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Quantitative Thin Layer Chromatography Analysis, Antioxidant, and Anti-Inflammatory Activities of Polyherbal Formulation (Ammarit-Osot) Extracts

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Abstract

This study was carried out to develop a TLC method for quantifying one of bioactive, piperine, from polyherbal formulation (Ammarit-Osot) extracts. The extracts were prepared by different solvents such as methanol (ME) and water (WE). The mobile phase of toluene:acetone:formic acid (7:2:1, v/v/v) was used for separation. The calibration curve showed a good linearity (r^2 = 0.9972) in the range of 50 – 500 ng/spot. This assay was assessed by intra-and interday precision (RSD 0.25 – 2.49%), and accuracy (101.05 – 101.56 %). The piperine content was found to be 23.06 ± 0.06 and 0.44 ± 0.03% w/w for ME and WE, respectively. Both ME and WE demonstrated significant activity in DPPH and FRAP assays, indicating their antioxidant potential. The TPC of ME and WE were 53.40 ± 2.21 and 101.18 ± 0.46 (mg GAE/g extract), respectively. The TFC of ME and WE were 43.35 ± 3.94 and 25.31 ± 1.74 (mg catechin/g extract), respectively. In addition, the cytotoxicity of extracts on RAW264.7 was investigated by MTT assay. The ME and piperine showed IC_{50} with 162.40 ± 7.84 and 115.68 ± 11.96 µg/mL, respectively. While the WE exhibited low cytotoxicity (IC_{50} > 1,000 µg/mL), the anti-inflammatory activity was assessed by LPS-stimulated NO overproduction. The ME and piperine displayed the ability to inhibit NO production, but WE not inhibited. The results indicated that the different solvent extracts of Ammarit-Osot have the potential to be antioxidant, anti-inflammatory and immune-stimulate agent.

Keywords**:** *Ammarit-Osot; TLC; piperine; antioxidant; anti-inflammatory activity*

1. Introduction

Polyherbal formulations encompassing bioactive compounds play a crucial role in influencing clinical efficacy. Following the World Health Organization (2000)'s guidance, the standardization of the active chemical marker through chromatographic fingerprint analysis has become an increasingly effective and essential process in the context of polyherbal formulations. Several methods for fingerprint analysis of polyherbal formulation are represented in chromatographic techniques such as HPLC (Ahmad et al., 2014; Bhope et al., 2011), TLC (Mian et al., 2023; Patel et al., 2010; Thatipelli et al., 2023), and GC (Shalini, & Ilango, 2021). Recently, TLC has emerged as a potent and simplified technique for assessing medicinal plants and polyherbal formulations (Shivatare et al., 2013). Validation involves conducting laboratory studies to confirm that

the performance characteristics of an analytical method satisfy the specified requirements for its intended applications.

The Ammarit-Osot formulation, a traditional Thai medicine, is composed of 18 plant ingredients and 4 pharmaceutical materials. This formula has been documented for its efficacy in addressing bloating, flatulence, and abdominal pain (Srithat et al., 2022). Those symptoms refer to gastropathy or gastritis motivated from erosion and/or inflammation in the stomach lining (Muszyński et al., 2016). *Piper nigrum* L. serves as a primary component, followed by *Cannabis sativa* L. Within *Piper nigrum* L. (Piperaceae family), the major active compound is piperine. Piperine is conventionally used in medicine for managing pain, reducing fever, addressing hypertension (Wang et al., 2021), providing anti-inflammatory effects (Abdel-Daim et al., 2019), alleviating poor digestion (Mehmood, & Gilani, 2010), and stimulating appetite (Gregersen et al., 2013).

2. Objectives

The aims of this work were to develop and validate a TLC method for the identification and quantification of piperine and to determine the antioxidant and anti-inflammatory activities of polyherbal formulation (Ammarit-Osot) extracts.

3. Materials and methods

3.1 Sample preparation

The fine powder of Ammarit-Osot formulation (5.0 g) was extracted using different solvent ratios 1:20 (w/v), sonicated with methanol for 30 min, and boiled with water for 10 min. The extracts were passed through filter paper (Whatman no. 1). The methanol extract (ME) and water extract (WE) were dried using an evaporator and freeze drying. The ME and WE were weighted (2.5 mg) and dissolved with ethanol 5 mL (0.5 mg/mL) for working solution of chromatography analysis.

