 and Appendix A, respectively. The proposed method satisfied all the requirements (Shabir, 2005).

Table 4.2: System suitability results

Parameter	**Acceptance Criteria**	**Results**	
		Methyl Salicylate	**Thymol**
System precision	i) % RSD of standard retention time ≤ 1%	0.048	0.036
	ii) % RSD of standard peak area ≤ 1%	0.429	0.589
	iii) % RSD of standard peak height ≤ 1%	0.576	0.590
Theoretical plate count	≥ 2000	8943	9194
Tailing factor	≤ 2	1.08	1.06
Resolution	≥ 2	-	11.130

4.2.2 Limit of Detection and Limit of Quantitation

LOD is the lowest concentration of analyte that can be detected by the analytical method but not necessarily quantitated as an exact value. On the other hand, LOQ is the lowest concentration of analyte that can be reliably quantitated with suitable accuracy (ICH, 2005). In this present study, the LOD and LOQ for both compounds together with relevant parameters are given in Table 4.3. From the results, it was observed for both compounds that the LOD and LOQ values were almost similar. Additionally, the LOD and LOQ values for methyl salicylate from this study were comparable with findings by Shabir and Bradshaw (2011). Conversely, LOQ for thymol were found to be higher than that of a previous study conducted by Hajimehdipoor et al. (2010), which was established at 8.6 µg/mL. Although the LOQ was higher, it was compensated by the comparable LOD value. Thus, the obtained detection limit and quantitation limit were found to be appropriate for routine analysis of methyl salicylate and thymol in traditional medicines.

Table 4.3: LOD and LOQ results

Parameter	**Results**	
	Methyl Salicylate	**Thymol**
Coefficient of determination, R^2	0.9994	0.9994
Slope, *b*	14.1120	6.2734
Slope standard deviation, S_b	0.0898	0.0376
Intercept, *a*	-6.8308	-9.1750
Intercept standard deviation, S_a	8.7282	3.5878
SS_{reg}	6919152.021	1723871.456

Table 4.3, continued

Parameter	Results	
	Methyl Salicylate	**Thymol**
S_{res}	16.7477	7.8599
LOD (µg/mL)	3.5603	3.7587
LOQ (µg/mL)	11.8677	12.5289

4.2.3 Linearity and Range

Linearity of the analytical method is defined by its ability to obtain a proportional relationship of response versus analyte concentration over the working range. In this present study, six concentrations of calibration solutions for methyl salicylate and thymol were analysed and the calibration curves were constructed from 25.10 to 149.68 µg/mL for methyl salicylate and 14.97 to 150.10 µg/mL for thymol using regression analysis. The results for linearity studies are summarised in Table 4.4 and Table 4.5 for methyl salicylate and thymol, respectively. The linearity plot for both compounds was a plot of mean response (peak area) as a function of the analyte concentration (µg/mL) as presented in Figure 4.4 and 4.5.

Aside from visually inspecting the constructed calibration curve, statistical analysis namely regression analysis by least squares method, Student's t-test and F-test were also conducted. The summary of regression analysis for methyl salicylate and thymol are shown in Table 4.6. The coefficient of determination (R^2) of both calibration curves were more than 0.997, which indicated acceptable fit of the data to the regression line. In terms of the working range,

six concentration levels of the working range for both compounds were assessed from data established from linearity analysis, thus meeting the requirements set by ICH guidelines (Shabir et al., 2007).

