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Antioxidant and xanthine oxidase inhibitory activities of rosmarinic acid-rich extract from perilla seed meal

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ABSTRACT: Hyperuricemia can lead to various diseases, including diabetes, renal disorders, and gout. Xanthine oxidase (XO), a crucial enzyme, synthesizes uric acid from xanthine; and concurrently generates reactive oxygen species, causing oxidative stress. This study aimed to validate the biological function of perilla seed meal (PSM), a by-product of cold-pressed perilla oil, concerning phytochemical contents, antioxidants, and XO inhibitory activities. PSM extraction employed 70% ethanol, and the solvent partition technique enriched the rosmarinic acid (RA)-rich fraction. Total phenolic and flavonoid contents, along with antioxidant capabilities, were determined. The reducing property of the PSM fraction was confirmed through the FRAP assay, while HPLC quantified RA. In addition, the inhibitory efficiency of the PSM fraction against XO was investigated. The ethyl acetate (EA) fraction exhibited the highest levels of phenolics, flavonoids, and RA. ABTS and DPPH assays revealed the most effective radical scavenging by the EA fraction, with IC_{50} values of 6.51 ± 0.11 µg/l and 20.43 ± 0.18 µg/l, respectively. Moreover, the EA fraction demonstrated the highest FRAP value of 3778.05 ± 6.8 µM Fe^{2+}/g. The XO inhibition of the EA fraction exhibited significant inhibition compared to the ethanol extract. Mixed-type inhibition of XO was identified in PSM. Our results suggest that PSM exhibits high phytochemical compounds, antioxidant capacities, and XO inhibition, meriting further exploration for clinical applications.

KEYWORDS: perilla seed, antioxidant, xanthine oxidase, inhibitory effect, rosmarinic acid, flavonoids

INTRODUCTION

Gout is a pathological disorder of joint inflammation occurring due to the increased formation of uric acid by the catalytic oxidation of purine nucleotides to hypoxanthine, xanthine, and then uric acid [1]. The production of uric acid is a catalytic reaction using xanthine oxidase (XO) with the formation of reactive oxygen species (ROS) [2]. Hyperuricemia leads to uric acid crystallization as well as ROS formation, causing damages and excruciating effects on joints, tendons, and surrounding tissues in gouty arthritis patients [3]. Hyperuricemia is an adverse risk factor that may lead to kidney stone formation, atrium injury, atrial fibrillation, cardiac arrhythmia, stroke, and heart failure [4]. Several drugs, including synthetic allopurinol, a hypoxanthine isomer purine analog, have been clinically used to treat gout and arthritis by inhibiting XO activity [5]. Moreover, some natural XO inhibitors from plants have been found to be effective in ameliorating gout conditions [6]. Perilla, or *Nga-mon* in Thai, is a common herb grown in northern Thailand [7]. It belongs to the Lamiaceae family, with many varieties, such as *P. frutescens var. crispa*, *P. frutescens var. frutescens*, and *P. frutescens var.* acuta, cultivated in Asian countries. Our laboratory previously reported that perilla leaves and seeds contained high levels of rosmarinic acid (RA), a powerful antioxidant and anti-inflammatory compound [8]. The aim of the present study was to determine the antioxidant capacity and XO inhibitory effect of RA-enriched extract from perilla seed meal (PSM). Here is the first report of the PSM fraction with XO inhibitory activity and the investigation on the type of inhibition.

MATERIALS AND METHODS

PSM preparation and extraction

Perilla seed was harvested from perilla plants that were cultivated in Phayao Province. The voucher specimen of the plant material was prepared by Asst. Prof. Dr. Wittaya Chaiwangyen and certified by Sanga Saphasri Research and Development Center, the Botanical Garden Organization, Ministry of Natural Resources and Environment, Thailand, under the herbarium code QBG-93756.

The residual PSM from oil removal was dried and ground. One hundred grams of dried PSM was extracted with 1 liter of 70% ethanol for 12 h at room temperature. The extract was filtered through a paper filter, then evaporated using a vacuum rotary evaporator (BUSHI, Switzerland), and dried by a lyophilizer (Scanvac Coolsafe, Denmark). The PSM ethanol extract was stored at −20 °C until further use.