3.2 Standard preparation

Stock solution of piperine (Sigma Aldrich, USA) standard (1 mg/mL) was prepared by accurately weighing 10 mg and dissolving it in 10 mL of ethanol in a volumetric flask, then diluted to 0.1 mg/mL for a working solution for chromatography analysis.

3.3 TLC validation

3.3.1 Chromatography condition

The chromatographic analysis was conducted by loading the standard compound, formulation extracts onto pre-coated aluminum TLC plates of silica gel 60 F254 (20 x 10 cm, Merck) by Linomat V sample applicator (CAMAG, Switzerland) as 6 mm of bands length with 100 µL syringe. The constant application rate at 80 nL/s. The plates were developed with a mobile phase containing toluene-acetone-formic acid (7:2:1, v/v/v) for 80 mm distance in a TLC chamber saturated with the mobile phase (40 min). Then, the TLC plates were visualized at 254 and 366 nm, and densitometric scanning was performed using TLC scanner 4 in absorbance mode at 330 nm with slit dimension at 4.00 x 0.30 mm.

3.3.2 Linearity

A working solution of piperine (0.1 mg/mL) was prepared at standard concentration of 0.01 – 0.1 mg/mL. The 5 µL of each concentration was applied to the TLC plate, corresponding to 50 – 500 ng/spot. Linearity was obtained by linear regression analysis of peak area versus standard concentration.

3.3.3 Limit of detection (LOD) and limit of quantitation (LOQ)

LOD and LOQ were determined based on calibration curve using the following equation:

LOD = 3.3 x SD of intercept/slope

LOQ = 10 x SD of intercept/slope

3.3.4 Specificity

The band of piperine in the formulation extracts was confirmed by comparing the Rf values and spectra of standard piperine.

3.3.5 Precision

Precision was determined by analysis of the repeatability measurement (n=3) of peak area of standard piperine. The intra- and inter-day precision were carried out at three concentration levels within the day and three different days, respectively. The data were expressed in terms of the relative standard deviation (%RSD).

3.3.6 Accuracy

The accuracy of the method was assessed on formulation extract spiked with three values of standard (n=3). Accuracy was expressed in terms of percentage recovery.

3.3.7 Robustness

Robustness was determined in terms of RSD by changing chromatographic conditions. The mobile

phase composition was slightly changed (±5%), then %RSD of peak areas were calculated.

3.4 The total phenolic content (TPC)

TPC was estimated by Folin-Ciocalteu method with modification described by Oogarah et al., 2020. The reactions containing extract (20 µL) and 0.2 N Folin-Ciocalteu reagent (Sigma Aldrich, USA) (100 µL) were mixed and incubated for 6 min. Subsequently, 0.75% (w/v) Na_2CO_3 (Ajex-Finechem, Australia) (80 µL) was added and incubated for 1 hour in the dark. Then the reactions were measured at 765 nm. The TPC values were reported as milligrams of gallic acid (Sigma Aldrich, USA) equivalents per gram of extract (mg GAE/g extract).

3.5 The total flavonoid contents (TFC)

TFC was determined following by Herald et al., (2012). The mixture containing 25 µL of the extract, 100 µL of water and 10 µL of 5% (w/v) $NaNO_2$. After incubation for 5 min, 15 µL of 10% (w/v) $AlCl_3$ (Ajex-Finechem, Australia) was added. The reaction mixtures were incubated for 5 min and 1 N NaOH (Carlo Erba, Germany) and water was added to reaction for 50 µL of each. The absorbance of the reactions was measured at 510 nm. TFC was expressed as milligrams of catechin (Sigma, China) equivalent per gram of extract (mg catechin/g extract).

3.6 Antioxidant activity

3.6.1 The 2,2-diphenyl-1-picrylhydrazyl (DPPH) scavenging assay

The reaction was conducted as described in previous work with modification (Adli et al., 2024). One hundred microliters of the extracts (0.005 – 5 mg/mL) were mixed with 100 µL of DPPH (0.8 mg/mL) (Sigma, USA). After incubation for 30 min in the dark, the absorbance of the reactions was measured at 517 nm. The percentage of DPPH radical scavenging was calculated using the equation below:

$$\% \text{ DPPH radical scavenging} = \frac{(A_{blank} - A_{extract})}{A_{blank}} \times 100$$

A_{blank} is absorbance of the mixture without extract and $A_{extract}$ is the absorbance of the mixture with extract.