Table 4.4: Linearity data for methyl salicylate

No.	Concentration of methyl salicylate (µg/mL)	Mean response
1.	25.10	333.023
2.	40.33	578.571
3.	79.77	1118.389
4.	100.38	1413.174
5.	124.58	1746.243
6.	149.68	2105.586

Table 4.5: Linearity data for thymol

No.	Concentration of thymol (µg/mL)	Mean response
1.	14.97	84.886
2.	25.21	150.268
3.	75.25	460.251
4.	100.07	619.233
5.	125.28	776.181
6.	150.10	933.624

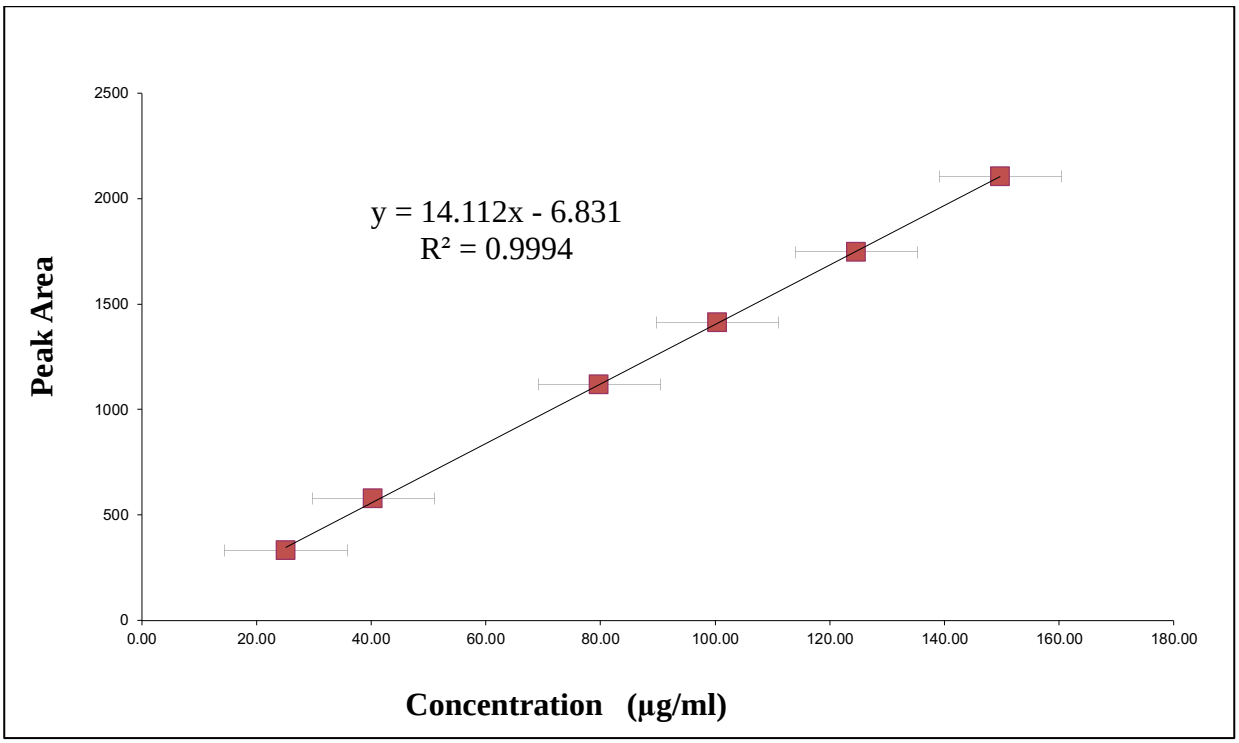

Figure 4.4: Linearity plot of methyl salicylate

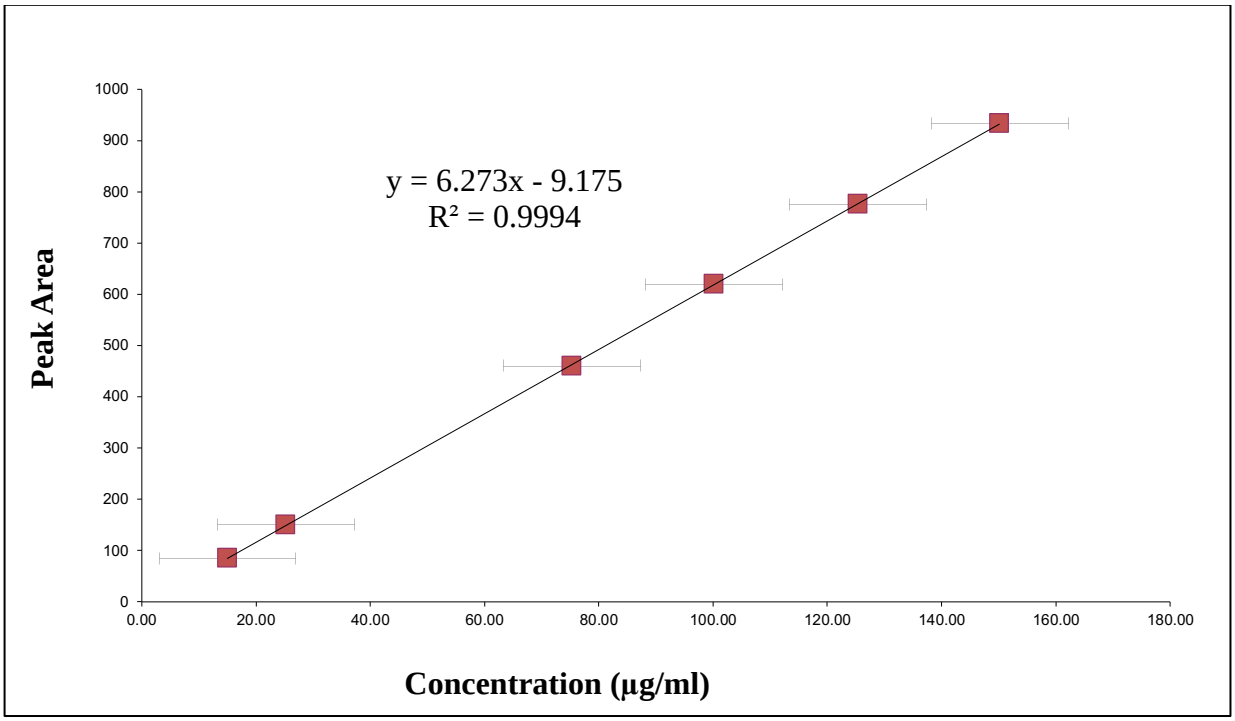

Figure 4.5: Linearity plot of thymol

Table 4.6: Regression analysis for methyl salicylate and thymol

Parameter	Results	
	Methyl Salicylate	**Thymol**
Coefficient of determination, R^2	0.9994	0.9994
Slope, *b*	14.1120	6.2734
Slope standard deviation, S_b	0.0898	0.0376
Intercept, *a*	-6.8308	-9.1750
Intercept standard deviation, S_a	8.7282	3.5878
Residual standard deviation, S_{res}	16.7477	7.8599

Both the regression and linearity models need to be accepted to confirm the linearity of the analytical method. As listed in Table 4.7 and Table 4.8, both methyl salicylate and thymol gave F_{reg} values higher than the critical value $F_{(0.95,1,n(p-1)).}$ This indicated that the variation of peak area was explained by the regression model. In terms of the linearity model, both compound's F_{lof} was lower than the critical value $F_{(0.95,n-2,n(p-1))}$. This signified the acceptance of the linearity model. Subsequently, confirmation of *y*-intercept was performed by calculating the *t* value for both compounds. As t_{cal} for methyl salicylate and thymol was lower than t_{crit}, it indicated that the *y*-intercept passed through the origin (zero). Table 4.9 shows the results for confirmation of *y*-intercept.

Table 4.7: Confirmation test of linearity (methyl salicylate)

Sources of variation	Sum of squares	Degree of freedom	Mean squares	Fisher ratio	*F* critical value
Regression	6919152.0210	1	6919152.0210	28008.93	4.75
Lack-of-fit	1523.3658	4	380.8415	1.54	3.26
Pure error	2964.4054	12	247.0338		

Table 4.8: Confirmation test of linearity (thymol)

Sources of variation	Sum of squares	Degree of freedom	Mean squares	Fisher ratio	*F* critical value
Regression	1723871.456	1	172381.456	21635.76	4.75
Lack-of-fit	32.3198	4	8.08	0.1	3.26
Pure error	956.1237	12	79.677		

