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Effect of Piperine and Its Derivatives on Peroxidase Enzyme

Piperin ve Türevlerinin Peroksidaz Enzimi Üzerine Etkisi

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ABSTRACT

In this study, the effects of piperine, a molecule isolated from the black pepper (*Piper nigrum*) plant, and its three derivatives piperic acid, piperic acid ethyl ester, and piperic acid methyl ester on peroxidase enzyme activity were investigated. Piperine is the primary bioactive compound of black pepper, which is traditionally used for spice and medicinal purposes. The objective of this research was to evaluate the impact of these four molecules on peroxidase activity. First, piperine was purified from black pepper and piperic acid, piperic acid ethyl ester and piperic acid methyl ester were synthesized by chemical methods. The activation effects of these compounds on peroxidase enzyme activity were assessed in vitro through spectrophotometric analysis. The results showed that each molecule significantly increased peroxidase activity. It was determined that derivatives of piperine showed a higher activation effect compared to piperine. These results indicate that piperine and its derivatives hold promise for potential applications in biotechnology and plant health, particularly in enhancing plant defense mechanisms. The results of the study suggest that piperine and its derivatives may be evaluated as natural peroxidase activators, which can be used especially in areas such as plant stress management and disease resistance.

Key Words

İsolation; derivatization; piperine; peroxidase; enzyme activity.

ÖZ

Bu çalışmada, kara biber (*Piper nigrum*) bitkisinden izole edilen piperin molekülü ve onun üç türevi piperik asit, piperik asit etil ester ve piperik asit metil ester üzerinde peroksidaz enzimi aktivitesinin etkileri incelenmiştir. Piperin, karabiberin birincil biyolojik aktif bileşenidir ve geleneksel olarak baharat ve tıbbi amaçlar için kullanılmaktadır. Bu araştırmanın hedefi, bu dört molekülün peroksidaz aktivitesi üzerindeki etkisini değerlendirmektir. Öncelikle, piperin karabiberden saflaştırıldı ve piperik asit, piperik asit etil ester ve piperik asit metil ester kimyasal yöntemlerle sentezlendi. Bu bileşiklerin peroksidaz enzimi aktivitesi üzerindeki aktivasyon etkileri, spektrofotometrik analiz yoluyla in vitro olarak değerlendirildi. Sonuçlar, her molekülün peroksidaz aktivitesini önemli ölçüde artırdığını gösterdi. Piperin türevlerinin piperine kıyasla daha yüksek bir aktivasyon etkisi gösterdiği belirlendi. Bu sonuçlar, piperin ve türevlerinin biyoteknoloji ve bitki sağlığı alanında, özellikle bitki savunma mekanizmalarını güçlendirme konusunda potansiyel uygulamalar için umut vaat ettiğini göstermektedir. Çalışmanın sonuçları, piperin ve türevlerinin özellikle bitki stres yönetimi ve hastalık direnci gibi alanlarda kullanılabilen doğal peroksidaz aktivatörleri olarak değerlendirilebileceğini göstermektedir.

Anahtar Kelimeler

İzolasyon; türevlendirme; piperin; peroksidaz; enzim aktivitesi.

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INTRODUCTION

Throughout history, natural products produced by plants, animals and microorganisms have served as a basis for in the discovery and development of modern medicine [1,2]. Natural products obtained from plants exhibit remarkable chemical diversity, encompassing a wide range of bioactive compounds. These natural products can be isolated and used directly, or they can serve as key molecules for the synthesis of more effective molecules. The synthesis of semi-synthetic derivatives from natural products is an important area of organic chemistry. Consequently, the ability to isolate piperine from black pepper (*Piper nigrum*) holds significant importance for pharmaceutical and biotechnological applications [3–7].

The importance of black pepper (*Piper nigrum*) is increasing due to its recognition as a source of natural compounds [8]. *Piper nigrum* L., known as black pepper, belongs to the Piperaceae family and is commonly found in the tropical regions of Asian countries and the east coast of India [9]. It is also used as a spice in many cultures. This species is also reported to be used in Ayurvedic herbal preparations (Trikatu) used to treat many ailments in traditional Indian medicine [10].

Piperine, the main active ingredient in this popular spice, is an alkaloid compound [11]. Piperine is used in Chinese traditional treatments as well as in Indian medicine. Piperine has been shown to increase blood circulation, stimulate salivation, and enhance appetite [12]. Piperine exhibits a versatile biological profile, including roles in pain management, hypotension, vascular cell modulation, and anti-inflammatory, antimicrobial, insecticidal, antiulcerogenic, antioxidant, and anticancer activities. Additionally, piperine enhances the absorption and bioavailability of other bioactive compounds [13–16]. The data obtained so far indicate that piperine paves the way to become a privileged scaffold for the development of bioactive compounds with therapeutic application on many human diseases.

