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# Inhibition of xanthine oxidase by Caulerpenyne from *Caulerpa prolifera*

## [Ksantin oksidazın *Caulerpa prolifera*'dan elde edilen caulerpenyne tarafından inhibisyonu]

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**ABSTRACT**

**Objective:** Seaweeds synthesize secondary metabolites to protect themselves against many factors such as epiphytic colonizations and herbivorous fishes. A significant amount of active metabolites in seaweeds have industrial importance in pharmacy, cosmetics and medicine. Among seaweeds, *Caulerpales* have recently aroused interest of many scientists because of its dominant sesquiterpenoid metabolite, caulerpenyne. So far, its anticarcinogenic, antiproliferative and antimicrobial properties have been reported in many papers. The present study aims at evaluating the potential of *Caulerpa* species as a natural remedy for gout.

**Material and Method:** Xanthine oxidase inhibition by caulerpenyne was evaluated using Michaelis-Menten and Lineweaver-Burk plots.

**Results:** Caulerpenyne inhibits xanthine oxidase with an $IC_{50}$ value of 26.92 μM. According to the results of this study, the type of caulerpenyne inhibition on xanthine oxidase was observed as an irreversible inhibition.

**Conclusion:** Members of *Caulerpa* Genus might be considered as a natural source of caulerpenyne with its inhibitory effect on xanthine oxidase. Further investigations are warranted on possible chemical modification of caulerpenyne to have better inhibitory activities.

**Key Words:** *Caulerpa* species, inhibition kinetics, caulerpenyne, xanthine oxidase.

**Conflict of Interest:** The authors have no conflict of interest

**ÖZET**

**Amaç:** Makroalg türleri; kendilerini epifitik kolonizasyon ve herbivor balıklar gibi çeşitli faktörlere karşı korumak için sekonder metabolitler sentezlerler. Deniz alglerindeki bu aktif metabolitlerin önemli bir kısmı farmasötik, kozmetik ve ilaç sanayinde endüstriyel öneme sahiptir. Deniz algleri arasında *Caulerpa*'lar; caulerpenyne adlı seskiterpenoid yapıdaki baskın metabolitinden dolayı son günlerde pek çok araştırmacının dikkatini çekmektedir. Günümüze kadar, bu türlerin antikanserojenik, antiproliferatif ve antimikrobiyal özellikleri pek çok çalışmada raporlanmıştır. Bu çalışma *Caulerpa* türlerinin, gut için doğal bir ilaç olarak potansiyelini değerlendirmeyi amaçlamaktadır.

**Materyal ve Metod:** Caulerpenyne ile ksantin oksidaz inhibisyonu Michaelis-Menten ve Lineweaver-Burk grafikleri kullanılarak değerlendirilmiştir.

**Bulgular:** Caulerpenyne ksantin oksidazı, $IC_{50}$ değeri 26,92 μM olacak şekilde inhibe eder. Çalışmanın sonuçlarına göre, ksantin oksidaz üzerinde caulerpenyne inhibisyonunun türü tersinmez inhibisyon olarak gözlenmiştir.

**Sonuç:** *Caulerpa* türleri; ksantin oksidaz üzerinde inhibitor etkisine sahip caulerpenyne için doğal bir kaynak olarak düşünülebilir. Caulerpenyne'nin daha iyi inhibitör aktivitesine sahip olabilmesi için gerekli olası kimyasal modifikasyonları üzerinde daha ileri araştırmaların yapılması gerekmektedir.

**Anahtar Kelimeler:** *Caulerpa* türleri, inhibisyon kinetiği, caulerpenyne, ksantin oksidaz.

**Çıkar Çatışması:** Yazarların çıkar çatışması bulunmamaktadır.

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## Introduction

Xanthine oxidoreductase (XOR), one of the members of the molybdoenzyme family, was first identified in milk by Schardinger [1]. This enzyme consists of two interconvertible forms with difference in functionality: xanthine dehydrogenase (XDH) and xanthine oxidase (XOD) [2]. The two forms of this enzyme catalyze the last two steps of purine degradation, namely the conversion of hypoxanthine to xanthine and xanthine to uric acid [3]. Under physiological conditions, the majority of XOR is found as XDH and a small part of it as XOD. The reaction of XOD generates uric acid and reactive oxygen species or hydrogen peroxide [4]. XDH is transformed into XOD via posttranslational modifications under inflammatory conditions. Besides, XDH may also exhibit XOD activity in some conditions such as ischemia and hypoxia which cause a decrease in the level of $NAD^+$ [3].

