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Original Research Article

Comparative Analysis of Phytochemicals and Antioxidant Activity of Ethanol Extract of *Centella asiatica* Leaves and its Nanoparticle Form

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ABSTRACT

The high level of flavonoid compounds in pegagan (*Centella asiatica*) that has been found wildly in Indonesia is considered profitable. The aims of this study were to perform comparative analysis of phytochemical and antioxidant activity of pegagan extract (PE) and pegagan nanoparticle form (PN), as well as predict compounds having antioxidant activity through metabolite profiling. *Centella asiatica* leaves were extracted using 70% ethanol. Nanoparticle form was made from PE using ionic gelation method. Antioxidant activity was done using DPPH method, with ascorbic acid as positive control. The metabolite profiling process was done using liquid chromatography – mass spectrometry (LC-MS). The result from phytochemical test showed that there were 50 compounds identified in PE and 43 compounds identified in PN. The antioxidant activity in both samples was indicated by IC_{50} with 56.57 µg/mL for PE 44.67 µg/mL for PN. The findings showed that PN dosage form had better antioxidant activity than PE dosage form, proving that nanoparticle preparations from natural product had potential to be used in pharmaceutical dosage forms. The research concluded that compounds with known antioxidant properties were determined to be responsible for antioxidant activity in *Centella asiatica* leaves from both PE and PN.

***Keywords*:** Antioxidant, *Centella asiatica*, Herbal, Nanoparticles

Introduction

Pegagan (*Centella asiatica*) is one of the plants that are found wild in Indonesia. *C. asiatica* contains high flavonoid and triterpenoid compounds so that this plant is considered to have antioxidant and neuroprotective activity.[1] Usually, the researcher uses nanoparticle processing to overcome the solubility problems of the active substance. Nanoparticles are known to be able to improve the bioavailability of a compound so that it can be optimally absorbed by the body due to the small size.[2] One of the methods of making nanoparticles is ionic gelation. Previous studies found that the method uses ionic interactions between positively charged amino chitosan groups and polyanion in the form of negatively charged sodium tripolyphosphate (STPP) and the results will reduce material size to nano size.[3,4]

The phytochemical properties of pegagan extract (PE) or pegagan nanoparticle form (PN) have not been completely confirmed yet even though *C. asiatica* is proven to have great potential as herbal medication. The aims of this study were to perform comparative analysis of phytochemical and antioxidant activity of PE and PN, as well as to predict compounds having antioxidant activity through metabolite profiling. Metabolite profiling was done using liquid chromatography – mass spectrometry (LC-MS). It has been known that LC-MS is a technique used for metabolite profiling which has improved performance of chromatographic resolution, speed and sensitivity analysis. The technique also is proven to have less time in profiling analysis and reduce solvent consumption.[5]

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Materials and Methods

Plant material

C. asiatica leaves were collected from UPT Materia Medica, Batu, Indonesia, in July 2020, and then identified in the same place with *Interagency Taxonomic Information System* (ITIS) number 29612. The fresh leaves were dried and blended to a fine powder.

Chemicals

The chemicals and reagents were taken from analytical grade and used as receivers. The ethanol as solvent, chitosan, STTP, aquadestilata, ascorbic acid, and DPPH were purchased from Biology Department, Faculty of Medical and Health Science, Maulana Malik Ibrahim State Islamic University. Methanol, acetonitrile, and formic acid as solvent and mobile phase on LC-MS were purchased from Chemistry Laboratory, Faculty of Science and Technology, Maulana Malik Ibrahim State Islamic University, Malang, Indonesia.

Synthesis of PE

The PE used 100 g of *C. asiatica* dry leaves. The substances were macerated using 500 mL of 70% ethanol for 24 h and the mixed compounds were stirred at 140 rpm using magnetic stirrer. This process was repeated to collect all the supernatants. Then, the mixed compounds were evaporated in a rotary evaporator to obtain 70% ethanol of PE.

Synthesis of PN

The PN used 3 mL of glacial acetic acid that was dissolved in 120 mL of distilled water. At the same time, 0.6 mg of STPP was dissolved in

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600 mL of distilled water. After becoming homogeneous, the two solutions then mixed and homogenized again using a stirrer with the addition of 3 mg chitosan and 0.6 mg of PE. Furthermore, the sample was stirred until it was homogeneous using a disperser at a speed of 10.000 rpm for 90 minutes. Then, 5 mL of acetonitrile were added. The sample was sonicated for 90 min at a frequency of 20 kHz and centrifuged for 30 min until the pellet was taken. To obtain nanoparticles, the pellet was incubated in the incubator until dry. Then, the pellet was crushed.

