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Effect Of Solvent On Flavonoid, Polyphenol Content And Biological Activities Of *Phyla Nodiflora* Extracts

Cao Thi Hue[1*], Nguyen Truong Quoc Anh[2], Truong Quynh Mai[3], Ha Thi Dung[4]

[1*]Thuyloi University, 175 Tay Son, Dong Da, Hanoi, Vietnam
[2]Vinschool Harmony, Phuc Dong, Long Bien, Hanoi, Vietnam
[3]Hanoi-Amsterdam High School for the Gifted, Cau Giay, Hanoi, Vietnam
[4]Hanoi University of Industry, 298 Cau Dien, Bac Tu Liem, Hanoi, Vietnam

***Corresponding Author:** Cao Thi Hue
*Thuyloi University, 175 Tay Son, Dong Da, Hanoi, Vietnam E-mail: caohue@tlu.edu.vn

Abstract
This study investigated the influence of different solvents on the total polyphenol and flavonoid content; and antioxidant and anti-inflammatory activity (NO inhibition) of *Phyla nodiflora* extracts. Food-grade ethanol 90° (ethanol), and ethanol/water mixtures at ratios of 7:3, 5:5, and 3:7 were used as the extracting solvents, yielding the corresponding extracts PN-E100, PN-EW73, PN-EW55, and PN-EW37, respectively. The results showed that the PN-EW73 extract had the highest total polyphenol content of 45.46 mg/g, while the PN-E100 extract had the highest total flavonoid content of 67.01 mg/g. The PN-EW73 extract also exhibited the best antioxidant activity as determined by the phosphomolybdenum and DPPH methods, and the best ability to inhibit nitric oxide production with EC_{50} values of the assays were 96.17, 63.98, and 75.13 µg/mL, respectively. These results facilitate selecting suitable solvents in developing natural antioxidant and natural NO inhibitory products derived from *Phyla nodiflora*.

Keywords: *Phyla nodiflora*, extraction solvent, antioxidant, inhibit NO production.

1. INTRODUCTION

Vietnam is believed to own a highly rich biodiversity with more than 12,000 flora species distributed throughout the country. Several of them are used as raw materials for developing new functional foods and drugs [1].

Phyla nodiflora, which belongs to the family Verbenaceae, is a wild, herbaceous, and fast-growing herb. It is found in many nations including Australia, Central and South America, the Mediterranean, Africa, and Asia [2]. The plant is mainly used for medicinal purposes but in some countries, it is also used in landscaping and as animal feed [3]. The family *Verbenaceae* consists of nearly 100 genera and 2,600 species, most of which grow in tropical and subtropical regions. 26 genera and more than 130 species of this family have been found in Vietnam. Widely distributed in our country, *Phyla nodiflora* has been used for a long time as an herbal remedy both fresh and dried [1].

Numerous studies about the chemical composition, biological activities, and pharmacological effects of *P. nodiflora* have been reported [4]. Phytochemical screening of *P. nodiflora* indicated the presence of alkaloids, glycosides, tannins, flavonoids, saponins, and terpenoids [4]. In traditional medicine, the plant has a bitter taste and is used to treat various ailments such as asthma, bronchitis, and knee pain, and to stimulate internal hemorrhoids, heart disease, and hepatitis [4, 5]. According to Vo Van Chi (2021), *P. nodiflora* is used to clear heat, treat acute tonsillitis, promote diuresis, reduce swelling, treat purulent gingivitis, alleviate pain, treat toothache, detoxify, and cool the blood. By applying crushed fresh plants, *P. nodiflora* is usually used to treat a variety of skin diseases, including boils, burns, shingles, chronic eczema, and purulent dermatitis [6]. The different extracts from *P. nodiflora* have been demonstrated to possess cytotoxic activity against various cancer cell lines, as well as anti-inflammatory, antibacterial, and hepatoprotective activities. The aerial parts of *P. nodiflora* are commonly used as an ingredient in herbal teas to support the treatment of menstrual disorders and infectious diseases [5, 7].

Although various international studies have been carried out on the pharmacological effects and chemical composition of *P. nodiflora*, no publications on the phytochemical investigation and biological activities of this plant grown in Vietnam have been reported. Furthermore, the chemical constituents and pharmaceutical effects of plant extracts might be affected by the extraction solvents. So, the purpose of this research is to determine the influence of different solvents on the chemical profile, antioxidant content, and biological activities of *P. nodiflora* grown in Vietnam. To reach this goal, the solvents chosen for this study were ethanol 90° (100%) and an ethanol-water mixture in the ratios of 7:3, 5:5, and 3:7. The yield of extracts was calculated. The total polyphenol and flavonoid content of each extract was determined. The antioxidant activity of the *P. nodiflora* extracts was investigated by DPPH, reducing potential, and phosphomolybdenum assay. Furthermore, the NO-inhibitory was also determined using the LPS-induced RAW 264.7 macrophage model.