Liquid-liquid extraction of PSM ethanol extract

To prepare the RA-rich fraction, the PSM ethanol extract was solubilized in water:n-hexane mixture and sequentially extracted by solvent partition technique using n-hexane (Hex), dichloromethane (DCM), ethyl

acetate (EA), and water fractions, respectively [8]. These four extracts with different solvent compositions were further studied.

Determination of total phenolic content

Total phenolic content (TPC) was determined by Folin-Ciocalteu assay [9]. Briefly, the dilution of the individual four extracts with different solvent compositions were incubated with 10% Folin-Ciocalteu reagent and 7.5% sodium carbonate for 30 min at room temperature. The reaction was measured at 765 nm. Gallic acid was used as a standard. TPC was expressed as milligrams of gallic acid equivalent (GAE) per gram extract. All the samples were examined in triplicate.

Determination of total flavonoid content

Total flavonoid content (TFC) was examined by aluminum chloride colorimetric method [10]. Briefly, each extract was incubated with 75 µl of 5% $NaNO_2$ in the dark at room temperature for 5 min. After that, 150 µl of 10% $AlCl_3$ and 500 µl of 1 M NaOH were added and adjusted the volume up to 2.5 ml with deionized water, then further incubated for 10 min at room temperature. The absorbance was measured at 510 nm. The TFC of the samples was expressed as milligrams of catechin equivalent (CE) per gram extract. All the samples were examined in triplicate.

Determination of rosmarinic acid (RA) content

The content of RA in all fractions was determined using high-pressure liquid chromatography/ultraviolet detection (HPLC/UV) method, as described previously [9]. Dried PSM extract from the solvent partition technique was dissolved in HPLC-grade methanol and filtrated through a 0.22 µm filter. Samples were injected into the reverse-phase ZORBAX Eclipse plus C18 column (4.6 mm × 150 mm, 5 µm particle diameters). The mobile phase consisted of 0.1% trifluoroacetic acid (TFA) in acetonitrile with a flow rate of 1.0 ml/min. A UV detector was used to identify the RA at wavelength 280 nm. The peak area and retention time of the fractions were analyzed by comparing them with the RA standard and represented as mg/g of the extract. The highest RA-containing fraction was selected for further experiments.

DPPH radical scavenging assay

Free radical scavenging activity of the PSM extracts were determined by quenching the stable 1,1-diphenyl-2-picryl hydrazyl radical (DPPH) [10]. In brief, 20 µl each of the extracts and the Trolox (6-hydroxyl-2,5,7,8-tetramethylchroman-2-carboxylic acid) standard were incubated with DPPH solution (177.5 µM) for 30 min in the dark at room temperature. The absorbance was measured at 515 nm. The percentage of DPPH radical scavenging activity was calculated using the following Eq. (1):

$$\text{DPPH radical scavenging (\%)} = \frac{A_0 - A_s}{A_0} \times 100 \quad (1)$$

when A_0 = DPPH solution absorbance and A_s = sample absorbance.

ABTS radical scavenging assay

The activity of radical scavenging activity was determined by decolorization of ABTS cationic radical (2,2'-azinobis (3-ethylbenzothiazoline-6-sulfonic acid) radical, $ABTS^{\bullet+}$) assay [10]. The radical was developed by incubating 7.5 mM ABTS solution and 2.45 mM of potassium persulfate oxidant in the dark at room temperature for 12–16 h. The $ABTS^{\bullet+}$ solution was diluted with deionized water until the absorbance reached 0.7 ± 0.02 at 734 nm. For the sample test, the extracts and the Trolox standard were incubated with $ABTS^{\bullet+}$ for 6 min in the dark at room temperature. The absorbance was then measured at 734 nm. The radical scavenging activity was expressed as a percentage of inhibition, determined using the following Eq. (2):

$$ABTS^{\bullet+}\text{scavenging (\%)} = \frac{1 - A_s}{A_0} \times 100 \quad (2)$$

when A_0 = $ABTS^{\bullet+}$ solution absorbance and A_s = sample absorbance.