Metabolite profiling with LC-MS

The metabolite profiling was performed through simple, rapid, reliable and precise reversed phase of LC-MS method. The method was developed and validated according to the regulator guidelines. Solid phase extraction was chosen as PE and PN preparation. Then, 1 µL of each sample was injected into a Shimadzu LC-MS – 8040, with MS-focused ion type [M]+. Samples were separated on a Shimadzu Shim Pack FC-ODS (2 mm x 150 mm, 3 µm) column with 90% methanol as isocratic mobile phase and 0.0083 mL/s of flowrate. The desolvation gas flow was 0.02 ml/s at 350°C. The MS used ESI ionization and scanning at 0.6 sec/scan (m/z: 10-2000). The results of LC-MS analysis were processed using PubChem database to obtain the data of peak and m/z spectra of each detected peak.

Preparation of a 0.2 mM DPPH solution

The preparation of DPPH solution was done using 1.97 mg of DPPH powder. The substance was dissolved in 25 mL of ethanol and then placed in a 25 mL volumetric flask and mixed thoroughly.

Determination of the maximum wavelength of DPPH

The determination of the maximum wavelength was done by mixing 1.5 mL of a 0.2 mM DPPH solution with 1.5 mL of ethanol. The absorbance was measured using a spectrophotometer UV-VIS at a 400-800 nm wavelength. The blank solution that had been combined with this determination was used in the analysis process.

Analysis of samples

The analysis of samples process was performed with the use of 2 mg of PE and PN that were dissolved in 20 mL of ethanol. Then, the solutions with concentration of 20, 40, 60, 80, and 100 ppm were made. The next process was to add 1.5 mL of each sample solution to 1.5 mL of 0.2 mM DPPH solution. The mixture was stirred and incubated for 30 min. After incubation, the absorbance of the sample was measured at a wavelength of 517 nm. Ascorbic acid solution as a positive control was made with the same concentration.[6]

Results and Discussion

The development of pharmaceutical dosage form is currently geared to improve the effectiveness and efficiency of medicament. The extraction of PN from PE of *C. asiatica* leaves aims to maintain the quality and quantity of active ingredients in the *C. asiatica* leaves, as well as to overcome the problem of solubility of active ingredients when used orally. Nanoparticles formulation can improve poor bioavailability and absorbance of a macromolecular compound by the body. Furthermore, nanoparticles also improve the pharmacokinetic properties of the constituents and reduce the irritating effect of active substances on the gastrointestinal tract.[2,7] Another advantage of nanoparticle preparation is to make the dose proportional with smaller dosage form as the size of nanoparticles are very small. The small size of nanoparticles can provide advantages in better absorption activity when compared to only extracts. Nanoparticles with a size smaller than 500 nm have an absorption three times greater than extracts with molecular sizes above 1000 nm.[8,9] Nanoparticles can penetrate to the small capillary and are taken up by the cell, being efficient drug accumulation at the target sites in the body. Nanoparticles penetration to the human body can increase therapeutic efficacy, bioavailability and surface area resulting in a faster dissolution of the active agent in an aqueous environment.[8,10]

The PE and PN were analyzed by LC-MS to interpret the diversity of available phytochemicals and potential antioxidant of active compounds. Figures 1 and 2 show PE and PN. Table 1 summarizes all the compounds characterized in PE and PN, including retention times, percentage of area, measured m/z, molecular formula, and predicted compounds. There was a 50 peak of compounds identified in PE and 43 in PN. Seven compounds contained in PE were not found in PN, as seen in Table 1. The seven compounds were flavonoids group, such as luteolin derivates (luteolin-7-O-rutinoside, luteolin-7-glucoside and luteolin-4'-glucoside), apigenin derivates (apigenin-7-rutinoside-4'-transcaffeate, apigenin-7-O-glucoside and apigenin-7-O-rutinoside), and quercetin derivates (quercetin-3-arabinoside). These compounds were not obtained in PN as they might be degraded during the PN preparation.

The other flavonoid compounds detected in both PE and PN were apigenin, luteolin, kaempferol, quercetin and routine. Other compounds, such as triterpenoid compounds, like umbelliferone, asiatic acid, brahmoside, isotankunic acid, madecassic acid, terminolic acid, asiaticoside, centellasaponin, scheffuroside, and betulinic acid, were also detected. The obtained compounds PE and PN had antioxidant activity. Antioxidant activity tests performed on PE, PN, and ascorbic acid as comparator were shown with IC_{50} values, and can be seen in Figure 3.