2. MATERIALS AND METHODOLOGY

2.1. Material

The plant *Phyla nodiflora* was collected at Thang Loi commune, Van Giang district, Hung Yen province, Vietnam in May 2023 (Fig. 1). A specimen was scientifically identified by Dr The Cuong Nguyen, Institute of Ecology and Biological Resources, Vietnam Academy of Science and Technology. All aerial parts of *P. nodiflora* were collected and combined for analysis. After preliminary cleaning, the plants were dried in the shade. The voucher specimen (FN-HY-BioTLU2021) is deposited at the Biotechnology Laboratory, Thuyloi University (175 Tay Son Street, Dong Da District, Hanoi, Vietnam). The dried plant material was ground and stored in ziplock bags in a dry, dark place for further analysis.

Fig. 1. Sample *Phyla nodiflora* collected in Hung Yen, Vietnam

2.2. Chemicals

All solvents were purchased from Ducgiang Chemical Company (Hanoi, Vietnam) and used without further purification. Distilled water was prepared in the Biotechnology Laboratory, Thuyloi University (Hanoi, Vietnam) using a filter (Labaqua Bio, Biosan, Latvia). The chemicals used for phytochemical screening such as Na_2CO_3, $FeCl_3$, H_2SO_4, and NaOH were obtained from Xilong, China. The chemicals used for polyphenol and flavonoid total content determination and for biological assay such as aluminum chloride, potassium acetate, potassium ferricyanide, sodium phosphate, TCA, Folin – Ciocalteu reagent, acid gallic, quercetin, acid ascorbic, *et al*. were purchased from Sigma-Aldrich (Chicago, USA).

2.3. Preparation of different extracts from *Phyla nodiflora*

The crude extracts of *P. nodiflora* were prepared by extracting its dried ground powder (100 g) with 300 mL of different solvents (ethanol and ethanol/water mixes) through a process of using an ultrasonic wave three times (Elma S120, Germany). Each time, the procedure was performed at 50ºC for 30 minutes. The filtrates were combined three times and condensed under a reduced pressure (Model R-100, Buchi, Switzerland). The *P. nodiflora* was extracted using food-grade ethanol 90° (100%), as well as ethanol/water mixes at 7:3, 5:5, and 3:7 ratios, resulting in the extracts PN-E100, PN-EW73, PN-EW55, and PN-EW37, respectively. The dried extracts were weighed for yield estimation and then stored at 2-12ºC for further analysis in a refrigerator (Model VH-1009HP3, Sanaky, Vietnam).

2.4. Phytochemical screening and the antioxidant content determination

Phytochemical screening

Qualitative analysis for the presence of biological compounds such as phenolics, flavonoids, carotenoids, terpenoids, coumarins, and saponins was conducted following the method of Yadav *et al*. (2014) [8]with a modification.

Phenolic compounds: A volume of 0.5 mL of *P. nodiflora* extracts was added to a test tube, followed by 1.5 mL of 7.5% Na_2CO_3 solution (*w/w*), 0.5 mL of distilled water, and 2-3 drops of Folin reagent. The mixture was shaken and allowed to stand for 10 minutes. A blue-green or violet color indicated the presence of phenolic compounds.

Flavonoids: A volume of 0.5 mL of *P. nodiflora* extracts was added to a test tube, followed by 1.5 mL of 5% $FeCl_3$ solution (*w/w*). The mixture was shaken and allowed to stand for 10 minutes. A pink color indicated the presence of flavonoids.

Carotenoids: A volume of 0.5 mL of *P. nodiflora* extracts was added to a test tube, followed by 1 mL of concentrated H_2SO_4. The mixture was shaken and allowed to stand for 10 minutes. A blue or green color indicated the presence of carotenoids.

Terpenoids: A volume of 0.5 mL of *P. nodiflora* extracts was added to a test tube, followed by 1.5 mL of 10% H_2SO_4 and 0.5 mL of 70% ethanol. The mixture was shaken and allowed to stand for 10 minutes. A brown-red precipitate indicated the presence of terpenoids.

Coumarins: A volume of 50 µL of extract was mixed with 750 µL of 10% NaOH solution. A yellow color indicated the presence of coumarins.
Saponins: 50 µL of *P. nodiflora* extracts were dissolved in 2 mL of distilled water and a few drops of sunflower oil were added. The mixture was heated at 90°C for 30 minutes. The formation of a milky emulsion indicated the presence of saponins. The sunflower oil was obtained from the Colofic Company, Quang Ninh, Vietnam.

Estimation of total phenolic content
The total phenolic concentration of PN-E100, PN-EW73, PN-EW55, and PN-EW37 was estimated using the method of Ferreira *et al*. (2007) [9]. In brief, 1.0 mL of Folin-Ciocalteu's reagent was mixed with 1.0 mL of the samples and incubated for 10 min at room temperature. Then, 1.0 mL of a 35% Na_2CO_3 solution (*w/w*) was added to each sample, and the final volume was brought up to 10 mL with deionized water. All samples were incubated for 2 hours in the dark before being measured absorbance at 725 nm with a spectrophotometer (Genesys 10S UV-VIS, Thermo Scientific, USA**)**. Gallic acid was used as a standard for the calibration curve. The results were expressed as milligrams of gallic acid equivalent (GAE) per gram of extract.

