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Phytochemical Screening and High-performance Thin-layer Chromatography Quantification of *Vitex trifolia* Leaves Hydro-alcoholic Extract: Potential Anti-inflammatory Properties

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Authors' contributions

This work was carried out in collaboration among all authors. Author ATG carried out the research, wrote and revised the article. Author MHH conceptualized the central research idea and provided the theoretical framework. Authors MHH, YZ, and AHJ designed the research, supervised research progress, author MHH anchored the review, revisions, and approved the article submission. All authors read and approved the final manuscript.

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ABSTRACT

Aims: The present study aims to evaluate the phytochemical composition of *Vitex trifolia* (*V. trifolia*) leaves hydro-alcoholic extract and to report for the first time, its phenolic content using a validated high-performance thin-layer chromatography (HPTLC) method.
Study Design: Qualitative phytochemical analysis and HTLC densitometric quantitative analysis.
Place and Duration of Study: The study was carried out at the Faculty of Pharmacy, Universiti Teknologi MARA (UiTM), Malaysia, from March 2020 to December 2020.
Methodology: The preliminary phytochemical screening was carried out qualitatively. The HPTLC analysis was performed on glass-backed 60 F_{254} silica gel plates using a two steps gradient elution

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method of the mobile phase. In the first step, methanol was used to develop the plates until 40 mm of developing distance, while in the second step, plates were developed with n-hexane:ethyl acetate:acetic acid (20:9:1, v/v/v) until 80 mm of developing distance. Detection and quantification were performed by densitometric analysis at 254 nm. The method was validated as per the International Council for Harmonization of Technical Requirements for Pharmaceuticals for Human Use (ICH) guideline in terms of linearity, precision, accuracy, the limit of detection (LOD), and the limit of quantification (LOQ).

Results: The preliminary phytochemical screening of *V. trifolia* leaves hydro-alcoholic extract showed the presence of alkaloids, flavonoids, phenols, phytosterols, and terpenoids. The developed HPTLC method was proved to be linear, precise, and accurate. The LOD and LOQ of the method were determined to be 2.01 µg/band and 6.08 µg/band, respectively. The total phenolic content of the extract was calculated from the standard gallic acid calibration plot and found to be 136.94 ± 4.02 mg gallic acid equivalent (GAE)/g of dried extract.

Conclusion: This preliminary study revealed that *V. trifolia* has a considerable amount of phenolic compounds, which can potentially contribute to its anti-inflammatory, antioxidant, and anticancer activities. Further pharmacological investigations are being carried out to support the folkloric claims.

Keywords: Vitex trifolia; phytochemical screening; phenolic content; HPTLC.

1. INTRODUCTION

In recent years, the pharmaceutical application of plant-based natural products has gained interest. Reports from the World Health Organization (WHO) indicate that near 65% of the world's population use plants derived natural products for their health care. Most of these products are used accompanied by conventional drugs. Approximately around 150000 plant species have been evaluated so far and many of them have been reported to possess therapeutic properties. The therapeutic effects of them are due to the presence of different secondary metabolites. However, exploring their complex chemical compositions has always been challenging for researchers [1].

Vitex trifolia (*V.trifolia*), a species from the family Verbenaceae, is a multi-purpose medicinal plant that is widely distributed in tropic and sub-tropic regions including Malaysia, Thailand, Indonesia, Australia, India, and Sri Lanka [2-3]. The plant is a shrub with trifoliate leaves and purple inflorescences [4-5]. It is traditionally used to treat inflammation, pain, fever, and allergy [3,6]. *V. trifolia*'s infusion and decoction are used to treat intestinal complications, tuberculosis, and amenorrhea [2]. It is also used as an emmenagogue as it stimulates the blood flow in the pelvic. Moreover, In Unani medicine, the plant is known as sambhalu and it is used to increase libido. The inner bark of the plant is useful for the treatment of diarrhoea, cough, hypertension, and sinusitis while the root is used as anthelmintic and diuretic medicines [7]. The fruits and leaves are used to treat inflammation and the flowers are useful to treat fever. The anti-inflammatory and antioxidant effects of *V. trifolia* were reported by several studies [8–10]. The plant can exhibit its anti-inflammatory effect by suppressing the expression of pro-inflammatory mediators such as cytokines, cyclooxygenase, and inducible nitric oxide synthase through modulation of inflammation-related signalling pathways including, nuclear factor kappa B (NF-κB) and mitogen-activated protein kinases (MAPKs) signalling pathways [9,11-12]. On the other hand, the plant's free radical scavenging ability is responsible for its antioxidant activity [2,13]. Additionally, *V. trifolia* can also possess antibacterial [14-15], antiviral [16-17], anticancer [18-19], hepatoprotective [20], antiasthmatic [7], antihistaminic [21-22], anti-diabetic [23], and anthelminthic [24] properties.