Table 4.9: Confirmation of *y*-intercept

Parameters	Methyl salicylate	Thymol
t_{cal}	0.783	2.557
t_{crit}	2.776	2.776

4.2.4 Specificity

The specificity of an analytical method refers to its ability to measure accurately the desired analyte response in a sample matrix (Chandran & Singh, 2007). Regression analysis by least-squares method were conducted to determine the slope (*b*), standard deviation of the slope (S_b), intercept (*a*) and standard deviation at intercept (S_a). Summary of regression analysis for methyl salicylate and thymol in both matrices (liniment and ointment) are presented in Table 4.10 and Table 4.11, respectively. Subsequently, two hypothesis testing utilising t-test was conducted for confirmation of specificity. Table 4.12 lists the results for specificity confirmation for methyl salicylate and thymol. From the results, all calculated values of t_1 and t_2 were lower than t_{crit}. This indicated that the slope and intercept were statistically not different from 1 and 0, respectively, which signified the overlap line was equivalent to $y = x$, confirming the analytical method's specificity.

Table 4.10: Regression analysis for methyl salicylate specificity studies

Parameter	**Results**	
	Liniment	**Ointment**
Coefficient of determination, R^2	0.9998	0.9985
Slope, *b*	1.0033	0.9674
Slope standard deviation, S_b	0.01	0.0264
Intercept, *a*	-0.8345	-0.3036
Intercept standard deviation, S_a	0.9322	2.4584

Table 4.11: Regression analysis for thymol specificity studies

Parameter	Results	
	Liniment	**Ointment**
Coefficient of determination, R^2	0.9996	0.9998
Slope, *b*	1.0366	1.0349
Slope standard deviation, S_b	0.0143	0.0096
Intercept, *a*	0.3227	0.0868
Intercept standard deviation, S_a	1.3052	0.8787

Table 4.12: Confirmation of specificity

Parameter	Methyl salicylate (liniment)	Methyl salicylate (ointment)	Thymol (liniment)	Thymol (ointment)
t_1	0.3342	1.2341	2.5593	3.6226
t_2	0.8951	0.1235	0.2473	0.0987
t_{crit}	9.9248	9.9248	9.9248	9.9248

Additionally, specificity for methyl salicylate and thymol were also demonstrated by the absence of any interferences from other excipients and sample components in blank solution (methanol), mixed standard solution ,blank sample solution and spiked sample solution. All of these findings were in line with requirements set by Shabir (2005). Appendix B, C, D, E, F and G illustrates chromatogram of blank solution (methanol), mixed standard solution, blank sample solution (liniment), blank sample solution (ointment), spiked sample solution (liniment) and spiked sample solution (ointment), respectively.

For the analytical method, the matrix effect was determined by analysing three sets (a set of calibration solution in methanol and two sets each in both sample matrices). All sets comprised of six concentration levels as established in the linearity studies. The parameters for least squares regression, F-test and t-test were computed as listed in Table 4.13 and Table 4.14. The F-test was used to test equivalence of all matrices' residual variances and t-test was conducted to statistically check for significant differences between the slopes of the calibration lines. For both compounds in both matrices, all residual variances were not different as illustrated in the lower value of F_{cal} when compared to the F_{crit}. Likewise, the calculated *t* value for all sets was less than the critical *t* value. This indicated that both slopes which referred to the methanol-liniment matrix or methanol-ointment matrix were not different. Hence, calibration can be conducted in solvent as there was no matrix effect observed for methyl salicylate and thymol.