Determination of ferric ion reducing antioxidant power (FRAP)

FRAP assay was used to determine the ability of the PSM extract in reducing the ferric tripyridyltriazine (Fe^{3+}-TPTZ) salt solution to Fe^{2+}-TPTZ form. The absorbance of the blue color Fe^{2+}-TPTZ was measured at 593 nm [11]. The FRAP value was expressed as µM equivalents of ferrous ion/g of PSM extract.

Determination of XO inhibitory activity

The XO inhibitory activity of the PSM ethanol and the EA extract were determined by separately adding different concentrations of the two extracts to 0.1 M phosphate buffer and XO (0.2 U/l) and incubated for 5 min at 25 °C. Then, 1 ml of xanthine substrate solution (0.15 mM) was added. The mixtures were further incubated for 10 min, then the reactions were suspended by 1M HCl and the absorbance of the individual reaction products was measured at 295 nm [12]. Allopurinol was used as positive control. The percentage of inhibition was calculated by the following Eq. (3):

$$\text{XO inhibition (\%)} = \frac{1 - A_s}{A_0} \times 100 \quad (3)$$

when A_0 = absorbance with no sample and A_s = sample absorbance.

Determination of XO inhibitory type

The EA fraction having the highest XO inhibitory activity was used to verify its type of action. The enzyme reaction was performed with different concentrations of EA extract (0–25 µg/l) and particular xanthine substrate (100, 200, 400, 600 µM) as described above. The Lineweaver-Burk plot was created by plotting $1/\nu$ (ΔOD/min) against 1/[xanthine]. The K_m and V_{max} values were determined by plotting a graph [13].

RESULTS

Quantitative determination of total phenolics, total flavonoid, and rosmarinic acid

The ethanolic extract of the PSM was partitioned with Hex, DCM, EA, and water, respectively. The sequential extraction yield of PSM fractions was shown in Table 1. The highest yield of extract was in water fraction (29.63) followed by Hex (12.29), EA (6.14), and DCM (3.13) fractions. Accordingly, total phenolics, flavonoids, and RA contents were determined. As shown in Table 2, the EA fraction depicted the highest value in TPC (48.32 ± 0.43 mg GAE/g extract), TFC (37.62 ± 0.43 mg CE/g extract), and RA (436.04 ± 52.37 mg/g extract). However, the Hex fraction showed the lowest contents in both TPC (3.01 ± 0.05 mg GAE/g extract) and TFC (1.01 ± 0.02 mg CE/g extract). Hence, our results demonstrated that EA was able to dissolve the highest amount of phenolics, flavonoids, and RA in the PSM.

Quantification of rosmarinic acid rich extract

From HPLC analysis, the RA standard depicted the major peak at a retention time of around 4.160 min (Fig. 1A). The RA content in 70% ethanolic extract was 67.81 ± 3.12 mg/g extract (Fig. 1B). The RA content in Hex, DCM, EA, and water fractions was 42.24 ± 1.15, 24.91 ± 0.44, 436.04 ± 52.37, and 36.16 ± 0.62 mg/g extract, respectively. As indicated in Table 3 and Fig. 1 (A–E), it could be summarized that the EA fraction contained the highest concentration of RA. This fraction was termed an RA-rich extract for further determination of its biological activities.

Free radical scavenging activity and reducing property of EA fraction from PSM

Free radical scavenging activity of the fractions was determined by DPPH and ABTS assay. Among the fractions, the EA fraction showed the lowest IC_{50} in scavenged DPPH (20.43 ± 0.18 µg/l) and ABTS radical (6.51 ± 0.11 µg/l), but the highest FRAP value (3778.05 ± 6.8 (µM Fe^{2+}/g) (Table 2). In contrast, the Hex fraction exhibited the highest IC_{50} value of DPPH and ABTS scavenging activities. The results emphasized the semi-polar property of the EA fraction containing the strongest activity in free-radical scavenging and reducing properties.