The best IC_{50} values were sequentially shown by ascorbic acid at 37.93 µg/mL, PN at 44.67 µg/mL and PE at 56.57 µg/mL. *C. asiatica* leaves have antioxidant activity due to the high content of flavonoid compounds, which is shown from the results of LC-MS analysis. Flavonoids are known to have compounds that have high antioxidant activity and are classified as polyphenolic compounds representing the majority of plant secondary metabolites and have been shown to possess remarkable health promoting effects, including antioxidant activity[11]. An antioxidant is a molecule capable of inhibiting the oxidation of another molecule. It breaks the free radical chain of reactions by sacrificing their own electrons, without becoming free radicals themselves. Antioxidants are nature's way of defending cells against attack by reactive oxygen species (ROS).[11,12] In addition, from LC-MS data, it was known that *C. asiatica* leaves also contain other compounds, such as centellasapogenol, centellasaponin, ferulic acid, p-coumaric acid, ascorbic acid, and caffeic acid which have antioxidant properties.[13]

The antioxidant activity was tested using DPPH method, which is very simple and also quick for manual analysis of antioxidant contents. This method can be used for solid or liquid samples and is not only specific to any particular antioxidant, but also applies to the overall antioxidant capacity of the sample. The DPPH analysis is a fast and uncomplicated test thereby ensuring reliable results. Furthermore, it requires only a UV-Vis spectrophotometer to perform, which explains its widespread use in screening antioxidant properties. Reaction with DPPH was adapted for measuring radical quenching kinetics.[11,14]

The IC_{50} comparative analysis results show that PN dosage forms are more active than the PE. The results obtained compared PN and PE antioxidant activity and was found that PN had more antioxidant activity than PE. It can be said that a form of extraction had less antioxidant activity than nanoparticles form. The higher antioxidant activity in PN can be made possible by the effect of particle size. The size of particles can affect antioxidant activity. The smaller the particle size leads to the wider expansion to the surface of a particle. There will be more contact between the particle and free radicals as well.[15]

However, the antioxidant activity of PE or PN is not necessarily caused by the effect of a compound alone. Due to the compounds found in PE and PN, it is possible that antioxidant activity and other activities arise due to the synergistic effect of several compounds at once along with the target receptor. *C. Asiatica* is one of the herbal medicine components. Multiple components of herbal medicine offer great potential for synergistic actions, have better curative effects and perform positive interactions between components by using whole plant extracts.[16,17] Herbal medicines are less expensive than the synthetic drug and useful for the treatment of different diseases. Furthermore, herbal medicines are widely used for enhancing beauty and for curing skin-related issues.[18]

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Table 1: Predicted compounds of PE and PN