3.6.2 The ferric reducing antioxidant power (FRAP)

The reaction was conducted as described in previous work with modification (Han et al., 2024). The mixture containing the extracts (20 µL) was reacted with 180 µL of FRAP reagent (300 mM acetate buffer, pH 3.3: 10 mM 2,4,6-Tri(2-pyridyl)-s-triazine (TPTZ) (Sigma, Switzerland): 20 mM $FeCl_3$ (Ajex-Finechem, Australia) in a ratio of 10:1:1 (v/v/v). After incubation at 37ºC for 15 min, the absorbance of the mixtures was measured at 593 nm. The results were expressed as millimolar Fe^{2+} equivalents per gram of extract (µmol Fe^{2+}/g extract).

3.7 Anti-inflammatory activity

3.7.1 Cell viability assay

The effects of ME, WE and piperine on cell viability were measured using a colorimetric 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) (Invitrogen, USA) assay (Ketkomol et al., 2024). The RAW264.7 cells were cultured in Dulbecco's Modified Eagle Medium (DMEM) (Gibco, USA) (10% fetal bovine serum and 1% penicillin/streptomycin) at 37ºC with 5% CO_2 incubator. The cells were inoculated at 1 x 10^4 cells/well in 96 well-plates and incubated overnight. Then, cells were treated with various concentrations of piperine (3.9 – 250.0 µg/mL), ME (3.9 – 250.0 µg/mL), and WE (31.29 – 2000.0 µg/mL) dissolved in 0.1% v/v DMSO in DMEM and incubated for 24 h. After the culture mediums were discarded, the cells were exposed to MTT solution (0.5 mg/mL) for 4 h. Then, dimethyl sulfoxide (DMSO) (Loba Chemie, India) was added after the medium was removed. Cell viability was measured at 570 nm.

3.7.2 NO assay

The NO assay was performed as described in a previous report with modification (Baek et al., 2020). The RAW264.7 cells were plated in 96 well plates at 5 x 10^4 cells/well and incubated overnight. The cells were pretreated with ME and piperine for 1 h. Subsequently, cells were stimulated with lipopolysaccharide (LPS) (Sigma, Israel) (1 µg/mL) and incubated for 24 h. The culture mediums were collected and reacted with Griess reagent for NO level measurement. The absorbance was determined at 540 nm using a microplate reader. The NO concentration was calculated based on the standard curve of $NaNO_2$ (Ajex-Finechem, Australia) solution (1.56 – 2000 µM).

3.8 Statistical analysis

The results are presented as the mean with SD. Statistical analysis was performed using one-way ANOVA with Dunnett's post hoc test, and p-values < 0.05 were considered statistically significant.

4. Results and discussion

4.1 Method validation

The TLC analysis method is as effective as HPLC and used for routine quantitative and qualitative analysis of plant extracts and polyherbal formulations (Sobhani et al., 2020; Urakova et al, 2008; Zhang et al., 2020). It offers several advantages over HPLC, such as using a smaller quantity of organic solvents, allowing the simultaneous analysis of many samples, and being cost-effective and rapid. Additionally, it requires a shorter equilibration time and does not need a clean-up process. The quality control of polyherbal medicinal formulations is a primary requisite for safety and efficacy assurance. The chromatography validation of phytochemical markers is a basic strategy in standardization and quality control. Previous studies have demonstrated that TLC analysis of piperine from plants (Mgbeahuruike et al., 2018; Pundarikakshudu et al., 2014; Ramesh et al., 2015) and polyherbal formulations (Chewchinda et al., 2018; Patra, & Kumar, 2010; Rajopadhye et al., 2012; Wetwitayaklung et al., 2010) exhibits a similar linear range (100 - 600 ng/spot) to this study, but with lower LOD and LOQ values. Piperine is a major component in the Ammarit-Osot extracts, then it used to be a marker for TLC-densitometric analysis in this work. The toluene-acetone-formic acid (7:2:1, v/v) was used as mobile phase to separated piperine with an Rf value of 0.52 ± 0.01. Validation of TLC method was carried out according to ICH guidelines (ICH, 2005). Linearity was found in the range of 50 – 500 ng/spot for piperine with correlation coefficients (r^2) of 0.9972. The sensitivity of the TLC method was measured through LOD and LOQ were found to be 24.45 and 74.08 ng/spot, respectively. Regression and analytical parameters were summarized in Table 1. The precision of analytical procedure was evaluated in terms of intraday and interday precision. The RSD values of response were less than 2% and 5% for intraday and interday precision, respectively (Table 1) indicating the method is repeatable and reproducible (Sharma, et al., 2021).