Xanthine oxidase Inhibition Activity

The XO activities of EA fraction, ethanol extract, and perilla oil were determined. As shown in Fig. 2, the EA fraction at 200 µg/l exhibited a higher percentage of inhibition at 88.47 when compared to ethanol extract. The EA fractions demonstrated significantly higher inhibition activity than the ethanol extract ($p < 0.01$). However, perilla oil (250–2000 µg/l) has a lower inhibitory activity of XO as shown in Fig. 3. Allopurinol, a positive control exhibited the highest percentage inhibition at a value of 100. These results confirmed that the EA fraction of PSM has a potential inhibition effect on XO activity.

Xanthine oxidase inhibitory type of action

According to the XO activity, the EA fraction was further analyzed for the type of enzyme inhibition. Lineweaver-Burk plots depicted a decrease in V_{max} values but an increase in K_m values with increasing concentration of the EA fraction, as shown in Fig. 4. This finding of alteration indicated that the inhibition pattern of EA fraction against XO was a mixed-type inhibition. The pattern suggested that flavonoids in the EA fraction might bind to other sites of the XO enzyme rather than directly to the active site.

DISCUSSION

Medicinal plants have been developed as supplements for the treatment of hyperuricemia, a common cause of gout, urinary stone formation, and other health problems [14, 15]. One of the widely used food supplement from plants is perilla seed [16]. After oil removal from the perilla seed by cold compression, the waste meal was found to contain high levels of compounds such as RA, apigenin, and luteolin [17]. The extract was initially prepared using 70% ethanol, chosen for its effectiveness in extracting RA, a polar and polyphenolic compound. It has been suggested that 55–75% ethanol could be optimal for extracting RA. This solvent was selected for its ability to dissolve a wide range of phytochemicals, including phenolics and flavonoids, which are known for their high antioxidant activity [18–20].

Our study was to determine RA content in PSM and its inhibitory effect against free radical capacity and XO activity. The partitioning technique from nonpolar solvents to polar solvents was applied to get an enriched RA fraction from the PSM [8]. The HPLC profile revealed that the EA fraction contained the highest peak of RA when compared with other solvent fractions. The findings of our study that solvent partitioning with EA effectively enriched RA was consistent with previous reports [21–23]. Since EA is more effective than ethanol in dissolving bioactive compounds, including phenolics and flavonoids [24, 25], we also found that the EA fraction contained the highest levels of these compounds.

Table 1 Percentage yield of the PSM fractions. The PSM ethanol extract was solubilized in water:hexane mixture and sequentially extracted by solvent partition technique by using Hex, DCM, EA, and water fractions, respectively.

Fractions extracted by solvents	Starting weight (g)	Obtained weight (g)	Yield (%)
70% Ethanol	1000	51.0	5.10
n-Hexane (Hex)	10	1.23	12.29
Dichloromethane (DCM)	10	0.31	3.13
Ethyl acetate (EA)	10	0.61	6.14
Water	10	2.96	29.63

Table 2 Total phenolics, total flavonoid, and rosmarinic acid contents of the PSM extracts.

PSM extracts by different solvents	Total phenolics (mg GAE/g extract)	Total flavonoid (mg CE/g extract)	Rosmarinic acid (mg/g extract)
70% Ethanol	12.15 ± 0.03	5.78 ± 0.05	67.81 ± 3.12
n-Hexane	3.01 ± 0.05	1.01 ± 0.02	42.24 ± 1.15
Dichloromethane	12.11 ± 0.03	2.59 ± 0.16	24.91 ± 0.44
Ethyl acetate	48.32 ± 0.43	37.62 ± 0.43	436.04 ± 52.37
Water	6.76 ± 0.65	3.09 ± 0.22	36.16 ± 0.62

The results are expressed as mean ± SD of triplicate samples. GAE = gallic acid equivalent; CE = catechin equivalent.

