Inhibition of catechol-O-methyltransferase (COMT) by some plant-derived alkaloids and phenolics

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ABSTRACT

In this study, as an alternative to the medicines, natural compounds extracted from plant species (*Peganum harmala*, *Cistus parviflorus* and *Vitex agnus-cactus*) were investigated in order to inhibit the catechol-*O*-methyltranferase (COMT) activity. In fluorometric enzyme assay, S-adenosylmethionine (SAM) and aesculetin (ES) were used as methyl donor and acceptor substrates, respectively. Their K_m values were determined as $3.5 \pm 0.3\,\mu M$ and $6.4 \pm 0.4\,\mu M$ in absence of inhibitor. Inhibition performances of the plant-derived polyphenolics and alkaloids were determined. Inhibitory effect of alkaloids extracted from *P. harmala* seeds was found the highest among the plant extracts; however, it was lower than that of 3,5-DNC. In case of inhibition mechanism, mixed type inhibition was observed for alkaloid extract whereas uncompetitive inhibition was observed for 3,5-DNC. In case of polyphenolic extracts obtained from *C. parviflorus* and *V. agnus-cactus* leaves, mechanism were also explained as mixed type inhibition and their αK_i values were calculated as $1.99 \pm 0.35\,\mu g/ml$ and $9.48 \pm 0.58\,\mu g/ml$, respectively.

1. Introduction

Since ancient times, plants have been used as medicine against certain human diseases. In the last century, the use of natural sources as medicine has expanded and there is a growing interest in obtaining biologically active compounds from natural sources. These compounds have antioxidant, antimicrobial, antiinflammatory and neuroprotective properties and classified as phytochemicals include most of catecholic structures [1,2]. In administration of catechols, it is known that they are methylated by an enzyme known as catechol-*O*-metyhltranferase (COMT; EC 2.1.1.6) that plays an important role in Parkinsonism [3]. The primary function of COMT is to deactivate biologically active catechols like catecholamine neurotransmitters (dopamine, epinephrine), levodopa (L-DOPA), carbidopa and flavonoids [4,5].

In current therapies for Parkinson's disease, selective COMT inhibitors, mainly synthetic nitrocatechol compounds (entacapone, tolcapone), are used in combination with L-DOPA and dopa decarboxylase inhibitors, although there are several well-known adverse side effects of these drugs [4,5]. Therefore, researchers have focused on the potency of natural products having ability of inhibiting COMT. Among several natural sources, tea catechins have been mostly investigated compounds by several research groups [6,4,7].

All of them revealed that flavonoids in *C. sinensis* have great inhibitory potency against COMT. In this study, the natural compounds extracted from the leaves of *Cistus parviflorus* and *Vitex agnus-cactus* and from the seeds of *Peganum harmala* were used as natural inhibitors of COMT in order to investigate the inactivation performance of methylation of aesculetin to scopoletin.

2. Experimental

2.1. Materials

HCl used as alkaloid extraction solvent was purchased from Merck (Germany). Petroleum ether used during the liquid-liquid extraction of alkaloids was purchased from Riedel-de Haen (Germany). All other extraction solvents were purchased from Sigma–Aldrich Chemie GmbH (Germany). COMT from porcine liver was purchased from Sigma (Germany). Other reaction mixture components including SAM, aesculetin (ES), $MgCl_2$ anhydrous and L-cysteine non-animal source were purchased from Sigma (Germany). Potassium phosphate buffer solutions were purchased from Fluka and Merck (Germany). Dimethylsulfoxide (DMSO) used in reaction mixture was purchased from Carlo-Erba Reagents (Spain).

2.2. Preparation of raw plant materials and extraction procedure

The leaves of *C. parviflorus* and *V. agnus-cactus* were washed, dried and ground. The leaves of these two plants were extracted with 70% aqueous ethanol solution at 30 °C for 2 h. After evaporation

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of alcohol by using a rotary evaporator, the aqueous crude extracts were dried by using lyophilizator. The unshelled seeds of *P. harmala* were ground. The ground seeds were extracted with methanol by using a Soxhlet apparatus at 70 °C for 8 h. Then, methanol was evaporated to dryness. Dried particles were dissolved with 5% HCl solution and it was extracted two times with 30 ml petroleum ether, the acidic part was collected and basified to pH 9.0 with NH₄OH which was further extracted with 50 ml chloroform for four times. Finally, chloroform layer was collected and evaporated to dryness.