Table 4.13: Test for matrix effect (methyl salicylate)

Parameter	**Liniment**		**Ointment**	
	Solvent (Methanol)	**Sample matrix (Liniment)**	**Solvent (Methanol)**	**Sample matrix (Ointment)**
Slope, *b*	0.0026	0.0027	0.0026	0.0025
Standard deviation, *S*	0.0026	0.0099	0.0026	0.0026
Residual variance, S^2res	0.0000067	0.000098	0.0000067	0.0000069

Table 4.13, continued

Parameter	Liniment		Ointment	
	Solvent (Methanol)	Sample matrix (Liniment)	Solvent (Methanol)	Sample matrix (Ointment)
F_{cal}	0.0682		0.9721	
F_{crit}	5.0503		5.0503	
t_{cal}	0.0008		0.0112	
t_{crit}	2.306		2.3060	

Table 4.14: Test for matrix effect (thymol)

Parameter	Liniment		Ointment	
	Solvent (Methanol)	Sample matrix (Liniment)	Solvent (Methanol)	Sample matrix (Liniment)
Slope, *b*	0.00074	0.00076	0.00074	0.00077
Standard deviation, *S*	0.00057	0.0012	0.00057	0.00064
Residual variance, S^2res	0.00000032	0.0000014	0.00000032	0.00000042
F_{cal}	0.2248		0.7803	
F_{crit}	5.0503		5.0503	
t_{cal}	0.0009		0.003	
t_{crit}	2.306		2.306	

4.2.5 Precision

Precision is defined as the degree of agreement between results obtained from repeated analysis by using the same method (ICH, 2005). In this present study, precision was investigated at two different levels: repeatability and intermediate precision.

4.2.5.1 Repeatability

Repeatability or intra-assay precision of the analytical method is attained when the analysis is performed in a single laboratory by a single operator using single set of equipment and reagents over a short interval of time (Chandran & Singh, 2007). All the percentage RSD assay values for methyl salicylate and thymol at three concentration levels (low, medium and high concentration) were found to be lower than 1.65% and met the requirements set by Shabir (2005). Table 4.15 and Table 4.16 describes the repeatability data for both compounds in their respective matrices. The results indicated high repeatability of the method.

Table 4.15: Repeatability data for methyl salicylate

Parameter	**Liniment**			**Ointment**		
	24.74 µg/mL	79.81 µg/mL	150.05 µg/mL	24.74 µg/mL	79.81 µg/mL	150.05 µg/mL
Mean (µg/mL)	24.59	79.92	152.69	24.49	82.52	154.81
Standard deviation	0.40	0.98	0.30	0.07	0.33	0.28
RSD (%)	1.65	1.23	0.20	0.30	0.40	0.18
95% Confidence interval	1.45	3.52	1.07	0.27	1.19	1.00

Table 4.16: Repeatability data for thymol

Parameter	Liniment			Ointment		
	14.97 µg/mL	74.87 µg/mL	149.75 µg/mL	14.97 µg/mL	74.87 µg/mL	149.75 µg/mL
Mean (µg/mL)	15.47	75.60	154.53	15.09	72.25	154.16
Standard deviation	0.25	0.35	0.18	0.09	0.15	0.88
RSD (%)	1.63	0.46	0.12	0.62	0.20	0.57
95% Confidence interval	0.90	1.25	0.65	0.34	0.52	3.15

4.2.5.2 Intermediate Precision

Intermediate precision of the analytical method is attained when the analysis performed consists within-laboratory variations such as different analysts, different days, a different set of equipment and reagents (ICH, 2005). Its objective is to ascertain the within-laboratory variations that will impact the variability of the results and subsequently identify a mechanism to regulate them (Chandran & Singh, 2007). The results for the first analyst was obtained from repeatability testing while the results for the second analyst are shown in Table 4.17 and 4.18. The RSD values for both analysts were calculated and found to be less than 1.65% for each concentration level. These results exhibited excellent precision for the analytical method as the assay results obtained by two analysts using two different instruments on different days should have a statistical RSD $\leq$ 2% (Shabir, 2005).