The major catabolic function of XOD is to catalyze the oxidation of hypoxanthine to xanthine and xanthine to uric acid. The overproduction of uric acid can cause hyperuricemia. Besides, excess amount of uric acid can deposit in joints, a condition termed gout. Gout is a common disease that affects a substantial amount of the world population, especially the Western populations. There is a positive correlation between the living standard of peoples and the occurrence frequency of this disease [5]. The deposition of uric acid crystals in joints and kidneys may cause arthritis and renal disorders. Moreover, hyperuricemia might have been related to hypertension, diabetes, obesity and some kinds of cancers [6,7]. The main starting point of the treatment of this illness is to inhibit uric acid biosynthesis or to excrete the excess amount of uric acid. The inhibitors of XOD are more preferable due to having lower side effects than uricosuric drugs. The well-known commercial XOD inhibitor is allopurinol. Although it has been used in clinical applications largely, the use of it can cause severe side effects such as hypersensitivity syndrome, Stevens Johnson syndrome, renal toxicity, allergy and skin problems [7-9]. Thus, there is a need to develop new XOD inhibitors which are safer and more effective than the currently used drugs. It might be possible to use XOD inhibitors for the treatment of hepatitis and brain tumor where it was determined that the activity of XOD is increased [8,10]. Moreover, it was reported that XOD has a significant role in many diseases such as cardiovascular disorders, inflammations, chronic heart failure [3]. Therefore, the new XOD inhibitors might be useful against these diseases.

The most significant source of safer inhibitors is natural materials such as plants. There are a lot publications which evaluate the potential of terrestrial plants as XOD inhibitors [7-9,11-14]. The present study evaluates the potential of caulerpenyne (CYN) as a XOD inhibitor. CYN is the main secondary metabolite of *Caulerpa* species which was firstly isolated from *Caulerpa prolifera* [15]. So far, anticarcinogenic, antiviral and apoptotic properties of CYN have been reported [16-20]. These remarkable results motivated researchers to investigate the other properties of this metabolite. One of the important investigation areas is to identify the potential of CYN as an enzyme inhibitor. There are few studies in the literature which evaluate the enzyme inhibitory potential of CYN and its derivatives. It inhibits phospholipase $A_2$ [21], lipoxygenase [22,23], pancreatic lipase [24,25] and α-amylase [26,27].

In the light of above mentioned knowledge, the present study aimed at evaluating the inhibitory effects of CYN on XOD activity and to elucidate the type of inhibition from kinetic studies.

## Materials and Methods

### *Collection of algae*

*Caulerpa prolifera* (Forsskål) J.V. Lamouroux was collected from the coastline of Çeşme/İzmir-Turkey in May 2009. The geographical coordinates of the collection area are 38° 07' 56 82" N - 26° 50' 06 72" E. The alive algae were immediately transported to the laboratory within plastic bags to prevent CYN degradation [28], the materials were washed with tap water and then distilled water to remove salts and epiphytes. The samples were stored at -20°C until the isolation of CYN.

### *Chromatographic isolation of CYN*

The extraction and purification of CYN from *C. prolifera* were conducted according to previously described procedures [23,27,29]. The purified CYN was analysed by reverse-phase HPLC and $^1$H NMR spectroscopy in order to determine its purity. NMR data were identical with those reported in the literature [15].