Estimation of total flavonoid
The flavonoid content of PN-E100, PN-EW73, PN-EW55, and PN-EW37 was determined using the method outlined by Chang *et al*. (2002) [10]. Specifically, 0.5 mL of each extract was combined with 1.5 mL of 95% ethanol, 0.1 mL of 10% aluminum chloride, 0.1 mL of 1 M potassium acetate, and 2.8 mL of distilled water. Subsequently, all samples were incubated in a dark place for 1 h at ambient temperature. Following incubation, the absorbance of each sample at 415 nm was measured against a negative control using a spectrophotometer (Genesys 10S UV-VIS, Thermo Scientific, USA). Quercetin served as a standard for constructing a calibration curve.

2.5. Biological activity assays
Reducing potential assay
For this study, the Oyaizu method, which first appeared in the 1986 modification, was used for estimating the reducing capacity of the *P. nodiflora* extracts (PN-E100, PN-EW73, PN-EW55, and PN-EW37) [11]. The solution was prepared with 2.5 mL of each extract (concentrations ranging from 0 to 300 µg/mL), 2.5 mL of 1% potassium ferricyanide (w/w), and 2.5 mL of 0.2 M sodium phosphate buffer (pH 6.6). All samples were incubated at 50°C for 30 minutes. Additionally, adding 2.5 mL of 10% trichloroacetic acid to each mixture, and centrifuging at 3,000 rpm for 15 minutes were aimed to stop the reaction. Next, 2.5 mL of the supernatant was transferred to a new tube and mixed with 2.5 mL of deionized water and 0.5 mL of 0.1% ferric chloride. The absorbance at 700 nm was measured using a spectrophotometer (Genesys 10S UV-VIS, Thermo Scientific, USA). The extract concentration required to reduce the absorbance by 0.5 (EC_{50}) was calculated from a plot of absorbance at 700 nm against extract concentration. Ascorbic acid served as a positive control.

Antioxidant activity in total (TAA) assay
TAA of the *P. nodiflora* extracts (PN-E100, PN-EW73, PN-EW55, and PN-EW37) was measured using the phosphomolybdenum method [12]. In short, 1 mL of reagent solution (0.6 M sulphuric acid, 28 M sodium phosphate, and 4 mM ammonium molybdate) was mixed with 0.1 mL of each extract (concentrations ranging from 0 to 100 µg/mL). After that, all samples were incubated at 100°C for 1.5 hours before cooling to 25°C and strongly mixing them. The solution absorbance was measured at 695 nm using a spectrophotometer (Genesys 10S UV-VIS, Thermo Scientific, USA), with the negative control serving as a reference. The concentration required to inhibit antioxidant activity by 50% (EC_{50}) was calculated using ascorbic acid as a standard.

DPPH scavenging assay
According to the method described by Okawa *et al.* (2001) [13], the free radical scavenging of *P. nodiflora* extracts was tested by adding the reagent DPPH (1,1-diphenyl-2-picrylhydrazyl) to the solution and incubating it at room temperature for 30 minutes. The absorbance of all samples was measured at 517 nm using a Genesys 10S UV-VIS Spectrophotometer (Thermo Scientific, USA). Ascorbic acid was used as a positive control. The following formula was used to calculate the samples' DPPH radical scavenging activity (I%):

$$I\% = \frac{Ac - As}{Ac} \times 100$$

Where: Ac represents the absorbance of the blank, and As denotes the absorbance of the sample.

Nitric oxide (NO) inhibitory assay
To evaluate nitric oxide (NO) inhibition, the model system LPS-induced RAW 264.7 macrophage was used. RAW 264.7 cells were thawed from nitrogen liquid storage. Cells were cultured in DMEM supplemented with 2 mM L-glutamine, 10 mM HEPES, 1 mM sodium pyruvate, and 10% fetal bovine serum (FBS) in a humidified incubator at 37°C with 5% CO_2 for 3-5 days. Depending on cell growth, they were subcultured at a ratio of 1:3, and then seeded into 96-well plates at a concentration of 2×10^5 cells/well and incubated at 37°C with 5% CO_2 for 24 hours. The growth

culture medium was replaced with fresh DMEM without FBS for 3 h. Then, the medium was replaced with a fresh medium containing the sample in different concentrations, and they were incubated for 2 hours. The negative control contained only the cell culture medium without the sample. For the positive controls, dexamethasone at concentrations of 0.8, 4, 20, and 100 µM were used. Cells were stimulated with 1 µg/mL LPS for 24 hours to induce NO production. Next, 100 µL of supernatant from each well was transferred to a new 96-well plate and mixed with 100 µL of Griess reagent (containing 50 µL of 1% (w/v) sulfanilamide in 5% (*v/v*) phosphoric acid and 50 µL of 0.1% (w/v) N-1-naphthylethylenediamine dihydrochloride in water). After that, all mixtures were incubated at 25ºC for 10 min before measuring the absorbance at 540 nm using a microplate reader and DMEM without FBS as a blank. Following a standard curve of $NaNO_2$, the nitrite concentration in each well was determined, and expressed as a percentage of the negative control (LPS). The following formula was used to calculate the inhibitory activity of the extract samples:

$$\%\,\textbf{inhibitory}\,(\%) = \mathbf{100} - \frac{OD_1 - OD_{blank}}{OD_{DMSO} - OD_{blank}} * \mathbf{100}$$

The EC_{50} value, which is the concentration required to inhibit 50% of NO production, was determined based on the TableCurve 2Dv4 software [14, 15].