Table 4.17: Intermediate precision data for methyl salicylate (second analyst)

Parameter	Liniment			Ointment		
	24.80 µg/mL	80.01 µg/mL	150.41 µg/mL	24.80 µg/mL	80.01 µg/mL	149.85 µg/mL
Mean (µg/mL)	26.98	81.48	147.54	25.91	81.42	146.73
Standard deviation	0.07	0.20	1.34	0.05	0.14	0.49
RSD	0.27	0.24	0.91	0.20	0.18	0.34
95% Confidence interval	0.26	0.71	4.80	0.18	0.51	1.77

Table 4.18: Intermediate precision data for thymol (second analyst)

Parameter	Liniment			Ointment		
	15.03 µg/mL	75.15 µg/mL	150.30 µg/mL	15.03 µg/mL	75.15 µg/mL	149.90 µg/mL
Mean (µg/mL)	16.48	75.13	146.53	17.53	75.18	149.54
Standard deviation	0.03	0.07	1.24	0.05	0.05	0.28
RSD (%)	0.20	0.10	0.84	0.29	0.06	0.19
95% Confidence interval	0.12	0.26	4.43	0.18	0.17	1.00

4.2.6 Accuracy

Accuracy or trueness is described as the closeness of the achieved results to the true value (Chandran & Singh, 2007). Accuracy of the analytical method was assessed by the recovery of known amounts of analyte spiked into the sample matrix. From the results obtained, mean recovery values of methyl salicylate and thymol at three concentration levels for both sample matrices were within a range of 96.5 to 104.18%, which fulfilled the requirements set by AOAC International (2013). These results indicated that the accuracy of the analytical method was satisfactory at the working range and the establishment of a close agreement within the spiked and recovered values. The recovery results are summarised in Table 4.19 and 4.20.

Table 4.19: Recovery results for methyl salicylate

Parameter	**Liniment**			**Ointment**		
	24.74 µg/mL	79.81 µg/mL	150.05 µg/mL	24.74 µg/mL	79.81 µg/mL	150.05 µg/mL
Mean recovery (%)	99.39	100.13	101.76	98.98	103.39	103.17
Standard deviation	1.64	1.23	0.20	0.30	0.42	0.19
RSD (%)	1.65	1.23	0.20	0.30	0.40	0.18
95% Confidence interval	5.86	4.42	0.71	1.08	1.49	0.67

Table 4.20: Recovery results for thymol

Parameter	Liniment			Ointment		
	14.97 µg/mL	74.87 µg/mL	149.75 µg/mL	14.97 µg/mL	74.87 µg/mL	149.75 µg/mL
Mean recovery (%)	104.18	100.98	103.19	100.81	96.50	102.95
Standard deviation	0.35	0.47	0.12	0.63	0.19	0.59
RSD (%)	0.34	0.46	0.12	0.62	0.20	0.57
95% Confidence interval	1.26	1.68	0.43	2.25	0.69	2.10

4.2.7 Solution Stability

Due to the use of autosampler for HPCL analysis which may involve overnight runs, deterioration can occur in the form of analyte degradation. Therefore, the stability of methyl salicylate and thymol in standard and sample solutions were investigated at room temperature. The mixed standard solution and spiked sample solutions were found to be stable for 24 hours as apparent in the APDI values which were within a range of 0.478 to 1.083% and 0.748 to 1.911% for methyl salicylate and thymol, respectively. This indicated the stability of analytical solutions at room temperature as the APDI values were within the range set by Shailajan et al. (2015).

Moreover, the mixed standard solution stored at chiller temperature was also found to be stable after six days of storage as the APDI values of methyl salicylate and thymol were 0.526% and 0.66%, respectively. These results proved that the same standard solution can still be utilised for analysis after six

days of its initial preparation. Summary of the stability results is shown in Table 4.21 and 4.22.