### *XOD inhibition with CYN*

Milk butterfly XOD (EC. 1.17.3.2) within the superoxide dismutase kit (RANSOD) of RANDOX Laboratories Ltd was used and xanthine was purchased from Sigma Chemical Co., St. Louis, USA. Since xanthine could not be solved in water, it was solved in a NaOH solution (100 µL, 0.5 M), and then was adjusted to 0.025 M, pH=9.0 by adding phosphate buffer (0.05 M, pH=9.0). This solution was used as a stock solution in order to prepare various concentrations of substrate required for analyses. XOD activity was measured in phosphate buffer solutions (0.05 M, pH 9.0) by measuring the increase in absorbance at 292 nm related to uric acid formation. In inhibition experiments, the enzyme solution was incubated with CYN (1:1 ratio) at 37 ºC for 1 hour which was previously determined as optimum incubation time. The samples were assayed in duplicate and the results were reported as the mean of two different experiments.

### *Inhibition kinetics of XOD in the presence of CYN*

The stock solution of CYN in methanol (10 mg.mL$^{-1}$) was diluted with phosphate buffer to prepare the required inhibitor concentrations (15, 25, 50 and 100 µM). The enzyme solution was incubated with various concentrations of CYN at 37 ºC, 200 rpm for 1 hour prior to addition into substrate solution. The final enzyme activity in the reaction medium was 10 U.L$^{-1}$ and the concentration was changed between 0.015 to 0.2 mM for substrate solution which was prepared freshly for each set. The control experiments were carried out by using methanol in assay conditions, and it was found that there was no effect on the activity of XOD. The results were expressed as mean ± SD of two independent experiments.

### *Statistical Analysis*

Student t test was used to evaluate the experimental data. The error bars in the figures show the standard deviation (±SD). The statistical significance was set at $p<0.05$.

## Results

The inhibitory effect of CYN on XOD activity was measured in the present study. The results showed that CYN inhibited the activity of XOD in a dose dependent manner ($IC_{50}$: 26.92 µM) (Figure 1).

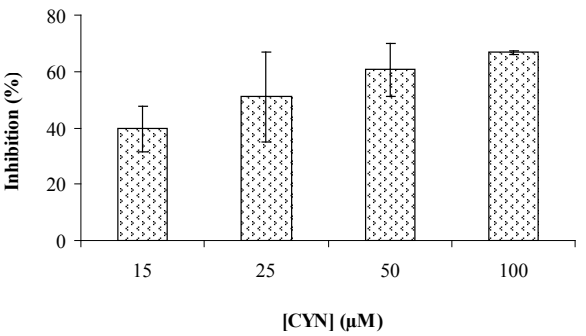


**Figure 1.** Inhibition (%)-concentration bar plot for XOD inhibition by CYN. The results are presented as mean ± SD of two independent experiments

The rates of the reactions were determined for all sets of the assays and the Lineweaver-Burk plot was drawn (Figure 2). The kinetic parameters, Michaelis–Menten constant ($K_M$) and maximum velocity ($V_{max}$) values, were calculated from this plot.

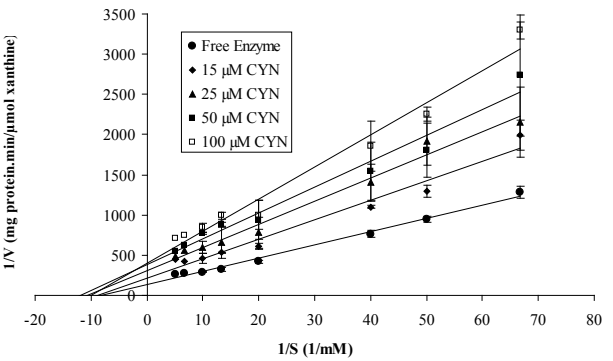


**Figure 2.** Lineweaver–Burk plot of XOD reaction with variable xanthine concentrations in the presence of CYN. The results were presented as mean ±SD of two independent experiments.

The maximum velocity values of XOD reaction were decreased with the increase of CYN concentrations, while Michaelis–Menten constant remained nearly the same. The $K_M$ and $V_{max}$ values of free XOD enzyme were determined as 0.13 mM and of 0.0077 µmol xanthine/mg protein.min, respectively. Apparent $V_{max}$ and $K_M$ values in the presence of different CYN concentrations are listed in Table 1.