Piperine derivatives exhibit sedative-hypnotic and muscle relaxant effects. In addition, it has been stated that it modulates the activity of various targets related to neurological disorders, including epilepsy, depression, Parkinson's disease and pain-related disorders [8,17–20]. Additionally, piperic acid ester derivatives have been reported to support melanocyte formation, which is beneficial for managing conditions such as vitiligo [21]. Although the use of piperine as a scaffold for bioactive compounds is still in its early stages, continued exploration of this structure could see remarkable advances in drug discovery programs.

Piperine, known for its diverse biological properties, also influences enzyme systems [2,11,22]. Piperine functions as an inhibitor for certain enzymes and serves as a plant activator by modulating the activities of catalase and glutathione peroxidase enzymes [12,23,24]. Plant activators are chemicals that activate defense genes in plants. As plant activators lack pesticide or antibiotic activity, their potential adverse effects on human health and the environment are minimal [25].

Plants respond to rapid production and accumulation of reactive oxygen species (ROS) by activating defense mechanisms. They carry out these defense mechanisms with enzymatic and non-enzymatic antioxidants. Among antioxidant enzymes, peroxidase plays a significant role [26]. Peroxidase enzyme (POD; EC 1.11.1.7) catalyzes the reaction between compounds that can donate hydrogen atoms and the H_2O_2 compound that accepts these atoms and is included in the oxidoreductase group [27,28]. POD enzymes are ubiquitous in nature and perform various critical physiological functions. Key functions include regulating hormonal activity in plants, protecting against tissue damage and pathogenic infections, and modulating indoleacetic acid levels [29–31].

This study investigated the effects of piperine (1), isolated from black pepper, piperic acid (2), derived from the hydrolysis of piperine, and piperic acid methyl (3a) and ethyl (3b) esters, synthesized through esterification of piperic acid, on peroxidase enzyme (CPOD) activity purified from cauliflower *(Brassica oleracea* L.).

MATERIALS and METHODS

Isolation of piperine

Five liters of chloroform were added to one kilogram of freshly ground black pepper, and the mixture was left at room temperature for 24 hours. The mixture was then filtered, and the solvent was removed by evaporating the filtrate. Piperine was purified from the obtained extract using an ethyl acetate-hexane mixture on a silica gel column. Piperine (1) was crystallized from an ethyl acetate-hexane mixture to obtain a pure product.

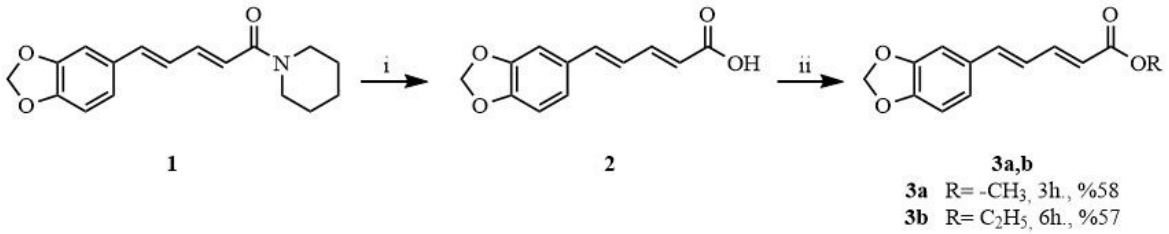

Figure 1. Synthetic route for piperine derivatives (i) KOH/MeOH, reflux, 72 h., (ii) H2SO4/MeOH or EtOH reflux, 3-6 h.

Synthesis of piperic acid

To a solution of 3 g (10.5 mmol) piperine (1) in methanol, 7 g (125 mmol) potassium hydroxide was added, and the magnetically stirred solution was refluxed for 72 hours. The mixture was then evaporated under reduced pressure, and the reaction mixture was neutralized with diluted HCl. The solid was filtered and recrystallized from ethanol [32]. Piperic acid (2) was obtained as 1.95 g (8.95 mmol) with a yield of 85%.

Synthesis of methyl and ethyl esters of piperic acid

A solution of 0.44 g (2 mmol) piperic acid (2) in 20 mL alcohol was added 1 mL concentrated H_2SO_4. The reaction mixture was stirred magnetically at reflux temperature for 3-6 hours. At the end of the reaction, the mixture was evaporated at reduced pressure to remove solvent. The solid was dissolved in 20 mL dichloromethane and the solution was washed with diluted Na_2CO_3 solution. The separated organic phase was dried with Na_2SO_4 and the solvent was removed by evaporation. The crude product was purified on silica gel column using ethylacetate-hexane [33]. Methyl ester of piperic acid (3a) was obtained using methanol as the solvent, yielding 0.27 g (1.2 mmol) in 58%, and ethyl ester of piperic acid (3b) was obtained using ethanol, yielding 0.28 g (1.1 mmol) in 56%.