No	RT	% Area		m/z	Molecular Formula	Predicted Compounds	PE	PN
		PE	PN					
1	1.684	1.24997	1.39803	162.03	$C_9H_6O_3$	Umbelliferone	√	√
2	1.839	2.73683	3.06101	164.04	$C_9H_8O_3$	p-coumaric acid	√	√
3	3.166	0.10524	0.11771	176.03	$C_6H_8O_6$	Ascorbic acid	√	√
4	3.173	1.19644	1.33817	176.04	$C_{10}H_8O_3$	7-methoxycoumarin	√	√
5	4.643	3.68297	4.11923	180.04	$C_9H_8O_4$	Caffeic acid	√	√
6	5.026	0.52849	0.59109	192.07	$C_{11}H_{12}O_3$	Myristicin	√	√
7	5.043	3.06581	3.42896	194.05	$C_{10}H_{10}O_4$	Ferulic acid	√	√
8	9.365	1.89761	2.12261	270.05	$C_{15}H_{10}O_5$	Apigenin	√	√
9	10.265	4.69566	5.25188	286.04	$C_{15}H_{10}O_6$	Luteolin	√	√
10	10.322	4.08474	4.56858	286.04	$C_{15}H_{10}O_6$	Kaempferol	√	√
11	11.427	2.94681	3.29587	302.04	$C_{15}H_{10}O_7$	Quercetin	√	√
12	12.421	5.27953	5.90491	354.09	$C_{16}H_{18}O_9$	Chlorogenic acid	√	√
13	14.432	3.30663	3.69831	418.09	$C_{20}H_{18}O_{10}$	Kaempferol-3-arabinoside	√	√
14	17.163	0.65401	0.73148	414.38	$C_{29}H_{50}O$	β-sitosterol	√	√
15	20.062	2.41398	2.69992	432.10	$C_{21}H_{20}O_{10}$	Kaempferol-7-rhamnoside	√	√
16	20.063	3.08793	3.45370	432.10	$C_{21}H_{20}O_{10}$	Kaempferol-4'-rhamnoside	√	√
17	20.093	0.85934	-	432.10	$C_{21}H_{20}O_{10}$	Apigenin-7-O-glucoside	√	-
18	21.429	4.07410	4.55669	432.10	$C_{21}H_{20}O_{10}$	Kaempferol-3-O-rhamnoside	√	√
19	21.432	2.04594	-	434.08	$C_{20}H_{18}O_{11}$	Quercetin-3-arabinoside	√	-
20	22.174	3.73710	4.17977	448.10	$C_{21}H_{20}O_{11}$	Quercetin-3-O-rhamnoside	√	√
21	22.185	1.69692	1.89793	448.10	$C_{21}H_{20}O_{11}$	Kaempferol-5-glucoside	√	√
22	22.187	1.32292	-	448.10	$C_{21}H_{20}O_{11}$	Luteolin-4'-glucoside	√	-
23	22.623	2.48447	2.77876	448.10	$C_{21}H_{20}O_{11}$	Kaempferol-3-O-glucoside	√	√
24	22.628	1.69506	-	448.10	$C_{21}H_{20}O_{11}$	Luteolin-7-glucoside	√	-
25	23.199	0.12472	0.13949	450.35	$C_{31}H_{46}O_2$	Phylloquinone (Vit K)	√	√
26	23.517	0.72722	0.81337	456.36	$C_{30}H_{46}O_2$	Betulinic acid	√	√
27	24.001	1.89777	2.12257	463.08	$C_{21}H_{19}O_{12}$	Quercetin-3-glucoside	√	√
28	25.928	5.82484	4.17793	488.35	$C_{30}H_{48}O_5$	Asiatic acid	√	√
29	27.766	1.69719	1.89823	488.35	$C_{30}H_{48}O_5$	Madasiatic acid	√	√
30	27.767	2.51351	2.81125	488.35	$C_{30}H_{48}O_5$	Centellasapogenol A	√	√
31	28.051	2.81238	3.14551	502.36	$C_{31}H_{50}O_5$	Methyl asiatate	√	√
32	28.092	1.52961	1.71080	504.34	$C_{30}H_{48}O_6$	Isothankunic acid	√	√
33	28.097	1.69117	1.89150	504.34	$C_{30}H_{48}O_6$	Madecassic acid	√	√
34	28.099	2.19347	2.45329	504.34	$C_{30}H_{48}O_6$	Brahmoside	√	√
35	28.105	1.29041	1.44327	504.34	$C_{30}H_{48}O_6$	Terminolic acid	√	√
36	31.102	0.27396	0.30641	536.43	$C_{40}H_{56}$	β-carotene	√	√
37	33.439	0.98143	-	578.16	$C_{27}H_{30}O_{14}$	apigenin-7-O-rutinoside	√	-
38	34.003	1.08782	1.21667	594.15	$C_{27}H_{30}O_{15}$	Kaempferol-7-rhamnoside-4'-glucoside	√	√
39	34.01	0.89277	-	740.19	$C_{36}H_{36}O_{17}$	apigenin-7-rutinoside-4'-transcaffeate	√	-
40	34.019	0.70390	-	594.15	$C_{27}H_{30}O_{15}$	luteolin-7-O-rutinoside	√	-
41	35.517	3.37806	3.77821	610.15	$C_{27}H_{30}O_{16}$	Rutin	√	√

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42	49.968	2.41817	2.70461	828.45	$C_{42}H_{68}O_{16}$	Centellasaponin B	√	√
43	62.161	1.53072	1.71203	942.51	$C_{48}H_{78}O_{18}$	Asiaticoside F	√	√
44	62.17	1.89788	2.12269	958.51	$C_{48}H_{78}O_{19}$	Asiaticoside	√	√
45	62.177	0.92920	1.03926	959.51	$C_{48}H_{78}O_{19}$	Centellasaponin C	√	√
46	62.178	0.63501	0.71022	959.51	$C_{48}H_{78}O_{19}$	Centellasaponin D	√	√
47	62.18	0.93048	1.04069	959.51	$C_{48}H_{78}O_{19}$	Centellasaponin A	√	√
48	62.183	1.23013	1.37585	970.51	$C_{49}H_{78}O_{19}$	Scheffuroside B	√	√
49	62.185	0.92927	1.03934	974.50	$C_{48}H_{78}O_{20}$	Asiaticoside B	√	√
50	62.186	1.03017	1.15220	974.50	$C_{48}H_{78}O_{20}$	Asiaticoside A	√	√