2.6. Statistical analysis

Each determination was performed in triplicate. Mean values were presented as mean ± SD. Statistical analysis was done with two-way ANOVA, and Tukey's multiple comparisons test using Prism GraphPad 7.0.

3. RESULTS AND DISCUSSION

3.1. Extraction yield of different extracts from *Phyla nodiflora*

Generally, the extraction yield is influenced by various factors, including the plant material, particle size, and extraction technique (solvent, solvent-to-solid ratio, extraction time, and extraction method). As results in Fig. 2, the highest extraction yield was observed in the 100% ethanol extract (FN-E100) (12.51%), and the lowest extraction yield was observed in the 3:7 ethanol-water extract (FN-EW37) (8.29%). Thus, increasing the ethanol content in the ethanol/water mixture will enhance the extraction yield. Ethanol is well known as an efficient solvent for extracting polyphenols, flavonoids, and other polar compounds from plant tissue [16]. However, in addition to yield, it is essential to evaluate the phytochemical composition and biological activities of the extracts, such as antioxidant and anti-inflammatory to fully assess the potential use of *P. nodiflora* in various fields of the food and pharmaceutical industry.

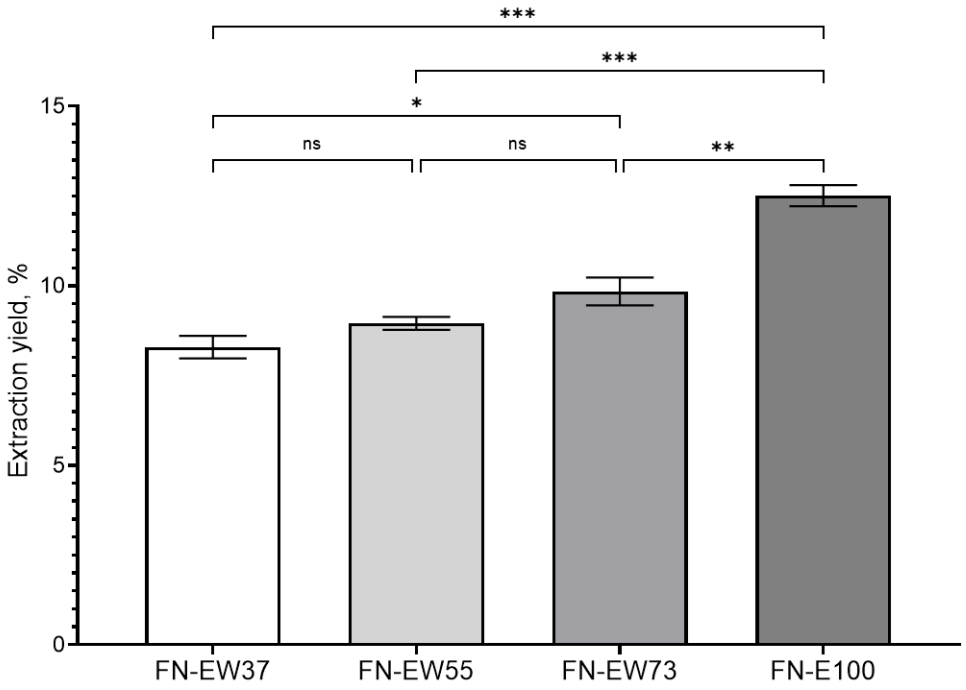

Fig. 2. The yield of different extracts from *P. nodiflora*

(Values are mean ± SD; the symbol " * " among the samples exhibited a significant difference by Tukey's test with $p < 0.05$).

3.2. Phytochemical screening of different extracts from *Phyla nodiflora*

Evaluating of the phytochemical composition is very important because it can greatly affect the biological activities of extracts, such as the antioxidant and anti-inflammatory properties. As shown in Table 1, the preliminary phytochemical screening of the PN-E.100, PN-EW.73, PN-EW.55, and PN-EW.37 extracts from the aerial parts of *P. nodiflora* pointed out the presence of flavonoids, terpenoids, phenolics, and alkaloids.

Table 1: The phytochemical screening of different extracts of *P. nodiflora*

The plant extracts	Biochemicals					
	Polyphenol	**Flavonoid**	**Terpenoid**	**Coumarin**	**Saponin**	**Alkaloid**
PN-E.100	+++	++	+	-	-	+
PN-EW.73	++	++	+	-	-	+
PN-EW.55	+	+	+	-	-	+
PN-EW.37	+	+	+	-	-	+

"+" : Presence; "-": Absence

These compounds were also reported in the different extracts from *P. nodiflora* by Yen *et al.* (2012) [17]. Polyphenols and flavonoids were present in the PN-E100 and PN-EW73 extracts with greater intensity compared to the other extracts. Terpenoids, quinones, and alkaloids were observed at comparable levels in all four samples. On the other hand, courmarins and saponins were absent in all extracts of *P. nodiflora* (Table 1). Consequently, solutions with 100% ethanol and the 7:3 ethanol-water mixture were the most suitable solvents for extracting flavonoids and polyphenols.