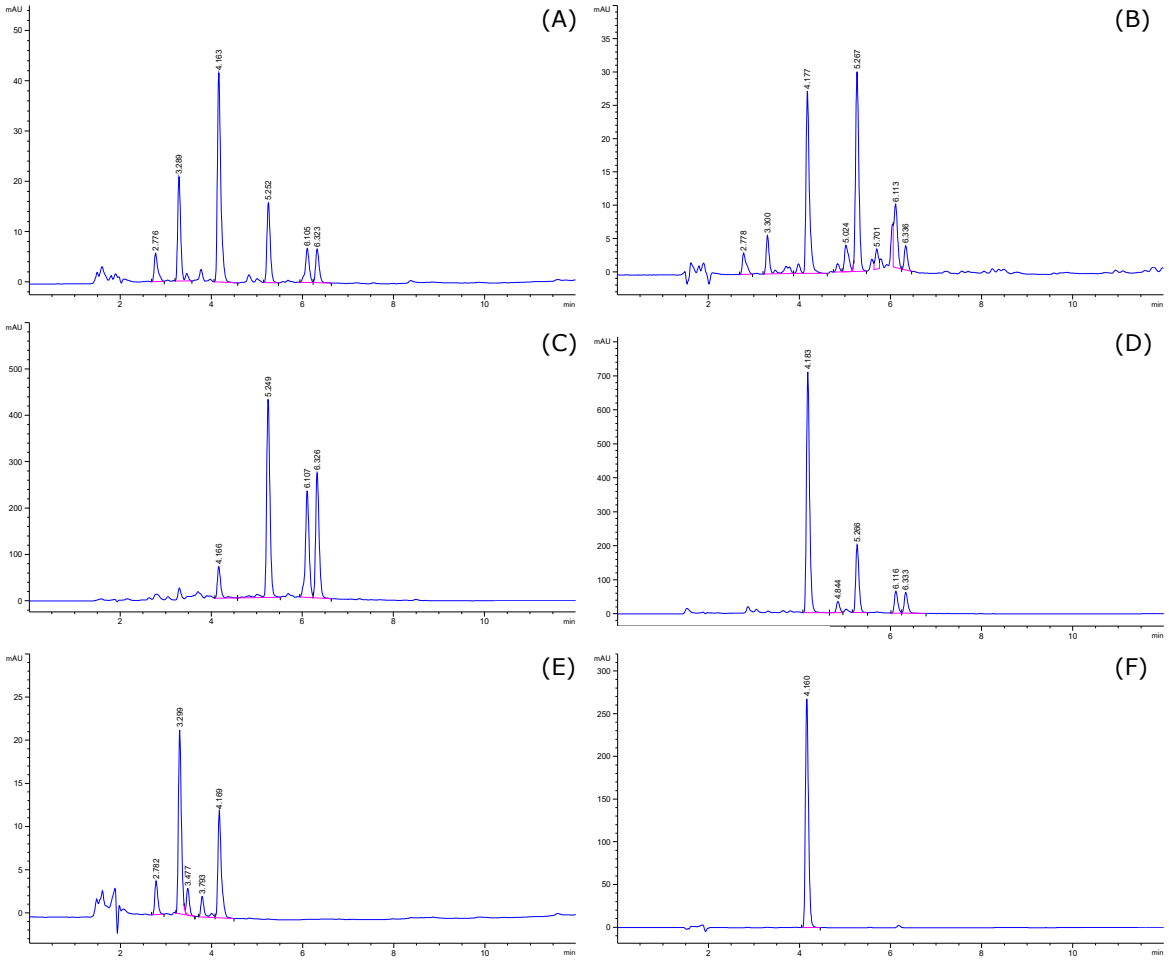

Fig. 1 HPLC chromatograms of the PSM extract fractions: (A), 70% Ethanol; (B), Hex; (C), DCM; (D), EA; (E), water; and (F), RA standard.

Table 3 Determination of antioxidant activities of the PSM extracts by different methods.

PSM extracts by different solvents	IC_{50} value of radical scavenging activities (µg/l)		FRAP value (µM Fe^{2+}/g)
	DPPH	ABTS	
Hexane	505.91 ± 2.53	243.80 ± 0.37	245.80 ± 8.17
Dichloromethane	31.43 ± 0.56	6.84 ± 0.40	126.77 ± 5.04
Ethyl acetate	20.43 ± 0.18	6.51 ± 0.11	3778.05 ± 6.86
Water	433.05 ± 3.85	67.16 ± 2.47	632.72 ± 3.12