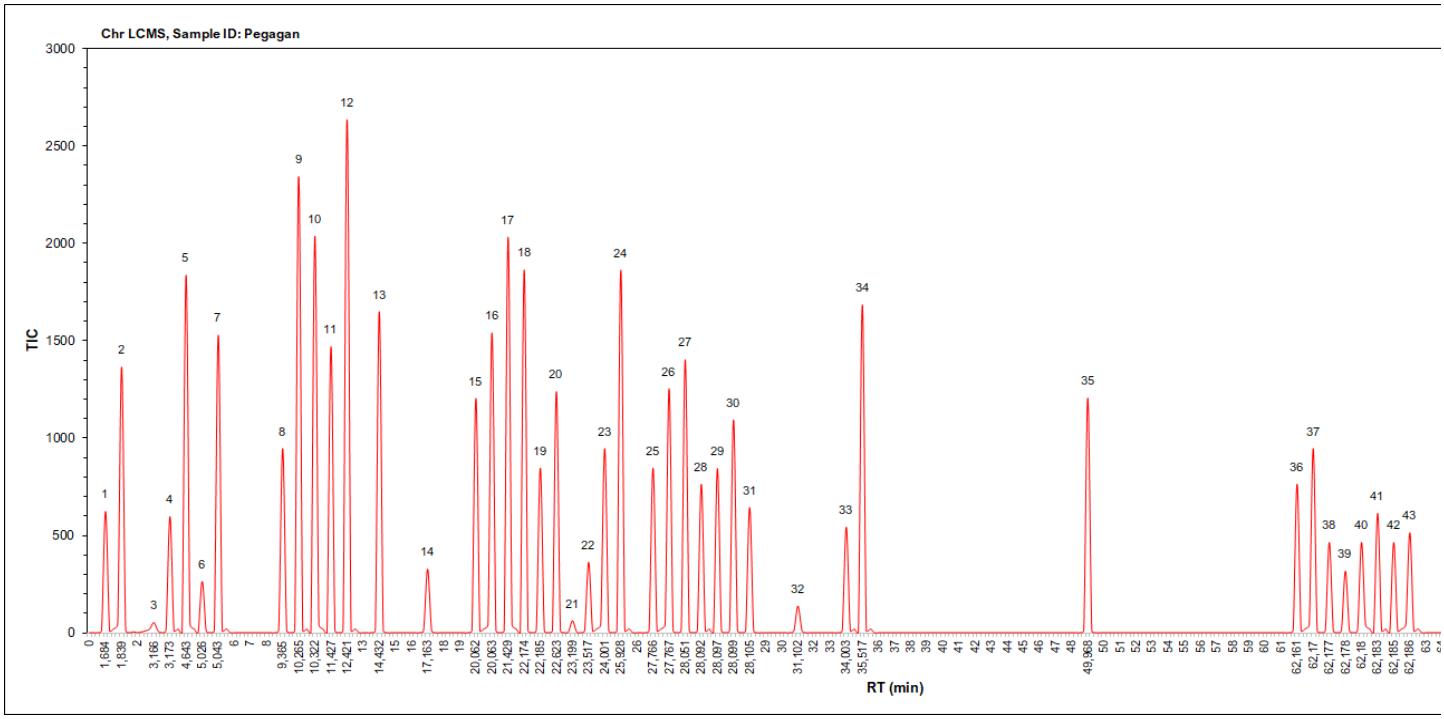

Figure 1: TIC of PE, which have 50 predicted compounds

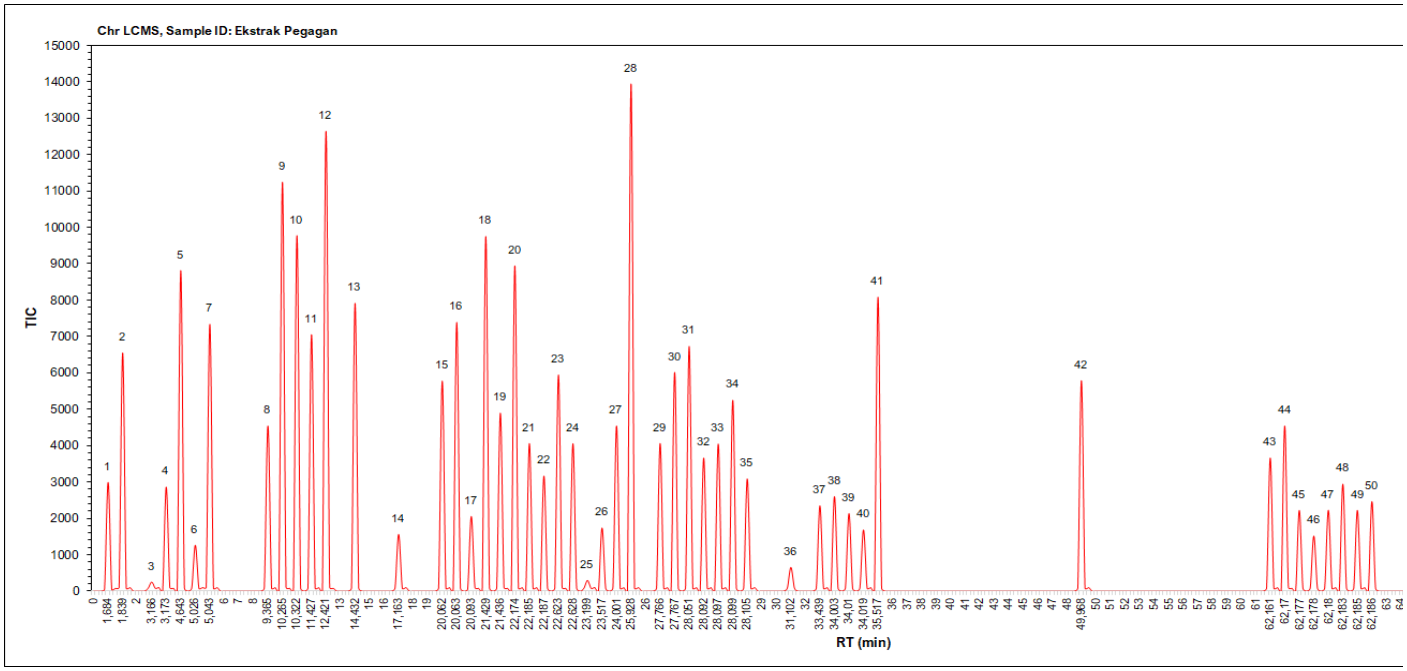

Figure 2: TIC of PN, which have 53 predicted compounds

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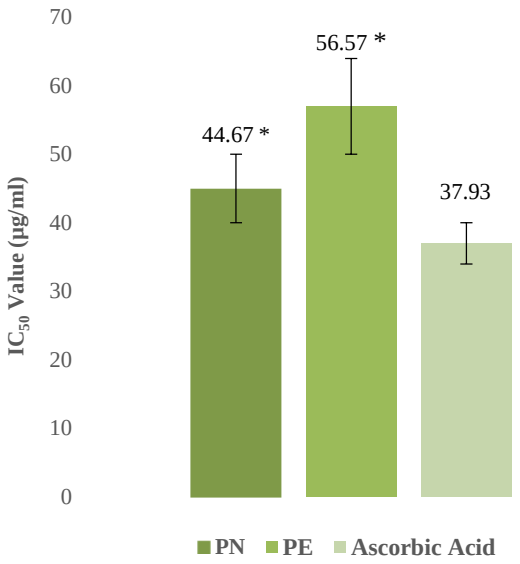

Figure 3: The IC_{50} values of PE, PN, and ascorbic acid. Each value is expressed as mean ± SD. *Significant differences compared to ascorbic acid at $p < 0.05$.

Conclusion

There were 50 compounds identified in PE and 43 in PN from the LC-MS results. The compounds found in *C. asiatica* leaves were known to possess antioxidant properties. The PN dosage form had better antioxidant activity than PE, proving that nanoparticle preparations from herbal medicine have the potential for use in pharmaceutical dosage forms.

Conflict of interest

The authors declare no conflict of interest.

Authors' Declaration

The authors hereby declare that the work presented in this article is original and that any liability for claims relating to the content of this article will be borne by them.

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