3.3. The flavonoid and polyphenol content of different extracts from *Phyla nodiflora*

Flavonoids are secondary plant compounds primarily composed of a benzopyrone ring with phenolic or polyphenolic groups attached at various positions [18]. Flavonoids have a variety of medicinal traits including anticancer, antioxidant, anti-inflammatory, and antiviral effects. Moreover, they possess neuroprotective and cardioprotective properties. These biological activities are influenced by the specific type of flavonoid, its potential mode of action, and its bioavailability [19].

Polyphenols are the major active compounds present in teas and some plants [20], and these compound groups have proven antioxidant, anti-inflammatory, anti-aging, antimicrobial, and supporting activity in solar photoprotection [21].

Total phenolic and flavonoid contents of PN-E100, PN-EW73, PN-EW55, and PN-EW37 samples were calculated in gallic acid and quercetin equivalent with the standard curve equation $y = 0.0066x - 0.0049$ ($R^2 = 0.9902$) and $y = 0.0147x - 0.0236$ ($R^2 = 0.9906$), respectively.

According to results in Table 2, the ethanol extract (FN-E100) of *P. nodiflora* contained the highest total flavonoid content (67.01 ± 2.12 mg QE/g), while the FN-EW73 extract was characterized by the highest polyphenol content among the four extracts. The flavonoid content in the *P. nodiflora* extracts varied with solvent polarity, following the order FN-E100 > FN-EW73 > FN-EW55 > FN-EW37, with corresponding values of 67.01 ± 2.12, 55.69 ± 1.47, 46.65 ± 0.94, 24.53 ± 1.18 mg QE/g, respectively. On the other hand, the polyphenol content in the *P. nodiflora* extracts followed the order FN-EW73 > FN-E100 > FN-EW55 > FN-EW37, which were the corresponding values of 45.46 ± 1.28, 32.44 ± 1.11, 26.48 ± 1.66 and 24.50 ± 1.27 mg GAE/g, respectively.

Table 2: The total phenolic and flavonoid contents of different solvent residues from *P. nodiflora*

Extracts	**FN-E100**	**FN-EW73**	**FN-EW55**	**FN-EW37**
Total phenolics (mg GAE/g)	32.44 ± 1.11^a	45.46 ± 1.28^b	26.48 ± 1.66^c	24.50 ± 1.27^c
Total flavonoid (mg QE/g	67.01 ± 2.12^a	55.69 ± 1.47^b	46.65 ± 0.94^c	24.53 ± 1.18^d

(Values are mean ± SD; different letters within the same raw have a significant difference by Tukey's test with $p < 0.05$).

Most of the previous studies about polyphenol and flavonoid content of *Phyla nodiflora* crude extracts have focused only on the methanolic residue of the aerial part of this plant. According to Sudha *et al*. (2013) [22], the methanolic extract of *Lippia (Phyla) nodiflora* contained high phenolic compounds (98.31 ± 0.004 mg GAE/g), total flavonoids (60.88 ±0.001 mg QE/g), flavonols (27.46 ± 0.002 mg QE/g), total tannin 5.97 ± 0.021 mg TAE/g and saponin 3.52 ± 0.017 mg DE/g.

In conclusion, extracting using methanol results in high flavonoid and polyphenol content. However, methanol, an organic solvent, is extremely toxic even in small amount, and therefore it is not recommended for use in the food industry. The complete removal of methanol from the extract requires advanced techniques. Consequently, two benign solvents ethanol and water are used in functional foods and nutraceuticals.

Based on the flavonoid and polyphenol content of the four extracts mentioned above, we can conclude that ethanol solvent (100%) could be used to create a *P. nodiflora* extract rich in flavonoids. To create an extract with a focus on polyphenols, an ethanol-water solvent mixture at a ratio of 7:3 will be recommended. In the next step, the biological activity of FN-EW73, FN-E100, FN-EW55, and FN-EW37 extracts will be evaluated.

3.4. Biological activity of different solvent extracts of *Phyla nodiflora* extracts

3.4.1. Reducing potential (RP)

Thanks to the simplicity, a reducing potential is one of the most common assays used to evaluate the antioxidant activity of plant extracts and fractions. However, no previous reports have reported the reducing power of ethanol and aqueous extracts from *P. nodiflora*. Therefore, this study investigated the reducing power of different extracts of *P. nodiflora,* with the results shown in Fig. 3. Ascorbic acid was chosen as the standard.