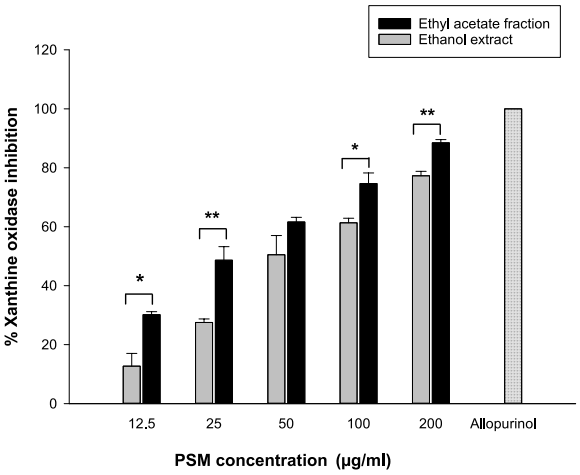

Fig. 2 Xanthine oxidase inhibition of ethyl acetate fraction and ethanol extract from perilla seed meal. Allopurinol (75 µg/ml) was used as a positive control. The results were expressed as mean ± SD of triplicate. * $p < 0.05$, ** $p < 0.01$ when compared with each group.

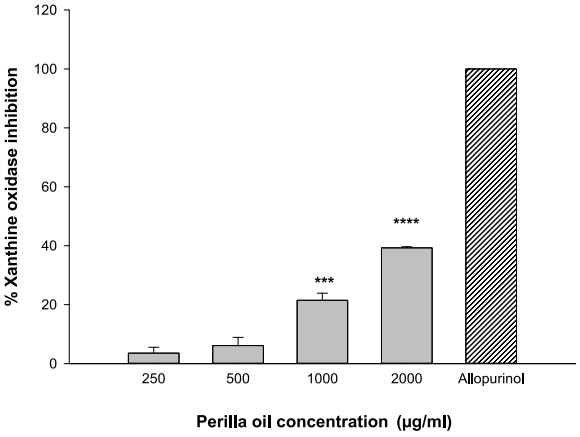

Fig. 3 Xanthine oxidase inhibition of perilla oil. Allopurinol (75 µg/ml) was used as a positive control. The results were expressed as mean ± SD of triplicate. *** $p < 0.001$ when compared with perilla oil at 250 µg/l.

Moreover, the EA fraction showed more potent inhibitory effects on both free radical scavenging and XO activities than the other fractions, in correlation with its RA content. In addition, EA fraction from perilla leaves with high RA content showed the highest activity in free radical scavenging and reducing power [26]. A similar study in another herb, *B. ciliata*, with antioxidant and a-glucosidase inhibitory activity and ethyl acetate extraction of *M. napaulensis* herb yielded the highest phenolics, free radical scavenging activities, and reducing power [27, 28]. We also found that RA from PSM scavenged superoxide anions which were generated during uric acid production. It suggested that RA was responsible for the antioxidant property and XO inhibition. Previous studies showed that RA in medicinal plants, such as *R. officinalis* and *P. saccatus*, possessed high potent activity in reducing ROS and uric acid content [29, 30].

The biological activities of RA from perilla seed have been reported to include anti-allergic, anti-inflammatory, and antidepressant properties [31]. The RA-rich fraction of EA from perilla seed efficiently suppressed receptor activator of nuclear factor kappa-B ligand (RANKL)-mediated osteoclast maturation [32].

We found that the EA extract clearly showed higher XO inhibition activity as compared with the ethanolic extract indicating the EA extract had a higher content of flavonoids including RA, caffeic acid, and apigenin [9, 33]. The type of inhibition action was a mixed-type, as confirmed by the Lineweaver-Burk plot. As previously reported, the perilla leaves extract with different flavonoid components demonstrated various types of action including mix-typed inhibition and competitive inhibition [34, 35]. The inhibitory effect of PSM extract on XO activity had never been determined and reported; therefore, our report showing the PSM's mixed-type inhibition of XO activity was considered as a novel finding. As well-known XO inhibitors, allopurinol and febuxostat have distinct mechanisms of action. Allopurinol acts as a competitive inhibitor by binding to the active site of the enzyme, which increases the enzyme's affinity for its substrate (K_m) without affecting the maximum velocity (V_{max}) of the reaction. In contrast, febuxostat functions as a non-competitive inhibitor, binding to a site other than the active site and thereby reducing V_{max} without altering K_m [36]. Our findings of mixed-type inhibition of XO, where the inhibitor interacted with a site distant from the