The multi-pharmacological effects of *V. trifolia* are due to the presence of several secondary metabolites including polyphenols, terpenoids, glycoside [25], alkaloids, phytosterol [26], and iridoids [27]. Polyphenolic compounds are playing a major role in the plant's anti-inflammatory, antioxidant, and anticancer activities [28]. Several studies reported the isolation of several polyphenolic compounds from *V. trifolia*. Casticin or vitexicarpin is one of the main polyphenolic compounds of genus *Vitex* including *V. trifolia* and reports suggest that it can display potent anti-inflammatory and anticancer properties [10,12]. Additionally, artemetin, chrysosplenol D, luteolin, penduletin, persicogenin, quercetin, vitexin, and isovitexin

are among polyphenolic compounds that have been isolated from the leaves, fruits, and aerial parts of the plant [7,28–31]

However, as per our knowledge from the literature, so far there is no report about the quantification of total phenolic content (TPC) of *V. trifolia* leaves hydro-alcoholic extract which is important for further pharmacological investigations. Thus, this study was aimed to evaluate the chemical composition of *V. trifolia* leaves hydro-alcoholic extract and to report for the first time, its TPC using a validated high-performance thin-layer chromatography (HPTLC) method.

2. MATERIALS AND METHODS

2.1 Chemicals and Reagents

Gallic acid, ferric chloride, Dragendroff's reagent, and sodium hydroxide were provided from Sigma-Aldrich (Munich, Germany). Acetic acid, chloroform, ethanol, ethyl acetate, sulphuric acid, hydrochloric acid, and n-hexane were purchased from Merck (Darmstadt, Germany).

2.2 Sample Extraction

V. trifolia leaves were collected from Subang Jaya, Selangor Darul Ehsan, Malaysia, and the plant was authenticated and deposited at the Atta-ur-Rahman Institute for Natural Product Discovery (AuRINs), Universiti Teknologi MARA, Puncak Alam, Selangor Darul Ehsan, Malaysia with a voucher specimen no. 0215USJ. Clean leaves underwent shed drying for 5 days and grounded into a fine powder using an electrical blender. The powdered leaves (150 g) were macerated in ethanol and water (1:1, v/v) under sonication for 30 min at room temperature (23-26 ^{o}C) [32]. Next, a rotary evaporator was used to remove the solvent from the sample at 40 ^{o}C and 100 mbar of pressure, and the sample was then freeze-dried at -80 ^{o}C. The final extract was stored at -20 ^{o}C in a light protective container.

2.3 Qualitative Phytochemical Screening

The qualitative phytochemical screening for the presence or absence of alkaloids, flavonoids, phenols, phytosterols, and terpenoids was performed according to different qualitative methods [33-34]. The presence of alkaloids was evaluated by Dragendroff's test. 3 to 4 drops of Dragendroff's reagent (potassium bismuth iodide) were added into 4 ml of extract solution. An orange precipitate indicated the presence of alkaloids in the sample. While testing flavonoids, 2 ml of the 10% sodium hydroxide was added to 4 ml of extract solution to produce a yellow colour that indicated the presence of flavonoids. For the screening of phenols, 4 to 5 drops of 3% ferric chloride were added into 3 ml of extract's aqueous solution. The existence of phenols was indicated after a blue colour appeared. Liebermann-Burchard's test was used to test the occurrence of phytosterols. The extract (50 mg) was dissolved in 2 ml of acetic anhydride and 3 to 4 drops of sulphuric acid were added. The presence of phytosterols was displayed with the appearance of a brown colour. For testing the presence of terpenoids, 0.5 ml of chloroform and 3 to 4 drops of sulphuric acid were added into 3 ml of extract's ethanolic solution. A reddish-brown ring confirmed the presence of terpenoids in the sample.