Table 4.21: Stability results (room temperature)

Sample type	**Methyl Salicylate**			**Thymol**		
	Day 0	Day 1	APDI (%)	Day 0	Day 1	APDI (%)
SST solution	1291.527	1304.856	1.027	560.067	564.273	0.748
Spiked sample (liniment)	1256.795	1262.816	0.478	558.158	563.113	0.884
Spiked sample (ointment)	1365.658	1380.533	1.083	588.542	599.900	1.911

Table 4.22: Stability results (chiller)

Sample type	**Methyl Salicylate**			**Thymol**		
	Day 0	Day 6	APDI (%)	Day 0	Day 6	APDI (%)
Calibration solution	2101.093	2090.076	0.526	898.957	904.906	0.660

4.3 Screening of Methyl Salicylate and Thymol

The summarised results of sample screening are shown in Table 4.23. Based on the HPLC analysis of all the samples, five were detected with methyl salicylate and none with thymol. As there is no current regulatory limit exists for both compounds, peak area at LOD concentration level was utilised as the benchmark for screening analysis. Methyl salicylate peak was present in sample LU1, LU2, LU3, OR3, OR2 and OU1. Their respective chromatograms and UV spectrums are illustrated in Appendix H, I, J, K, L, M, N, O, P, Q and R. Although methyl salicylate peak was present in sample OR2, its peak area was lower than LOD level thus deeming it a negative sample. Other positive samples were detected with methyl salicylate as their methyl salicylate's peak area values were higher than the corresponding peak area value from the LOD standard solution.

Additionally, out of the five adulterated samples, unregistered traditional medicines made up the majority with four positive samples. Although these findings were expected considering the tendency of manufacturers to deliberately adulterate unregistered traditional medicines, it was alarming to detect an adulterated registered sample (OR3). This might be due to the manufacturer's lack of transparency in regards to the active ingredients, whereby it was not declared in the label or during product registration. Methyl salicylate was not declared as the manufacturer will be required to provide substantial quality control evidence concerning methyl salicylate in that particular product. It will be costly as quality control testing requires personnel training and also relevant analytical equipment. Most manufacturers of traditional medicines are small and medium-sized enterprises which have limited funding and facilities for quality control. Furthermore, regulatory requirements for traditional medicines are more likely to be less stringent compared to that of pharmaceutical products. This may create a loophole that can be manipulated by manufacturers. Although such incidence is remote, it can be

overcome by regular post-market surveillance of traditional medicines and facility inspection to ensure compliance towards Good Manufacturing Practice (GMP) principles. In terms of unregistered traditional medicines, the accompanying hazards can be minimised by Pharmacy Enforcement Division's active role in monitoring the sales of traditional medicines. This may include thorough investigation and laboratory analysis before any confiscation of operation and prosecution in a court of law (MyHEALTH, 2017). Consumers can also play a part by ensuring the registration status of any traditional medicine prior consumption. Any exaggerated claims on the label should be received with caution as it might be an adulterated traditional medicine.

It is noteworthy to mention only methyl salicylate was detected from the list of samples. Possibly, this was deliberately done by the manufacturers to ensure the effectiveness of their traditional medicines by the presence of methyl salicylate's analgesic effect. The preference towards methyl salicylate in contrast to thymol was also evident as it was listed as one of the main adulterants for traditional medicines throughout the world (Posadzki et al., 2012). Although thymol was not the adulterant in this study, this could be due to its presence in other dosage forms such as creams as evident in this year's cancellation of *Warisan Salju Langkawi Krim Susu Kambing Gamat Plus*'s product registration (National Pharmaceutical Regulatory Agency, 2019).

Table 4.23: Screening results of traditional medicines

No.	Sample Code	Dosage Form	Registration Status	Peak Area of Methyl Salicylate (Sample)	Peak Area of Methyl Salicylate (LOD solution)
1.	LR1	Liniment	Registered	Not detected	64.8268
2.	LR2	Liniment	Registered	Not detected	64.8268
3.	LU1	Liniment	Unregistered	375.7730	64.8268
4.	LU2	Liniment	Unregistered	285.5094	64.8268
5.	LU3	Liniment	Unregistered	336.2017	62.4743
6.	OR1	Ointment	Registered	Not detected	64.8268
7.	OR2	Ointment	Registered	20.8065 (<LOD)	64.8268
8.	OR3	Ointment	Registered	363.0748	62.4743
9.	OU1	Ointment	Unregistered	263.1386	64.8268
10.	OU2	Ointment	Unregistered	Not detected	62.4743