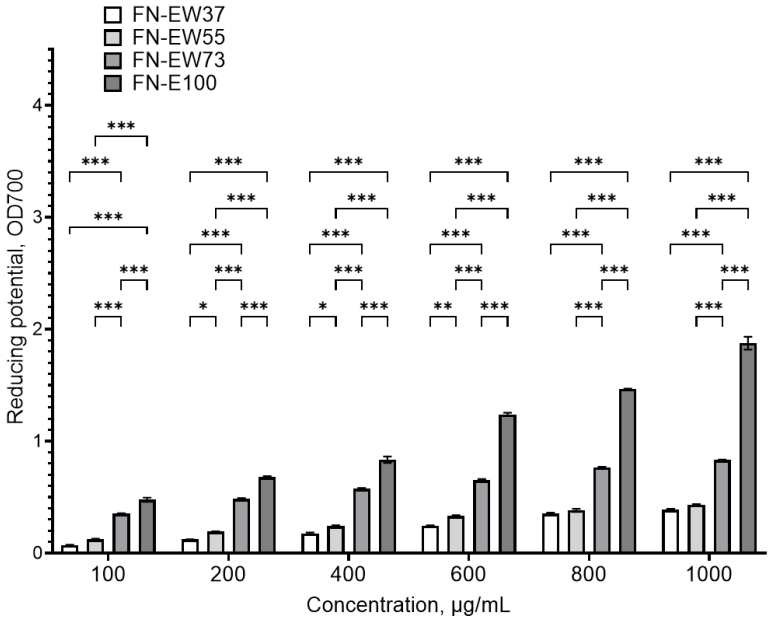

Fig. 3: Reducing potential of different solvent residues from *P. nodiflora*
(Values are mean ± SD; the symbol " * " among the samples exhibited a significant difference by Tukey's test with $p < 0.05$).

The results in Fig. 3 indicated that the RP of FN-E100, FN-EW73, FN-EW55, and FN-EW37 increased with an increase in their concentration. The extract with the highest value of OD_{700} to the fact that this extract has the most RP. The OD_{700} values of FN-E100, FN-EW73, FN-EW55, and FN-EW37 were 0.478, 0.352, 0.124, and 0.072, respectively. So, the RP was significantly affected by the polarity of the solvents.

According to the EC_{50} values in Table 3, the highest value of RP was observed in the FN-E100 with the EC_{50} of 118.86 ± 3.54 µg/mL. The FN-EW73 exhibited the medium RP, and the EC_{50} value was 300.60 ± 10.72 µg/mL. The FN-EW55 and FN-EW37 samples showed smaller values of RC with EC_{50} of 1296.33 ± 15.72 and 1147.50 ± 126.72 µg/mL, respectively. Ascorbic acid has been stable throughout these experiments and displayed strong reducing ability with an EC_{50} value of 65.48 ± 3.75 µg/mL. Ascorbic acid was also used as a positive control to antioxidant activity assay in several published studies [23, 24].

3.4.2. Antioxidant activity by phosphomolybdenum method

The phosphomolybdenum method is commonly used to determine the total antioxidant activity (TAA) of plant extracts, and often expressed as ascorbic acid equivalents [25]. However, the TAA of *P. nodiflora* using this method has not been previously reported. Thus, this study measured and compared them to ascorbic acid.

The results (Fig. 4) revealed that the TAA of ascorbic acid > FN-EW73 > FN-E100 > FN-EW55 > PN-EW37, with the corresponding percentage of TAA at 50 µg/mL were 93.23, 45.68, 33.12, 15.23 and 6.35%, respectively.

The EC_{50} values of ascorbic acid, PN-EW73, PN-E100, and PN-EW55 were 21.23, 69.97, 91.55, and 178.25 µg/mL, respectively. The PN-EW37 sample did not show the TAA within the concentration range of 0-100 µg/mL (Table 3). The notable TAA observed in FN-EW73 and FN-E100 extracts of *P. nodiflora* indicates a high concentration of antioxidants in these fractions, which could be attributable to the presence of phenolic and flavonoid compounds.

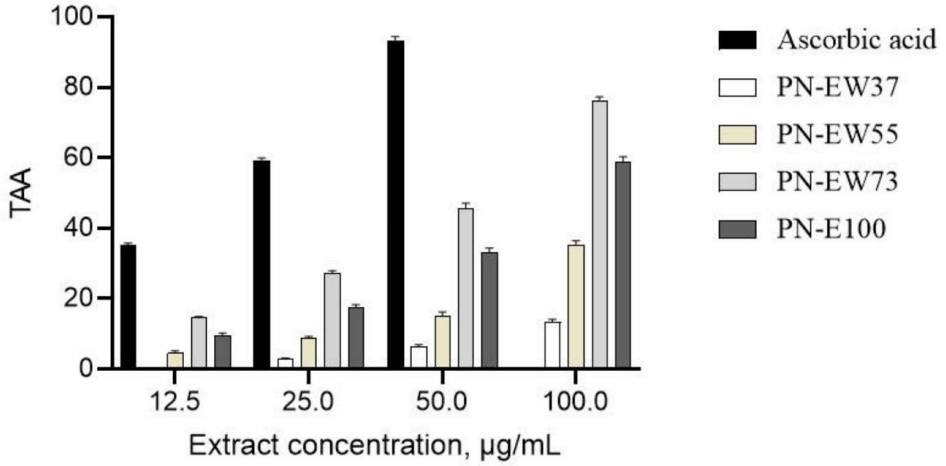

Fig. 4: Total antioxidant activity of different solvent residues from *P. nodiflora*

3.4.3. DPPH radical scavenging activity

Another popular method for evaluating the antioxidant activity of plant extracts and drugs is a free radical scavenging assay, which involves reducing an alcoholic DPPH solution in the presence of a hydrogen-donating antioxidant. Thus, the non-radical form of DPPH is formed, and the color of the samples changes from purple to yellow [26].