2.4 HPTLC Analysis and Phenolic Content Quantification

The HPTLC analysis of *V. trifolia* leaves hydro-alcoholic extract was carried out according to the method by Agatonovic-kustrin et al. [35]. Gallic acid standard and plant powder stock solutions were prepared by dissolving 10 mg of the standard compound and plant powder in 10 ml TLC grade methanol. The solutions were then centrifuged at 3000 rpm for 10 min and stored in light protective containers. Chromatography was performed using an HTPLC system (CAMAG, Muttenz, Switzerland) on HPLTC glass-backed plates (20 cm × 10 cm) pre-coated with silica gel 60 F_{254} with 0.20 mm layer thickness (Merck, Germany). The HPTLC plates were pre-washed with 100% methanol and dried at 100 ^{o}C for 30 min to be activated. 20 µl of *V. trifolia* leaves hydro-alcoholic extract solution and 2, 4, 6, 8, and 10 µl of standard gallic acid solution were applied on TLC plates as 8 mm bands using an automatic TLC sampler 4 (CAMAG, Muttenz, Switzerland) equipped with a 25 µl HPTLC syringe (Hamilton, Bonaduz, GR, Switzerland). The bands were applied with an 8 mm distance from the bottom edge, 14 mm distance from both sides, and a minimum of 2 mm of the distance was maintained between bands. An automatic developing chamber 2 (CAMAG, Muttenz, Switzerland) was used to develop the plates in the mobile phase after the sample application. For separation, a 2 step gradient elution method of mobile phase was used. In the first step, methanol was used to develop the plates until 40

mm developing distance, while in the second step, plates were developed using n-hexane, ethyl acetate, acetic acid (20:9:1, v/v/v) until 80 mm developing distance. The developed plates were photo-documented using a TLC visualizer (CAMAG, Muttenz, Switzerland) before derivatization under UV light at 254 nm wavelength and daylight, and after derivatization under daylight. The derivatization was carried out using a Chromatogram Immersion Device (CAMAG, Muttenz, Switzerland) by dipping the plates into the ferric chloride (1%) for 1 sec with an immersion speed of 5 cm/s. After derivatization, plates were dried at 100 ^{o}C for 10 min. Quantitative analysis was done with TLC scanner 3 (CAMAG, Muttenz, Switzerland) at 254 nm. The scanning speed was 20 mm/s while the slit dimension was set at 6.00 mm × 0.4 mm. The densitograms were analysed by WinCATS version 1.4.9.2001 (CAMAG, Muttenz, Switzerland).

2.5 Method Validation

The validation of the method was performed as per the International Council for Harmonization of Technical Requirements for Pharmaceuticals for Human Use (ICH) guideline [36], and the linearity, precision, accuracy, the limit of detection (LOD), and the limit of quantification (LOQ) of the method were assessed. To determine the linearity, a calibration curve was plotted using the concentration of the standard gallic acid (µg/band) against peak area (AU) and was assessed by linear regression analysis. The precision was determined by assessing the repeatability of the method using inter-day and intra-day precision methods. The repeatability was measured by measurement of peak area (AU) of standard gallic acid at three concentrations (4, 6, and 8 µg/band) under the same condition on the same day and three consecutive days and was assessed by the relative standard deviation (% RSD). The recovery rate of the standard solution was measured to determine the accuracy of the method. Spiked samples were prepared by adding known amounts of standard gallic acid at 50, 100, and 150% of the pre-analysed analyte. The samples were analysed under the same conduction in triplicate and the recovery rate was calculated. The LOD of the method is defined as the lowest concentration of the analyte in the sample mixture which is qualitatively detectable while the LOQ is the lowest amount of the analyte in the sample that can be can be quantified. The LOD and LOQ of the standard were calculated as $3.3\sigma/S$ and $10\sigma/S$ respectively, where σ is the standard deviation and S is the standard calibration curve slope.