Table 1 Analytical parameters for determination of piperine by the proposed TLC-densitometric method

Parameter	Values
Range of linearity (ng/spot)	50 - 500
Regression equation	y = 27.405x + 3277.9
Correlation coefficient (r^2)	0.9972
R_f	0.52 ± 0.01
Intraday precision (%RSD)	0.25 - 1.74
Interday precision (%RSD)	1.73 – 2.49
Recovery (%)	
ME	101.05 ± 3.70
WE	101.56 ± 3.69
LOD (ng/spot)	24.45
LOQ (ng/spot)	74.08

The average recoveries at three different levels of piperine from extracts were 101.05 – 101.56 % (Table 1). The robustness was determined by introducing a small change in mobile phase composition. The % RSD of peak area and Rf were found to be less than 2 % indicated the method is robustness. The specificity of the method was performed by comparing Rf and spectra of piperine band in each extract with standard. The overlay spectrum between the standard and extracts showed a good correlation at peak start, peak apex and peak end (Figure 1B) and the Rf value of piperine in extracts were 0.52 (Figure1C, 1D, and 1E).

The validated TLC method was used to measure the content of piperine in different extracts of Ammarit-Osot. There was no interference and well separated bands of piperine were observed. The piperine exhibits a quenching band under UV 254 nm (Figure 2A) and blue fluorescent under UV 366 nm at Rf 0.52 (Figure 2B). The ME and WE were found to contain piperine 23.06 ± 0.06 (% w/w) and 0.44 ± 0.03 (% w/w), respectively.

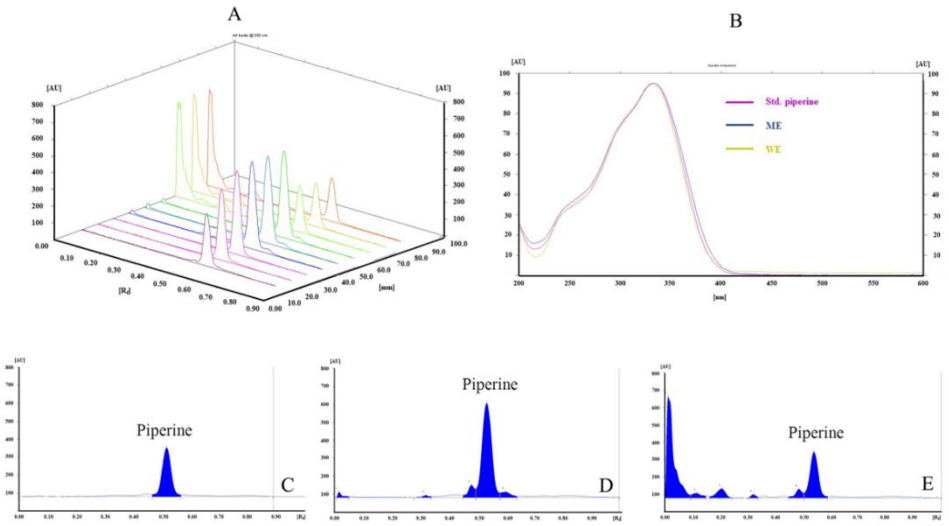

Figure 1 Three-dimension overlay of the TLC densitogram of standard piperine, ME and WE of formulation (A), Overlay of UV spectra scanning from 200 – 600 nm by TLC densitometric method (B), TLC densitograms of standard piperine (C) ME (D) and WE (E)