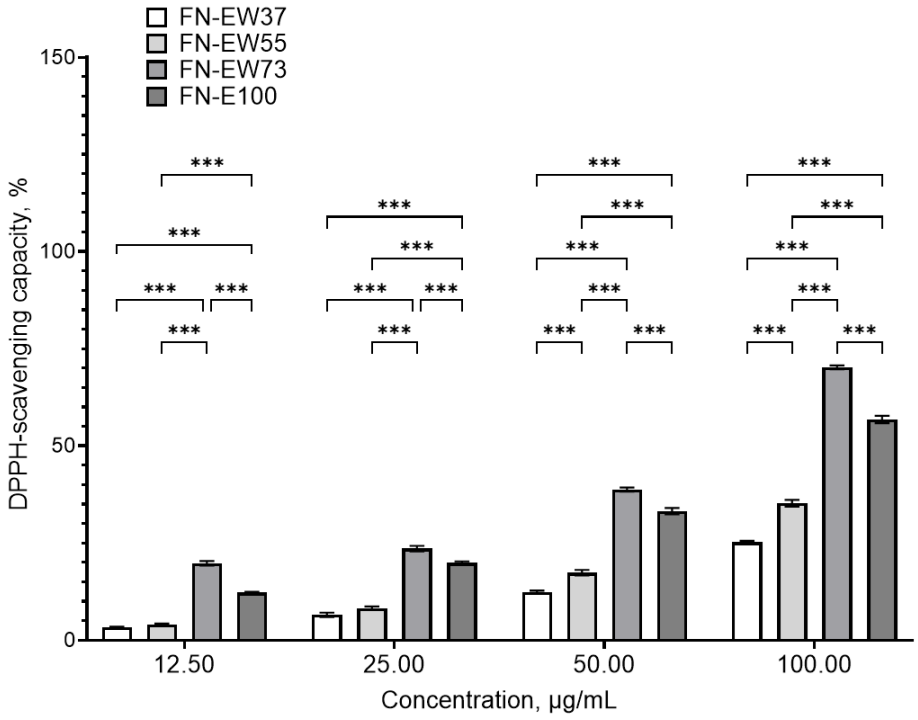

Fig. 5: DPPH scavenging capacity of different solvent residues from *P. nodiflora*

(Values are mean ± SD; the symbol " * " among the samples exhibited a significant difference by Tukey's test with $p < 0.05$).

Fig. 5 showed the scavenging activity of the PN-E.100, PN-EW.73, PN-EW.55, PN-EW.37 extracts on DPPH radical. The scavenging capacity was in the following order PN-EW73 > PN-E100 > PN-EW55 > PN-EW37. At the concentration of 100 µg/mL, the DPPH scavenging capacity values of PN-EW.73, PN-E.100, PN-EW.55, PN-EW.37 were 70.22, 56.78, 35.23 and, 25.23%, respectively. Ascorbic acid was used as positive control and showed significantly higher DPPH radical scavenging activity compared to the four extracts. At the concentration of 6.25 µg/mL, the DPPH ability of ascorbic acid was 50.99%.

Table 3: The EC_{50} values on DPPH radical, TAA, reducing power and NO-inhibition assay of different solvent extracts from *P. nodiflora*

$EC_{50, \mu g/mL}$				
Extracts	**DPPH scavenging assay**	**Phosphomolybdenum assay**	**Reducing capacity assay**	**NO inhibitory assay**
PN-E100	85.49 ± 1.79^{a}	82.82 ± 1.87^{a}	118.86 ± 3.54^{a}	130.19 ± 2.23^{a}
PN-EW73	67.02 ± 1.54^{b}	59.99 ± 1.23^{b}	300.6 ± 10.72^{b}	75.12 ± 1.05^{b}
PN-EW55	141.39 ± 2.35^{c}	144.08 ± 4.12^{c}	1295.33 ± 15.72^{c}	184.68 ± 2.35^{c}
PN-EW37	199.42 ± 2.79^{d}	350.12 ± 5.26^{d}	1147.50 ± 16.72^{d}	-
Ascorbic acid	5.22 ± 0.22^{e}	20.09 ± 1.87^{e}	65.48 ± 3.75^{e}	-
Dexamethasone	-	-	-	16.58 ± 0.68^{f}

("-" – Not active or not tested; values are mean ± SD; different letters within the same column have a significant difference by Tukey's test with $p < 0.05$).

As results in Table 3, the EC_{50} value of DPPH scavenging activity of PN-EW73, PN-E100, PN-EW55, and PN-EW37 samples were 67.02 ± 1.54, 85.49 ± 1.79, 141.39 ± 2.35 and 199.42 ± 2.79 µg/mL, respectively. Only two samples, PN-EW73 and PN-E100, were able to scavenge DPPH radical, having EC_{50} values below 100 µg/mL. Furthermore, the PN-EW73 extract exhibited the highest antioxidant activity. Therefore, the ethanol/water solvent mixture at a ratio of 7:3 was recommended for the preparation of extract with the best DPPH radical scavenging activity.
On the other hand, Shukla *et al.* (2009) [27] reported the DPPH scavenging activity of crude methanolic *P. nodiflora* extract. They found that the extract of the aerial parts exhibited weak activity with an EC_{50} value of 799.74 µg/mL, approximately 8-9 times lower than that of the PN-EW73 and PN-E100 samples, as shown in our results in Table 3. Therefore, *Phyla nodiflora* harvested in Hung Yen, Vietnam, extracted with ethanol or ethanol-water mixture (7:3) provides strong DPPH radical scavenging ability.
Overall, the DPPH radical scavenging activity of PN-E100, PN-EW73, PN-EW55, and PN-EW37 extracts was consistent with the above total antioxidant assay. Based on the results of TAA, RP, and DPPH assays, the PN-EW73 extracts from aerial parts of *P. nodiflora* showed the best outcome related to the high content of phenolics and flavonoids in this extract.