2.6 Statistical Analysis

All data were analysed statistically using Excel 2015 (Microsoft, USA) and the data were presented as mean ± SD. The chromatographs were analysed using WinCATS version 1.4.9.2001 (CAMAG, Muttenz, Switzerland).

3. RESULTS AND DISCUSSION

3.1 Preliminary Phytochemical Screening of *V. trifolia*'s Leaves Hydro-alcoholic Extract

The preliminary phytochemical screening revealed the presence of alkaloids, flavonoids, phenols, phytosterols, and terpenoids in *V. trifolia* leaves hydro-alcoholic extract Table 1. The presence of phytosterols, terpenoids, flavonoids, and phenols in the ethanol extract *of V. trifolia* leaves was also reported by a couple of different studies [13,26]. Moreover, *V. negundo* [13], *V. agnus-castus* [37], and *V. glabrata* [38] leaves extracts were also reported to have various secondary metabolites. This indicates that *V. trifolia* is rich in secondary metabolites which may contribute to its reported multi-pharmacological effects.

Table 1. Preliminary phytochemical screening of *V. trifolia* leaves hydro-alcoholic extract

Constitutes	Test Method	Results
Alkaloids	Dragendroff's test	+
Flavonoids	Alkaline test	+
Phenols	Ferric chloride test	+
Phytosterols	Liebermann-Burchard's test	+
Terpenoids	Salkowski's test	+

+ denotes the presence of the substance in the sample.

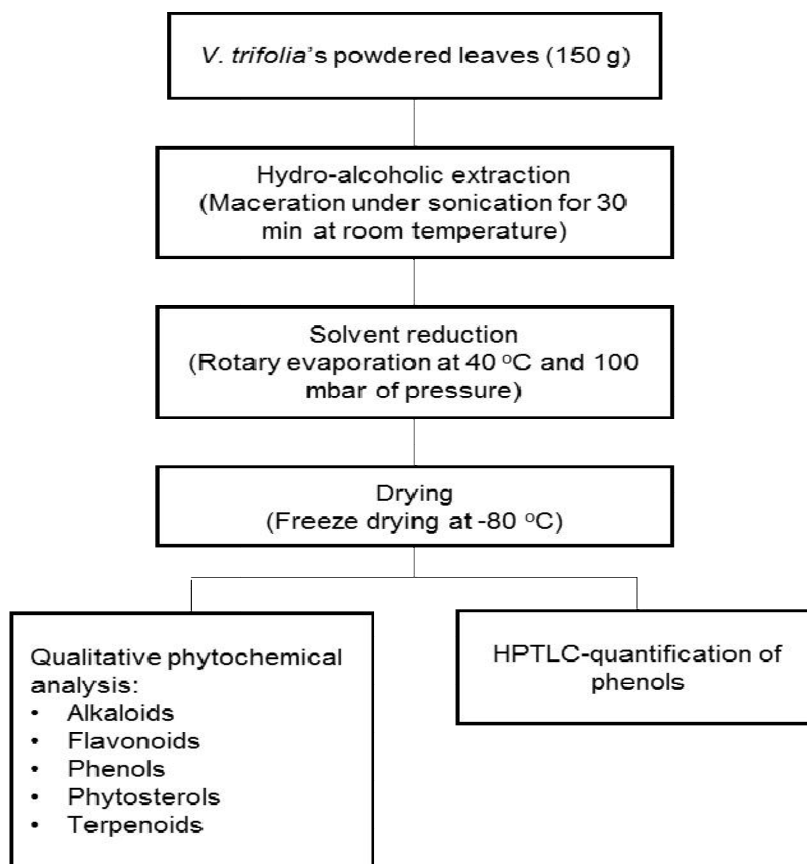

Fig. 1. The schematic diagram for extraction and phytochemical screening of *V. trifolia* leaves