Partial purification of peroxidase enzyme from cauliflower *(Brassica oleracea L.)*

Obtaining the cauliflower *(Brassica oleracea L.)* plant

Cauliflower (*Brassica oleracea* L.) plant was obtained from the local market in Erzincan. It was preserved at +4°C for use.

Preparation of peroxidase enzyme homogenate

It was taken from cauliflower (*Brassica oleracea* L.) (10 g) frozen and ground in liquid nitrogen. It was mixed with a magnetic stirrer for 15 minutes in 50 mL Na_2HPO_4 (0.1 M pH 7.0) buffer. The mixture was filtered through filter paper and centrifuged at 8.000 rpm in a refrigerated centrifuge at 8.000 rpm for half an hour. After centrifugation, the supernatant was stored at 4°C for use.

Ammonium sulfate precipitation and dialysis

The extract was filtered and centrifuged at 8.000 rpm for half an hour, and the supernatant was added to the supernatant in the ranges of 0-10% 10-20% 20-30% 30-40% 40-50% 50-60% 60-70% 70-80% 80-90%, respectively. Precipitation was carried out by adding ammonium sulfate. Calculation of the amount of ammonium sulfate was made with the formula given below.

$g[(NH_4)_2SO_4]=1.77*V*(S2-S1)/ 3.54-S2$

V = Supernatant
S1 = current ammonium sulphate saturation
S2 = desired ammonium sulphate saturation

For the precipitation process, ammonium sulfate was thoroughly dissolved in the crude homogenate for each interval. The suspension, to the determined saturation, was centrifuged at 10.000 rpm for 1 hour. After each centrifugation process, POD activity was checked in the precipitate and supernatant. The homogenate with the highest activity was selected and dialysis was performed. For this process, the dialysis bag was placed in phosphate buffer (10 mM, pH 7.0) on a magnetic stirrer at +4 °C for 12 hours.

Peroxidase Activity Assay

Guaiacol was used as a substrate to measure POD activity in the cauliflower sample. Concentrations were adjusted to a total volume of 1 mL. Finally, 17 µL of enzyme homogenate was added to the mixture containing 333 µL of 22.5 mM H_2O_2, 333 µL of 45 mM guaiacol, and 317 µL of phosphate buffer (pH: 7.0, 0.1 M). The change in absorbance was monitored at 470 nm for 1 min [34].

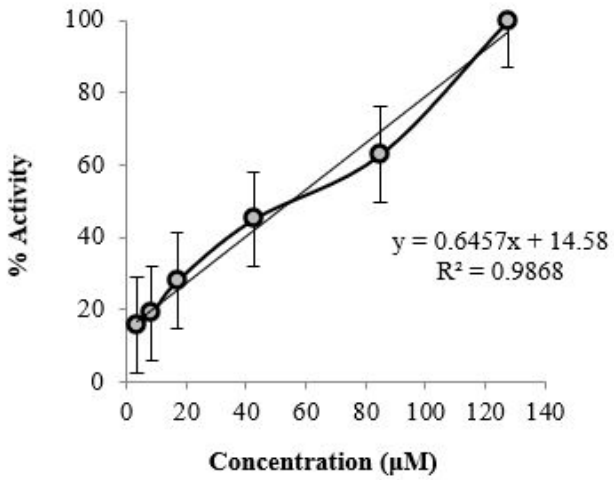

Figure 2. Activity effect of piperine on peroxidase enzyme. Each data point represents the mean ± SD of three replicates.

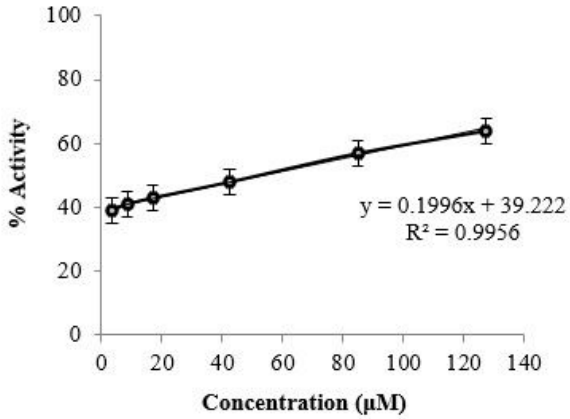

Figure 3. Activity effect on piperic acid peroxidase enzyme. Each data point represents the mean ± SD of three replicates.