3.4.4. NO inhibition activity

Nitric oxide (NO), which is a signaling molecule, plays a crucial role in the pathogenesis of inflammation associated with cancer. Moreover, NO is considered an anti-inflammatory mediator produced by various cell types, including macrophages, fibroblasts, and hepatocytes. Excessive NO production in pathological conditions is linked to inflammatory responses. NO is synthesized and released into endothelial cells through the catalytic action of nitric oxide synthase (NOs) enzymes, which convert arginine to citrulline during inflammation. If left untreated, cellular inflammatory responses can progress to chronic inflammation [28]. Therefore, the anti-inflammatory potential of the tested samples can be preliminarily assessed by evaluating their ability to inhibit NO production in RAW 264.7 cells. The results of this study are presented in Fig. 6.
The concentration-dependent inhibitory effects of the *P. nodiflora* extracts on NO production were evaluated using linear regression analysis. Among all samples in this study, only the FN-EW73 extract exhibited NO inhibition with the EC_{50} value < 100 µg/mL (75.12 ± 1.05 µg/mL). The EC_{50} values for FN-E100, and FN-EW55 were found of 130.19 ± 2.23, 184.68 ± 2.35 µg/mL, respectively (Table 3). The extract obtained using an ethanol-water solvent mixture (3:7) did not show NO-inhibition activity when the EC_{50} value was less than 200 µg/mL. Dexamethasone, a potent glucocorticoid, served as a positive control and exhibited an EC_{50} value of 16.58 ± 0.68 µg/mL.

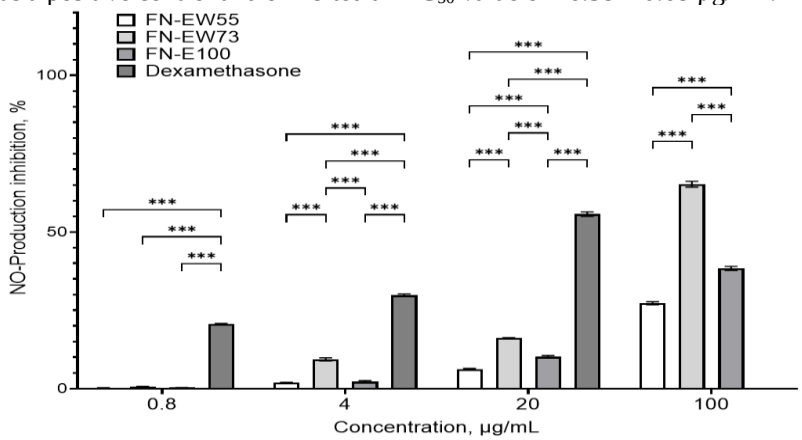

Fig. 6: NO-production inhibition of different solvent residues from *P. nodiflora*
(Values are mean ± SD; the symbol " * " among the samples exhibited a significant difference by Tukey's test with $p < 0.05$).

Based on the results of the antioxidant and NO-inhibition assays, it can be concluded that the ethanol/water solvent mixture (7:3) is the most suitable solvent for extracting bioactive compounds from *P. nodiflora*. Therefore, this plant may be a valuable source of natural compounds with potential therapeutic applications.

CONCLUSION

The influence of extraction solvent (ethanol, ethanol/water with different ratios) on the yield, composition of antioxidants, and biological activity of the *P. nodiflora* extracts was investigated in this work. Phytochemical screening of various extracts from the aerial parts of *P. nodiflora* collected in Hung Yen, Vietnam was tested. The results showed the presence of polyphenols, flavonoids, terpenoids, and alkaloids in *P. nodiflora* extracts. The highest total flavonoid content was found in the ethanolic *P. nodiflora* extract (FN-E100), whereas the FN-EW73 extract of *P. nodiflora* contained the highest polyphenol content. The FN-E100 and FN-EW73 extracts of *P. nodiflora* showed significant activity in reducing potential, DPPH radical scavenging, and phosphomolybdenum assay. Among these extracts, the FN-EW73 sample extracted by ethanol/water (7:3) showed NO inhibition with EC_{50} was 75.12 ± 1.05 µg/mL. As a result, the ethanol/water mixture (7:3) and ethanol (100%) were considered as the most suitable and safe solvents for extracting *P. nodiflora* powder to develop rich antioxidant and anti-inflammatory products, such as instant tea, tablets, animal feed and other preparations. Isolation of the chemical composition and structure of isolated compounds in *P. nodiflora* extracts, as well as formulation of products from those extracts, will be carried out in future research.

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