3.2 HPTLC Method Validation

With a 2 step gradient elution method of mobile phase using 100% methanol and n-hexane:ethyl acetate:acetic acid (20:9:1, v/v/v) the bands were found to be separated symmetrically without any diffuseness, which indicates the suitability of the mobile phase system for the separation of the sample. The validation values for the HPTLC method are shown in Table 2. The developed method was found to be linear in the range of 2-10 µg/band with coefficient determination (R^2) of 0.973 ± 0.011. The LOD and LOQ for gallic acid were found to be 2.01 µg/band and 6.08 µg/band, respectively which indicated that the developed method is quite sensitive for the simultaneous quantification of phenolic compounds. The intra-day and inter-day precision % RSD, shown in Table 4, was recorded less than 10% which shows the method is precise. The accuracy of the method, shown in Table 4, was evaluated in terms of the method's recovery rate, and the average recovery rate was found to be 99.71% which shows good accuracy of the method as it is within the range of 90 to 110%. Thus, the method was found to be linear, sensitive, precise, and accurate.

3.3 Quantification of Phenolic Content in *V. trifolia* Leaves Hydro-alcoholic Extract

The chromatograms, shown in Fig. 2, indicate that the constitutes of the sample were separated symmetrically without any diffuseness. The identification of phenols in the extract was determined by comparing the retention factor (R_f) values of the sample with that of the standard. The R_f value of standard gallic acid was found to be 0.4. The hydro-alcoholic extract of *V. trifolia* leaves showed 6 peaks and the fourth peak, with the R_f value of 0.39, was similar to the standard gallic acid's R_f value which indicates the presence of phenols in the sample Table 4. The total phenolic content of the plant was determined using gallic acid calibration curve (y= 2371.6x + 3781.1, R^2 = 0.973 ± 0.011), Fig. 4, and presented as mg gallic acid equivalents

Table 2. Method validation data for high-performance thin-layer chromatography quantification of gallic acid in *V. trifolia* leaves hydro-alcoholic extract

Validation parameter		Results
Regression line		y = 2371.6x + 3781.1
Linearity (coefficient determination)		0.973 ± 0.011
Precision (% RSD)		
	Intra-day	7.37
	Inter-day	3.02
Accuracy (% recovery)		99.71
LOD (μg/band)		2.01
LOQ (μg/band)		6.08

LOD, the limit of detection; LOQ, the limit of quantification; RSD, relative standard deviation

Table 3. Precision data of the developed high-performance thin-layer chromatography method for the quantification of gallic acid in *V. trifolia* leaves hydro-alcoholic extract (n=3)

Concentration (μg/band)	Intra-day precision Mean peak area ± SD	% RSD	Inter-day precision Mean peak area ± SD	% RSD
4	15882.37 ± 1540.47	9.69	15557.97 ± 347.68	2.23
6	21599.57 ± 1310.46	6.07	21602.97 ± 854.17	3.95
8	26495.7 ± 1682.07	6.35	26133.65 ± 749.04	2.87

SD, standard diversion; RSD, relative standard deviation

Table 4. Accuracy data of the developed high-performance thin-layer chromatography method for the quantification of gallic acid in *V. trifolia* leaves hydro-alcoholic extract (n=3)

Gallic acid in the analyte (mg/g dried extract)	Excess gallic acid added to the analyte	Theoretical content (μg/g dried extract)	Recovered content ± SD (μg/g dried extract)	Recovery rate (%)
137	50%	205.5	209.12 ± 1.59	101.76
137	100%	274	264.96 ± 3.25	96.7
137	150%	342.5	344.79 ± 24.04	100.67