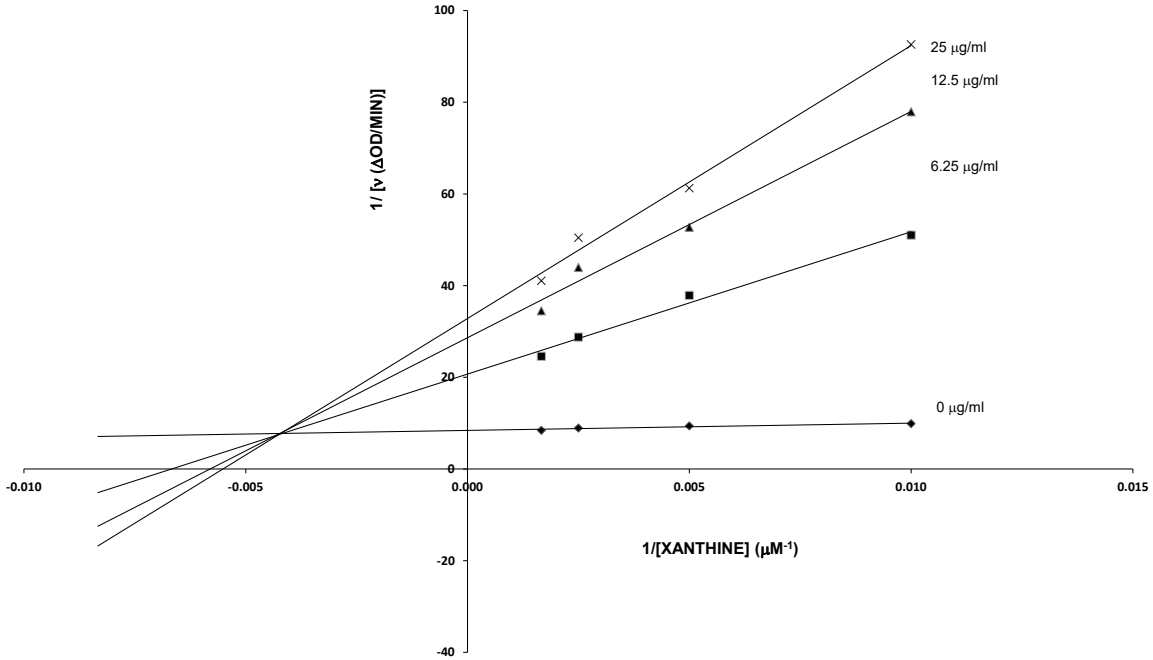

Fig. 4 Lineweaver-Burk plots for the kinetic analysis of xanthine oxidase activity inhibited by the PSM's EA fractions of different concentrations, 0 to 25 μg/ml.

active site, suggested a more versatile mechanism of action. Mixed-type inhibitors affect both the enzyme's affinity for its substrate and its catalytic turnover rate, leading to a more comprehensive and potent inhibition compared with other known XO inhibitors.

The flavonoids in the EA fraction might bind to sites other than the active site of the XO enzyme. This was supported by the observation of mixed-type inhibition (Fig. 4), where the inhibitor affected both the enzyme's affinity for the substrate and its catalytic turnover rate. Unlike competitive inhibitors, which bind directly to the active site, mixed-type inhibitors interact with both the enzyme and the enzyme-substrate complex, implying that they might bind to allosteric sites or other regions of the enzyme that influence its overall activity. The ability of flavonoids to cause such mixed-type inhibition indicated that they likely interacted with XO at sites distinct from the active site, modulating the enzyme's function in a more complex manner than simple competitive or non-competitive inhibition [37, 38]. The XO inhibition of the EA extract brought about the reduction of free radicals and uric acid formation [39].

The finding of this study suggested that PSM could be used in combination with standard drugs for the treatment of hyperuricemia and gouty arthritis.

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