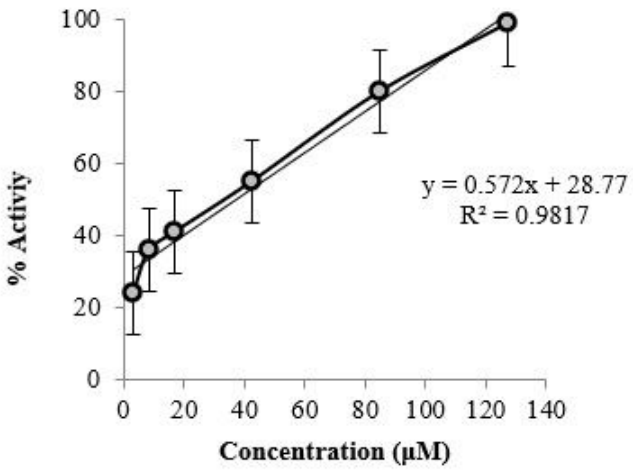

Figure 4. Activity effect on piperic acid methyl ester peroxidase enzyme. Each data point represents the mean ± SD of three replicates.

Three replicates were used in each application.

Activity Assay of Piperine and Piperine Derivatives on Peroxidase Enzyme

Solutions of piperine (1) and piperine derivatives (2, 3a-b) were prepared between 3.4 and 127.5 µM. The activity of the extract was measured without the addition of piperine (1) and its derivatives (2, 3a-b). Then, the activity of the POD enzyme was examined using different concentrations of piperine and piperic acid ester derivatives. Three replicates were used for each application. Standard deviation and AC_{50} (concentration at which 50% of maximum activity was observed) values were calculated.

RESULTS

Piperine was obtained from black pepper (***Piper nigrum***) as yellow crystals. Piperic acid was obtained with 85% yield by hydrolysis of piperine. Piperic acid methyl ester was synthesised with a yield of 58% and piperic acid ethyl ester was synthesised with a yield of 56% from piperic acid. Solutions of these compounds were prepared between 3.4 and 127.5 µM and their effects on peroxidase enzyme activity were recorded. Standard deviation and AC50 values were calculated by plotting the % activity versus concentration graph. The AC_{50} values for piperine and piperic acid ester derivatives are given in Table 1. It was observed that piperine, which contributes to the defence system in plants by increasing antioxidant activity, increased the activity of peroxidase by 100% at a concentration of 127.5 µM (Figure 2). At a concentration of 127.5 µM, piperic acid, piperic acid methyl ester and piperic acid ethyl ester showed 64% (Figure 3), 99% (Figure 4) and 98% (Figure 5) activity, respectively. As shown in the present research, piperine and piperic acid ester derivatives tested for POD activity act as activators.

DISCUSSION

In the 21st century, developing technologies are actively influencing health management, food quality and safety, in short, many aspects of human life. However, rapid population growth and increasing urbanisation have led to a decline in agricultural land. As a result, it is difficult to produce sufficient, efficient and high-quality food for everyone in the world [2,35–37]. In this sense, as an alternative to synthetic chemical pesticides in the management of plant diseases, many plant activators have been widely investigated for disease control [38,39]. And in line with this need, the trend towards finding new plant activators has increased [40].

Piperine, which gives black pepper its pungent flavour and aroma, is consumed as a dietary spice. Piperine acts as a bioavailability enhancer for many chemotherapeutic agents [41], and many studies suggest that piperine has synergistic effects when taken with different classes of drugs [13,24]. Studies have observed that piperine has various bioactivities in agriculture [37]. The reason why piperine was preferred in this study is the increasing demand for natural and safer antioxidants in food applications and the increasing consumer preference for natural antioxidants [42,43].

POD, an antioxidant enzyme, is widely distributed in living organisms including plants, animals and microorganisms [34,44,45]. This enzyme is one of the basic enzymes that control the growth and development of the plant. It is one of the fundamental enzymes that control plant growth and development. It is involved in various cellular processes, including the formation, stiffening and eventual lignification of cell walls, which protects tissues from damage and infection by pathogenic microorganisms [30,46]. In addition, POD can be used to modify a wide range of chemicals. It can also be used for applications such as the removal of phenolics from wastewater, the synthesis of various aromatic compounds, and the removal of peroxides from food and industrial waste [47]. This avoids the use of synthetic antioxidants such as butylated hydroxyanisole (BHA) and butylated hydroxytoluene (BHT), which are used to prevent the oxidation of lipids in foods and have been questioned for their toxic and carcinogenic effects [48,49].