CHAPTER 5: CONCLUSION

5.1 Conclusion

In this present study, an HPLC method for simultaneous determination of methyl salicylate and thymol in Malaysian traditional medicines was analysed and validated. At the moment, there are no available HPLC methods for simultaneous determination of methyl salicylate and thymol in traditional medicines. Instead, previously reported methods mainly focused on the determination of methyl salicylate and thymol as an individual compound or in conjunction with other compounds. The run time of this proposed method is the shortest (9 minutes) in comparison to other literatures. In turn, the rapid analysis of both compounds will help to save costs associated with electricity, reagents and labour. The sensitivity of the analytical method was reflected by the acceptable LOD and LOQ values for both compounds. The linearity of the proposed method was confirmed by the acceptance of regression and linearity models. Strong linear association between analyte concentration and detector response (R^2>0.997) was also demonstrated in a wide working range (25 to 150 µg/ml for methyl salicylate and 15 to 150 µg/ml for thymol). Specificity was also confirmed by t-test of the obtained slope and intercept as well as the absence of any interferences from excipients and sample components. Low percentage RSD values for repeatability and intermediate precision indicated the high precision of the analytical method. High degree of trueness was also exhibited as the recovery values for both compounds were within the range of 96.5 to 104.18%. From this study, it can be concluded that this sensitive, linear, specific, precise and accurate HPLC method can be utilised for routine quality control analysis of methyl salicylate and thymol in traditional medicines.

By applying this proposed method, selected Malaysian traditional medicines were screened for potential methyl salicylate and thymol adulteration. It should be noted that methyl salicylate was the only adulterant detected in the five positive samples. This showed the preference of manufacturers towards methyl salicylate in comparison to thymol. Moreover, the findings also revealed that unregistered traditional medicines were the main target for adulteration. This is not surprising as unregistered traditional medicines do not undergo crucial quality control and safety testing, which makes them susceptible to adulteration by unscrupulous manufacturers to gain lucrative profits.

5.2 Impact on Stakeholders

Although this study only screened for methyl salicylate and thymol adulteration, it is worth pointing out that this method can further quantitate methyl salicylate and thymol in traditional medicines, specifically in liniment and ointment. This will prove to be useful and practical once the limits are defined by regulatory bodies for both compounds. In another context, this proposed method will also be beneficial to traditional medicine manufacturers, who plans to conduct quality control testing for their methyl salicylate and thymol-based products. The application of this method in regulatory and industrial settings will help to ensure the safety and quality of traditional medicines, thereby safeguarding consumer's health. In addition, it can also be applied for future studies on the toxicity and safety of methyl salicylate and thymol in animals and humans.

5.3 Future Work

In future investigations, it might be possible to include menthol and camphor in the analysis. At the moment, only a gas chromatography method developed by Bachute and Shanbhag (2016) can simultaneously determine methyl salicylate, thymol, menthol and camphor in an ointment formulation. These compounds resemble closely in terms of their

physical and chemical properties as well as common active ingredients in many topical formulations. Unlike methyl salicylate and thymol, Drug Registration Guidance Document (DRGD) set a limit of 10% and 11% permitted content for menthol and camphor, respectively, when applied as active ingredients for external preparation (National Pharmaceutical Regulatory Division, 2016). The need for simultaneous determination of all these compounds is further highlighted by the product registration cancellation of *Minyak Seri Pala* as a result of undeclared menthol and thymol (National Pharmaceutical Regulatory Agency, 2019). Further studies on other sample matrices such as gels, lotions and creams will also need to be undertaken to ensure a comprehensive screening approach.

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LIST OF PUBLICATIONS AND PAPERS PRESENTED

List of Publications

1. **Adzib, M.S.M.** & Ilham, Z. (2020). Simultaneous analytical determination of methyl salicylate and thymol in selected Malaysian traditional medicines. *AIMS Medical Science, 7*(2), 43-56.