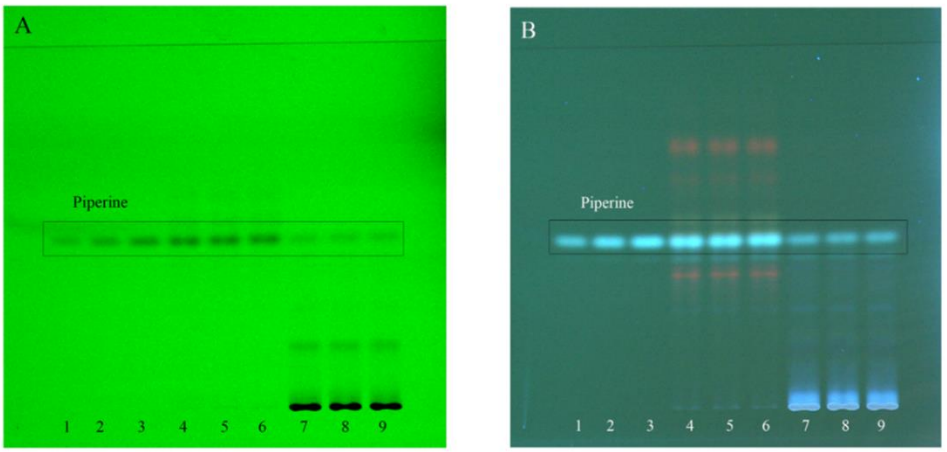

Figure 2 The silica gel F254 TLC plate of Ammarit-Osot extract was developed with toluene-acetone-formic acid (7:2:1, v/v). Lanes 1-3: standard piperine (100, 200, and 400 ng); Lanes 4-6: ME of Ammarit-Osot; Lanes 7-9: WE of of Ammarit-Osot detected under UV 254 nm (A), and 366 nm (B)

4.2 Antioxidant activity

The percentage of extraction yield, TPC, and TFC of the extracts obtained from Ammarit-Osot formula exhibited in Table 2. The yield and TPC were found to be higher in WE (~2 folds). The TFC, however, showed a higher level in ME. In previous work, the higher polarity solvent was potent in extracting phenolic compounds (Duan et al., 2023; Sasadara, & Wirawan, 2021). The results showed that the polarity of the solvent influenced phenolic extraction. In Table 2, the higher DPPH radical scavenging activity of ME is not only due to its high piperine content but also correlated with its flavonoid content (Cai et al., 2006; Seyoum et al., 2006). Previous reports have found that *P. nigrum* extract and piperine exhibit moderate DPPH and FRAP inhibitory activities (Adekoya et al., 2021; Qin et al., 2020; Zarai et al., 2013). Additionally, the FRAP assay was used to assess the ability of reductants to donate hydrogen atoms. The WE exhibited high reducing power, indicating that its activity is due to its polyphenolic content (Chen et al., 2020a; Loganayaki et al., 2013).

Table 2 Percent yield, total phenolic, total flavonoid, and antioxidant activities of different extracts of formular

Parameter	ME	WE
% Yield	9.81	25.70
TPC (mg GAE/g extract)	53.40 ± 2.21	101.18 ± 0.46
TFC (mg catechin/g extract)	43.35 ± 3.94	25.31 ± 1.74
DPPH (IC_{50}; mg/mL)	0.058 ± 0.080	0.349 ± 0.090
FRAP (μM Fe^{2+} equivalent)	741.54 ± 4.44	1221.95 ± 3.76

Data are expressed as mean ± SD.

4.3 The cytotoxicity and anti-inflammatory activity

The cytotoxicity of piperine, ME and WE on RAW 264.7 cells was investigated by MTT assay. The RAW 264.7 cells were incubated with various concentrations of piperine (3.9 – 250.0 μg/mL), ME (3.9 – 250.0 μg/mL), and WE (31.29 – 2000.0 μg/mL). As shown in Figure 3A-B, cell viability was significantly affected by piperine and ME with IC_{50} values 115.68 ± 11.96 and 162.40 ± 7.84 μg/mL, respectively. Similarity to previous studies, piperine promoted toxicity on Raw 264.7 cells at concentration greater than 40 μg/mL (Boonrueng et al, 2022; Duan et al, 2022). While all concentrations of WE did not show cytotoxic effects on RAW 264.7 cells (Figure 3C).

The anti-inflammatory activity was determined based on the inhibitory effect on NO production in LPS stimulated RAW 264.7 cell. Piperine acts as an anti-inflammatory agent, inhibiting the production of prostaglandin E2 (PGE2) and nitric oxide (NO) induced by LPS. It also suppresses the gene expression and translation of TNF-α, iNOS, and COX-2. (Gunsuang et al., 2019; Ying et al., 2013). In this study, after cells were induced by LPS and co-treated with piperine or ME, NO production was inhibited in a concentration-dependent manner. Figure 4A and 4B, the piperine and ME showed significantly reduced NO secretion from 41.3 ± 1.4 to 22.5 ± 2.0 μM (13 – 51 % inhibition) and 10.4 ± 2.5 μM (13 – 77 % inhibition), respectively. Typically, water extracts from plants contain hydrophilic compounds or polysaccharides known for their immune-enhancing activity (Chen et al., 2020b; Geum et al., 2020; Hong et al., 2017; Qin et al., 2019; Tabarsa et al., 2020). In this study, the WE contain a small amount of piperine but a high concentration of phenolic compounds. These compounds did not significantly impact cell viability (Figure 3C) or inhibit NO secretion (Figure 4C). Moreover, the WE acted as an immunomodulator, significantly enhancing NO production in a dose-dependent manner (Figure 4C).