SD, standard deviation

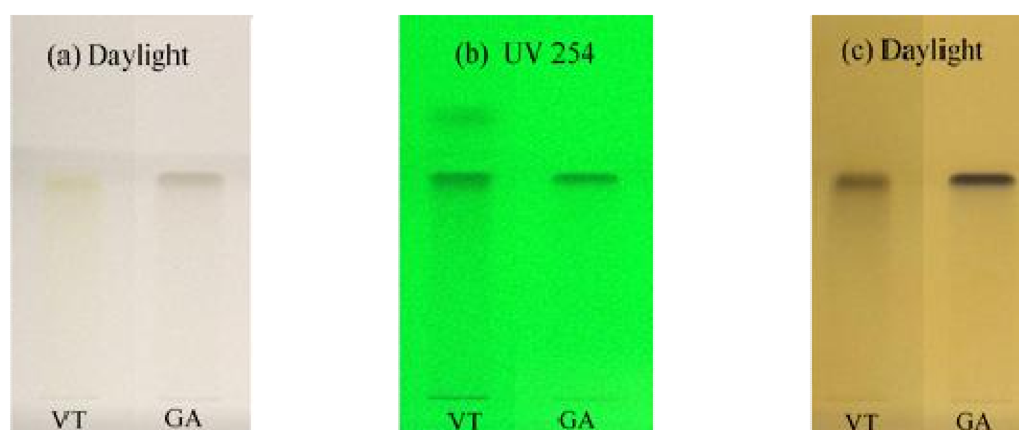

Fig. 2. Developed chromatograms of *V. trifolia* leaves hydro-alcoholic extract and standard gallic acid, (a) and (b) before derivatization, (c) after derivatization. VT, *V. trifolia*; GA, gallic acid

Table 5. R_f values for *V. trifolia* leaves hydro-alcoholic extract and standard gallic acid

Track	Peak	R_f	Max height	Area	Assigned substance
VT	1	0.00	443.4 AU	3755.6 AU	Unknown
	2	0.03	96.3 AU	2921.4 AU	Unknown
	3	0.33	223.7 AU	12846.7 AU	Unknown
	4	0.39	313.0 AU	10124.7 AU	Gallic acid
	5	0.57	211.5 AU	6149.9 AU	Unknown
	6	0.88	14.9 AU	383.2 AU	Unknown
GA	2	0.40	309.5 AU	13707.5 AU	Gallic acid

AU, peak area; GA, gallic acid; R_f, retention factor; VT, V. trifolia

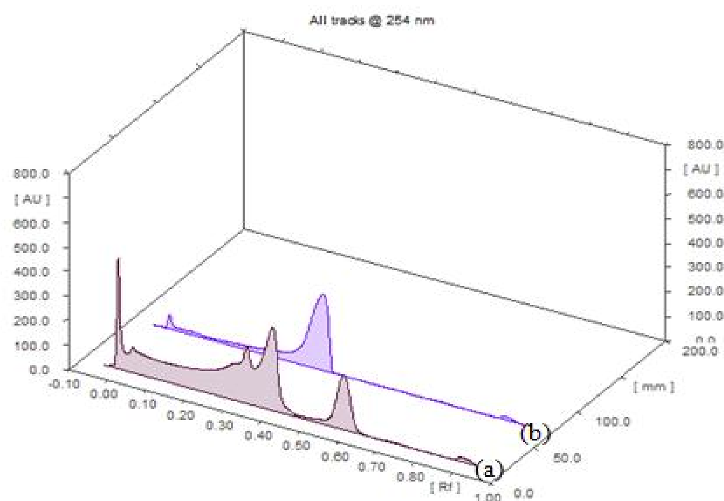

Fig. 3. 3-D chromatograms of *V. trifolia* leaves hydro-alcoholic extract (a) and standard gallic acid (b)