The results of Lineweaver-Burk plot can be interpreted as CYN non-competitively inhibited XOD. But it is known that the irreversible inhibition also leads to a decrease in the maximum velocity values similar to non-competitive inhibition. In order to be sure about the type of inhibition, the relationship between enzyme activity and its concentration was determined [30] (Figure 3). Enzyme solution was not pre-incubated with CYN before substrate addition during these experiments. The plot of the enzyme activity versus the concentrations of enzyme gave two straight lines with approximately same slopes and different abscissa intercepts. The results revealed that the inactivation of XOD by CYN occurred

**Table 1.** Kinetic values of XOD inhibition from Lineweaver–Burk plot

| Concentration of CYN (µM) | $V_{max}$ (µmol xanthine/mg protein.min) | $K_M$ (mM) | $R^2$ |
|---|---|---|---|
| 0 | 0.0077 | 0.13 | 0.99 |
| 15 | 0.0046 | 0.11 | 0.97 |
| 25 | 0.0032 | 0.09 | 0.98 |
| 50 | 0.0026 | 0.09 | 0.97 |
| 100 | 0.0025 | 0.10 | 0.97 |

irreversibly. Moreover, the abscissa intercept showed the amount of enzyme inhibited irreversibly.

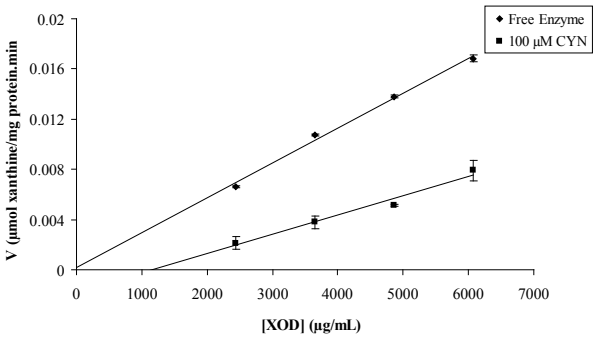


**Figure 3.** Diagram of $V_{max}$ versus $[E]_t$. The results were presented as mean ±SD of two independent experiments.

In order to determine the kinetic parameters of inhibition, the activity of XOD was measured after various preincubation periods with CYN (Figure 4). It can be clearly seen that the relation between the enzyme activity and preincubation time is linear which indicates that the reaction fits the pseudo-first order model. Apparent first-order inhibition rate constants ($k_{app}$) were calculated from the slopes of the lines. And the irreversible inhibitory constants ($K_I$ and $k_{inact}$) of CYN on XOD enzyme activity was calculated from the plot created by below equation:

$$\frac{1}{k_{app}} = \frac{K_I}{k_{inact}} \cdot \frac{1}{[I]} + \frac{1}{k_{inact}}$$

where $k_{app}$ is the apparent first-order inhibition rate constant, $K_I$ represents the dissociation constant for the first step of complex (EI complex) formation, $k_{inact}$ is the rate constant for the second step of complex (irreversible EI complex) formation and [I] is the inhibitor concentration [31]. The $K_I$ and $k_{inact}$ values of the inhibition of XOD by CYN were determined as 3.05 µM and 0.020 $min^{-1}$, respectively (Figure 5).

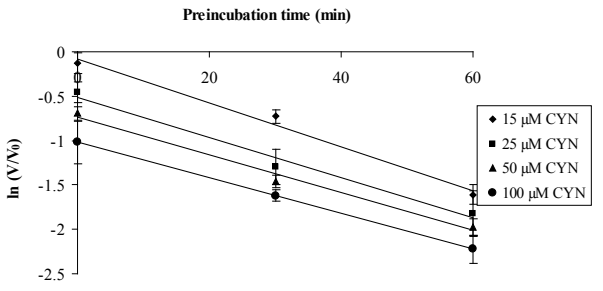


**Figure 4.** Time dependent inhibition of XOD by CYN. The results are presented as mean ± SD of two independent experiments.