In the current study, the activity of piperine and piperic acid ester derivatives on the peroxidase enzyme was investigated. Piperine, a natural product isolated from black pepper, has been shown to have potential health and medicinal uses. Further studies are needed to investigate such combination effects, and research should be intensified to develop piperine derivatives.

One study demonstrated the role of plant activators in inducing disease resistance in many crops using various compounds such as acibenzolar S-methyl [50]. Another study evaluated butylated hydroxytoluene (BHT) as a plant activator. The results showed that BHT application alleviated lipid peroxidation by increasing antioxi-

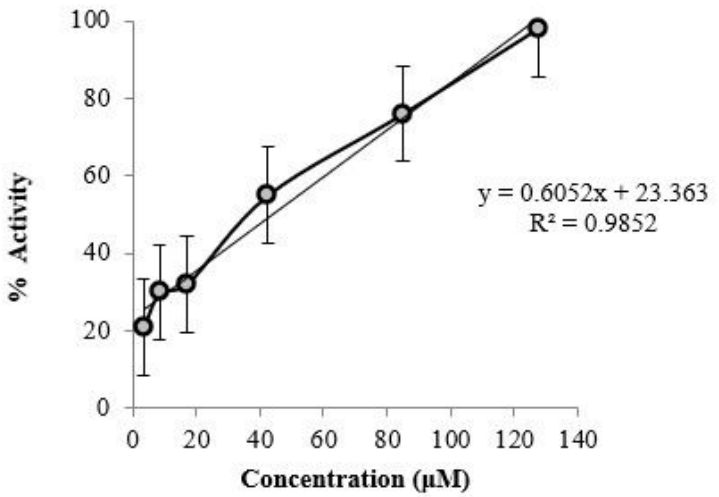

Figure 5. Activity effect on piperic acid ethyl ester peroxidase enzyme. Each data point represents the mean ± SD of three replicates.

Table 1. AC_{50} value of piperine and piperine derivatives

Sample	Piperine	Piperic acid	Piperic acid methyl ester	Piperic acid ethyl ester
AC_{50} ± SD µM	54.9 ± 0.006µM	54.0 ± 0.007 µM	37.11 ± 0.004 µM	44.0 ± 0.003 µM

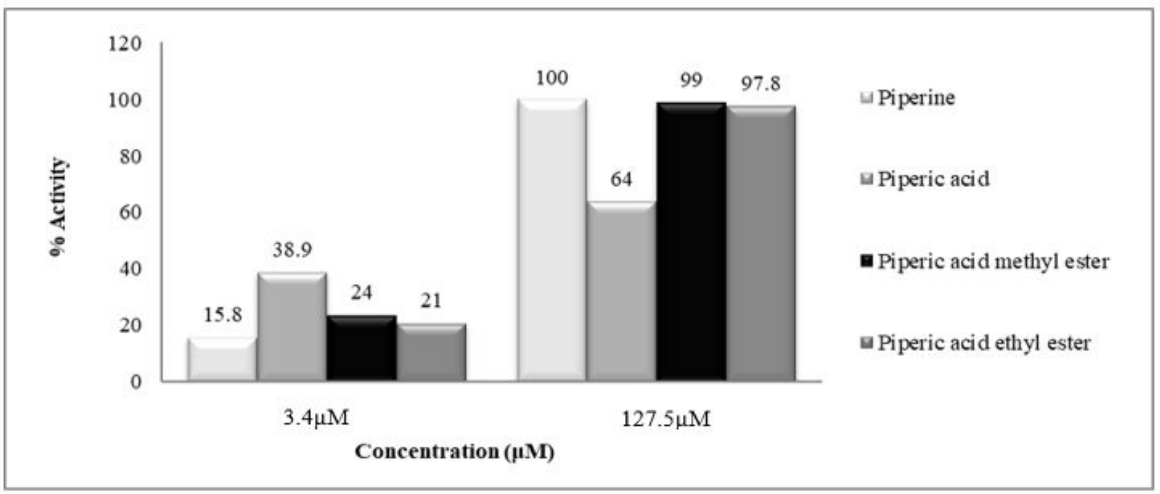

Figure 6. % activity of piperine and piperine derivatives at different concentrations

dant enzyme activities against *B. dothidea* in apple fruit and significantly increased the activity of four defence enzymes [51].

A study investigated the effect of basil and rosemary extracts on peroxidase enzyme and found them to be potent activators [52]. Basil and rosemary showed 204.7% and 205.8% activity respectively. However, here, the fact that piperine is isolated from a natural source and is a pure substance makes it special.

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