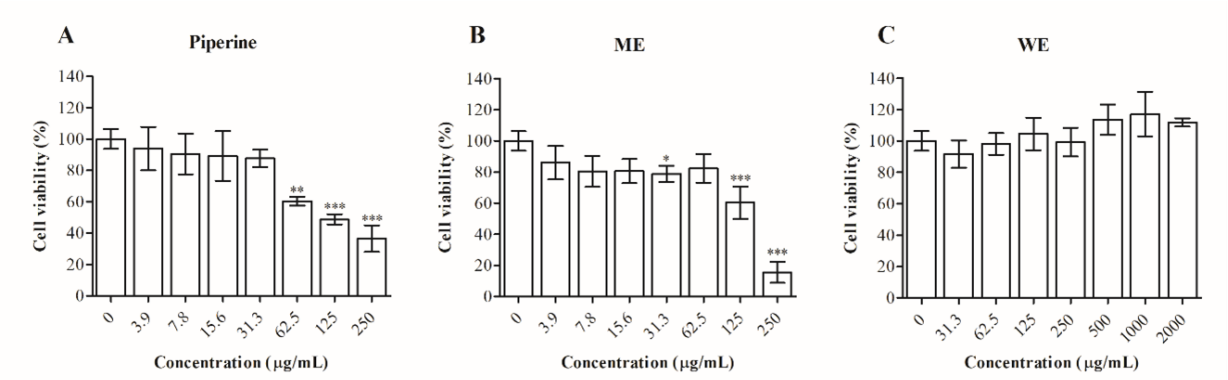

Figure 3 The effect of piperine (A), ME (B) and WE (C) at various concentrations on RAW264.7 cells viability for 24 h. The values expressed as the mean ± SD. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, significant difference compared to the control group

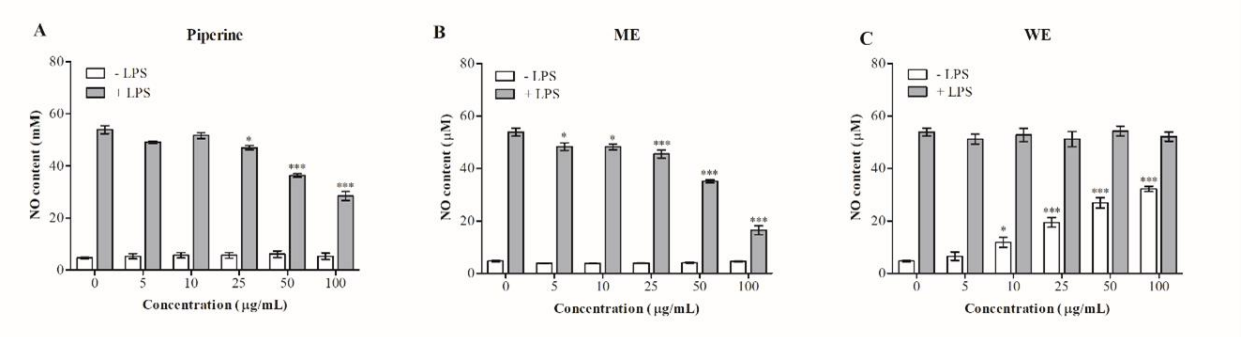

Figure 4 The Effect of piperine (A), ME (B) and WE (C) on the production of nitric oxide (NO). The values expressed as the mean ± SD. * $p < 0.05$ and *** $p < 0.001$, significant difference compared to the control group

5. Conclusion

The proposed TLC-densitometric method developed a simultaneous quantitative estimation of piperine as the active chemical marker from the poly-herbal Ammarit-Osot formulation. This method was found to be specific, reproducible, precise, accurate and robust according to ICH guidelines. The Ammarit-Osot formulation displayed reducing power and radical scavenging activity that related to a number of phenolic and flavonoid contents. The anti-inflammatory activity was found only in the ME, while the WE exhibited immune-enhancing activity. This result indicates that different solvent extraction affected chemical components and bioactivity. Therefore, the Ammarit-Osot formulation is a potential herbal formulation for therapeutic application.

6. Acknowledgements

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