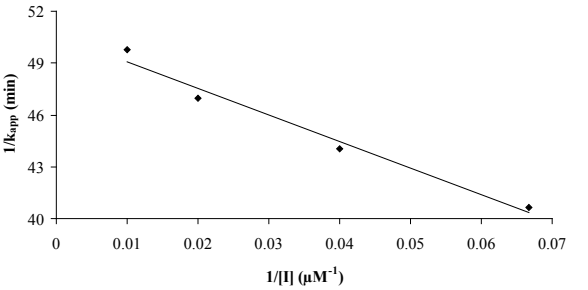


**Figure 5.** The plot of $1/k_{app}$ versus 1/[I] for determination of $K_I$ and $k_{inact}$ values.

## Discussion

Seaweeds like terrestrial plants synthesize various kinds of biochemical products that have significant properties for scientific investigations. It is known that various kinds of plants have been used to deal with some diseases since prehistoric times. The main purpose of the present study was to determine the potential of *Caulerpa* species as a natural remedy for gout. It was presented in the previous reports that CYN, the main secondary metabolite of *Caulerpa* species, has some unique properties such as anticarcinogenic, antiproliferative, apoptotic and antiviral effects [16-19]. Moreover, the enzyme inhibitory effects of CYN towards some medically important enzymes like phospholipase $A_2$, lipase, lipoxygenase and amylase have been shown by some researchers [21-27]. In the light of the knowledge above, the inhibitory effects of CYN on XOD enzyme activity were determined in this study.

XOD catalyse the conversion of hypoxanthine and xanthine which are formed in purine degradation metabolism to uric acid. Since the solubility of uric acid in water is very low, the excess amount of it is accumulated especially in joints and kidneys. The level of uric acid in blood might be reduced by inhibiting its formation with XOD inhibitors or increasing its excretion out of the body with uricosuric drugs. The inhibitors of XOD function in two different mechanisms as this enzyme contains two different binding sites for substrate binding. Therefore, the inhibition of XOD occurs either with interaction of purine binding sites, like allopurinol [32], or interaction of FAD cofactor binding sites, like benzimidazole [33].

Several side effects of commercially used drugs for the treatment of hyperuricemia and gout have been reported in the literature so far [7-9]. Therefore, it is necessary to find efficient inhibitors with minimum side effects. There have been increasing interests to find potential materials for solving these problems. In one of these reports, the type of XOD inhibition reaction with allopurinol was determined as competitive and the $K_i$ value of this inhibition was detected as 0.34 µM. It was also reported that apigenin, quercetin, myricetin,

isovitexin and genistein inhibited XOD competitively like allopurinol. The inhibitory constants of these inhibitors were determined as 0.61±0.31 μM, 1.40±0.78 μM, 2.17±1.13 μM, 5.22±2.02 μM and 3.23±1.01 μM, respectively [34]. There have been various studies that evaluate the inhibitory potential of natural polyphenols especially flavonoids. Chan et al. [35] determined the $IC_{50}$ values of XOD inhibition with caffeic and chlorogenic acid as 74.6 ± 11.04 μM and 126.28 ± 2.86 μM, respectively. The result found for caffeic acid was also consistent with the results of Nguyen et al. [36] ($IC_{50}$: 85.4 μM). The inhibitory effects of luteolin and apigenin were also evaluated in Nguyen et al's study [36], and the types of inhibition were determined as competitive. The competitive type of XOD inhibition with apigenin was also confirmed by Lin et al. [34]. In spite of these results, there were some studies indicating that luteolin, kaempferol and apigenin were the mixed type of inhibitors for XOD [37]. The differences among these results may be caused from the differences between used methods or the differences between concentrations of enzyme and substrate. The XOD inhibitory potential of structural analogs of coumarin was tested in another report conducted by Lin et al. [38]. It was reported that all of the studied components inhibited XOD in a competitive manner (Table 2). Besides, it was reported that the inhibitory effect of esculatin ($IC_{50}$: 10.84 μM) was comparable with the effect of allopurinol ($IC_{50}$: 1.07 μM). The antioxidant, anti-inflammatory, anticarcinogenic and also reactive oxygen species scavenging properties of these analogs were also determined in this investigation [38]. This observation is very important since the secondary metabolite of *Caulerpa* species have similar properties. There have been such studies using natural active compounds instead of synthetically produced components as an inhibitor in the literature. Unno et al. [12] isolated two active components, valoneic acid dilactone (VAD) and ellagic acid (EA), from the water extract of the leaves of *Lagerstroemia speciosa*. The $IC_{50}$ values of VAD, allopurinol and EA were determined as 2.5 μM, 10.4 μM ve 71.5 μM, respectively. These results revealed that the inhibitory effect of VAD was stronger than that of allopurinol. The kinetic analyses of VAD presented that the inhibition of XOD occurred in a non-competitive manner. This result was also well in line with the result of the study conducted by Hatano et al. [40], which presented the non-competitive inhibition of XOD with VAD from *Mallotus japonicus* [12]. The main purpose of the present study was to determine the inhibitory effect of CYN on XOD activity since it is a target enzyme for the treatment of gout and related symptoms. The $IC_{50}$ value of XOD inhibition with CYN was found as 26.92 μM and the type of inhibition was determined as an irreversible inhibition. Thus, the value of $V_{max}$ was decreased from 0.0077 μmol xanthine/mg protein.min to 0.0025 μmol xanthine/mg protein.min in the presence of 100 μM CYN. Generally, it is expected