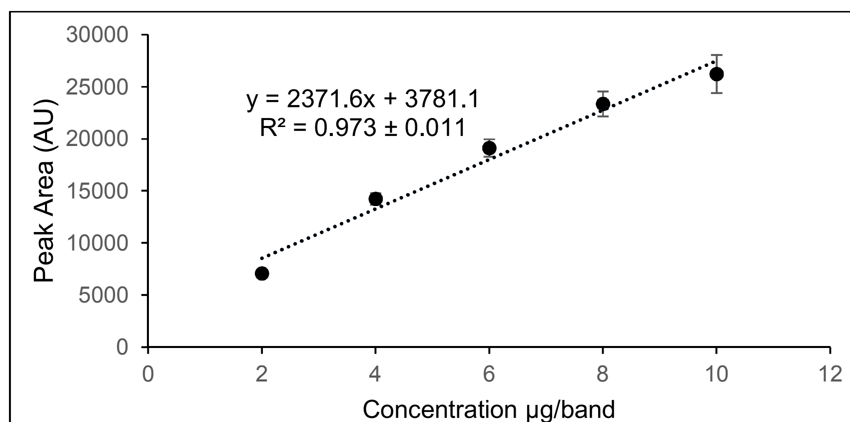

Fig. 4. Gallic acid calibration curve

(GAE)/g of dried extract. The TPC was found to be 136.94 ± 4.02 mg GAE/g dried extract which is a considerable amount of phenolic compounds in the plant. The TPC of other types of *V. trifolia* extracts was previously reported by a couple of studies. Saklani et al. [13] reported the TPC of *V. trifolia* leaves ethanol extract to be 77.20 ± 0.22 mg GAE/g of dried extract, while the TPC of the plant's leaves methanol extract was found to be 13.69 mg GAE/g of dried extract [2]. In both

studies, the plant's phenolic content was reported much lesser than what was found here in this study. This can be due to the use of different extraction methods and solvents. Furthermore, the amount of phenolic content in *V. trifolia* leaves hydro-alcoholic extract is more than the TPC of *V. agnus-castus and V. mollis* leaves methanol extracts and *V. agnus-castus*, *V. negundo*, and *V. megapotamica* leaves ethanol extracts which were reported to be 123.9 ± 2.81 [39], 33.70 [40], 53.33 ± 1.38 [37], 89.72 ± 0.14 [13], and 81.5 [41] mg GAE/g of dried extract, respectively. Polyphenolic compounds are a group of naturally occurred secondary metabolites in plants with more than one group of phenols [42-43]. They are widely distributed in plants and they are reported to possess several pharmacological effects including, anti-inflammatory [44], antioxidant, and anticancer [43] properties. Several polyphenolic compounds were isolated from *V. trifolia* and other *Vitex* genus including, casticin, artemetin, gallic acid, quercetin, penduletin, persicogenin, vitexin, and isovitexin [8,19,28,31]. These compounds are believed to be responsible for *V. trifolia*'s anti-inflammatory, antioxidant, and anticancer effects by acting on different signalling pathways related to the pathogenesis of inflammatory diseases, oxidative stress, and cancer [8,10,12, 28]. These secondary metabolites inhibit the expression of inflammatory mediators such as cytokines, leukotrienes, pro-inflammatory enzymes, and chemokines through modulation of NF-κB and MAPKs pathways [9,11-12]. Moreover, the antioxidant activity of phenols is expressed by their free radical scavenging ability [2,13]. This preliminary study indicates that *V. trifolia* leaves hydro-alcoholic extract can be a good source of polyphenolic compounds as we found that it has a good amount of phenolic content. However, further investigations are needed to determine the type of phenolic compounds and their specific pharmacological effects.

4. CONCLUSION

From the qualitative preliminary phytochemical analysis, various secondary metabolites such as alkaloids, flavonoids, phenols, phytosterols, and terpenoids were identified in *V. trifolia* leaves hydro-alcoholic extract. HPTLC analysis further confirmed the presence of phenols in the plant with a considerable amount. The TPC of the plant hydro-alcoholic extract was found to be more than that of several previously reported *Vitex* species alcoholic extracts. The presence of phenolic compounds in a high concentration, and other secondary metabolites can potentially contribute to the plant's antioxidant and anticancer activities especially anti-inflammatory attributes. More so in the COVID-19 pandemic situation that warrants more research to be carried out to boost the immune system. Thus, the plants which are rich in potential phytochemical composition should be considered for pharmaceutical and/or nutraceutical usage. On that note, further pharmacological investigations are needed to prove these claims.

DISCLAIMER

The products used for this research are commonly and predominantly use products in our area of research and country. There is absolutely no conflict of interest between the authors and producers of the products because we do not intend to use these products as an avenue for any litigation but for the advancement of knowledge. Also, the research was not funded by the producing company rather it was funded by personal efforts of the authors.

CONSENT

Not applicable.

ETHICAL APPROVAL

Not applicable.

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COMPETING INTERESTS

Authors have declared that no competing interests exist.

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