**Table 2.** The inhibitory characteristics of some compounds towards XOD.

| References | Inhibitory compound | Inhibition type | $K_I$, % inhibition, $IC_{50}$ |
|---|---|---|---|
| [8] | allopurinol | mixed | $K_i$: 7.3 μM |
| | liquiritigenin | | $K_i$: 14.0 μM |
| | isoliquiritigenin | | $K_i$: 17.4 μM |
| [38] | 4-hydroxycoumarins | competitive | $IC_{50}$: 78.13±3.11 μM |
| | esculetin | | $IC_{50}$: 10.84±0.14 μM |
| | 4-Methylesculetin | | $IC_{50}$: 75.79±1.98 μM |
| | allopurinol. | | $IC_{50}$: 1.07±0.01 μM |
| [39] | apigenin | - | $IC_{50}$: 1.50 μM |
| | quercetin | | $IC_{50}$: 2.08 μM |
| | naringin | | $IC_{50}$: 49.40 μM |
| [9] | Petroleum ether extract of *Erythrina stricta* Roxb. | - | $IC_{50}$: 30.2±2.2 μg.mL$^{-1}$ |
| [9] | Chloroform extract of *Erythrina stricta* Roxb. | - | $IC_{50}$: 21.2±1.6 μg.mL$^{-1}$ |
| [9] | Ethyl acetate extract of *Erythrina stricta* Roxb. | - | $IC_{50}$: 44.9±1.4 μg.mL$^{-1}$ |
| Present study | CYN | irreversible | $IC_{50}$: 26.92 μM<br>$K_I$: 3.05 μM<br>$k_{inact}$: 0.020 min$^{-1}$ |

that a drug should react selectively with a target pathway, without reacting with any other one. Unfortunately, not every drug shows this property. Therefore, there has been a great interest among researchers in finding such specific molecules. Due to the results of this study CYN can be classified as a good inhibitor for XOD and therefore, it can be recommended as a potential drug for XOD related diseases after in vivo studies. Unfortunately, CYN inhibits not only XOD but also phospholipase $A_2$ [21], lipoxygenase [22,23], pancreatic lipase [24,25] and alpha amylase [26,27]. Many drugs, which are currently used, act similarly and by reacting with different pathways they may have severe side effects. As Flemmig et al. [5] stated in their research, allopurinol, a well known inhibitor for gout, inhibits besides the enzymes of the purine metabolism also other enzymes. Hence, it causes undesirable side effects. Indeed, the present study is a first step and exhibits only the results of *in vitro* experiments. Consequently, *in vivo* experiments are strongly recommended as the next step.

## Conclusion

The present study revealed that the main secondary metabolite of *Caulerpa* species, CYN, inhibits XOD irreversibly. Further investigations are strongly recommended on the possible modification of CYN to have better inhibitory activities. Therefore, CYN might be an alternative material for the treatment of XOD activity related diseases or this metabolite can be used as a pristine molecule for producing new synthetic constituents.


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## Conflict of Interest

The authors have no conflict of interest.

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