2.3. Determination of enzyme activity and inhibition study

Enzymatic assays were performed according to the method reported by J. Veser's and M. Kurkela et al.'s studies with minor differences by using Microfluor white, 96 well plate [8,9]. The followed reaction which was catalyzed by COMT was conversion of aesculetin to scopoletin at 37 °C. Aesculetin was dissolved in dimethyl sulfoxide (DMSO) and diluted with aqueous buffer solution containing 100 mM phosphate, 5 mM MgCl₂, 20 mM L-cysteine (pH 7.4), for a final DMSO concentration of 2% in the 200 μl of reaction mixture. All other reagents were dissolved in the same buffer solution. Fluorometric measurements were performed at 355 nm emission and 460 nm excitation wavelengths for 2 h by using Thermo Varioskan Flash microplate reader. Enzyme concentration was kept constant at 11.0 μg/ml while five different SAM and five different aesculetin concentrations varied from 10 μM to 100 μM and from 2 μM to 6 μM, respectively. 3,5-DNC and the crude extracts obtained from plant species were dissolved in DMSO and diluted with the buffer solution for a final DMSO concentration of 2%. Final concentrations of inhibitors in 250 μl total reaction volume were given on resulting figures.

2.4. Curve fitting and data analysis

In data analysis, reciprocals of velocities and substrate concentrations gave the linear relationship by which K_m and V_{max} values could be calculated. The kinetic mechanism and inhibition constants were obtained by fitting the initial steady state velocity as a function of substrate concentration to the following equations defined in R.A. Copeland's book using nonlinear regression analysis program in GraphPad Prism 5.0 software [10]. The goodness of curve fit was evaluated statistically by one-way of ANOVA following with Tukey's multiple comparison test.

$$v = \frac{V_{max}[S]}{K_m + [S](1 + ([I]/\alpha K_i))} \quad \text{uncompetitive inhibition}$$

$$v = \frac{V_{max}[S]}{K_m(1 + ([I]/K_i)) + [S]} \quad \text{competitive inhibition}$$

$$v = \frac{V_{max}[S]}{K_m(1 + ([I]/K_i)) + [S](1 + ([I]/K_i))} \quad \text{noncompetitive inhibition}$$

$$v = \frac{V_{max}[S]}{K_m(1 + ([I]/K_i)) + [S](1 + ([I]/\alpha K_i))} \quad \text{mixed type inhibition}$$

where V_{max} is the maximum enzyme velocity without inhibitor. K_m is the Michaelis–Menten constant without inhibitor. K_i is the inhibition constant indicating the dissociation of EI complex. AlphaK_i (αK_i) is also inhibition constant indicating the dissociation of ESI complex. In this term, Alpha (α) determines the mechanism by revealing the binding degree of the inhibitor which explains the affinity of enzyme for substrate [10]. At very high α values that is much greater than 1, mechanism is explained by competitive inhibition and very low α values ($\alpha \ll 1$) indicate uncompetitive

inhibition. If it is around 1 ($\alpha < 1$ or $\alpha > 1$) at a certain value, mechanism can be described as mixed type inhibition which explains the deviation from noncompetitive and competitive nature [10]. If it is equal to 1, noncompetitive inhibition takes place.

3. Results and discussion

3.1. Kinetic analysis to determine enzyme–substrates relationship

Before performing inhibition study, a previous study was performed by changing substrates and enzyme concentrations in order to obtain the linear working concentration ranges of enzyme and also substrates. Enzyme concentrations were kept constant at 16.5–11.0–8.5 μg/ml for this experiment while seven different SAM concentrations varied from 10 μM to 600 μM were used against six different ES concentrations prepared at the range of 2–10 μM. As a result, it was found that the concentration of SAM should be kept lower than 200 μM and the lowest enzyme concentration was determined as 11.0 μg protein/ml. It was also revealed that the used concentration range of ES did not deviate from linearity. From this experiment, K_m values both for SAM and ES was obtained as 3.5 ± 0.3 μM and 6.4 ± 0.4 μM which was reported as 6.2 μM in Veser's study [8].

3.2. Kinetic analysis for inhibition study

In inhibition study, ES concentration was kept constant at 4 μM in 250 μl total reaction volume while SAM concentrations were changed from 10 μM to 100 μM to determine the inhibition performance with respect to SAM. Besides, inhibition with respect to ES was investigated by varying its concentrations from 2 μM to 6 μM at constant SAM concentration of 100 μM. Enzyme concentration was kept constant at 11.0 μg/ml for the whole assay. A representative percentage values of COMT inhibition obtained for the assay performed at 100 μM SAM concentration were given in Fig. 1. Inhibition performances of crude extract of alkaloids and their standards were found as comparable to that of obtained for positive control, 3,5-DNC. In Fig. 1, it was also revealed that among polyphenolic extracts, *C. parviflorus* leaf extracts showed higher inhibition than that of *V. agnus-cactus*. As a result, alkaloids were found to be more potent COMT inhibitors than the polyphenolics and it was thought that nitro groups content of them plays important role in COMT inhibition.

Michaelis kinetic calculations supported these findings. A sharp decrease of formation rate of scopoletin, V (nmol/min mg protein) was observed in presence of the positive control. The nonlinear fitting of data given in Fig. 2A obtained by using GraphPad software which was supported also by the reciprocal plot (Fig. 2B) demonstrated the uncompetitive nature of the positive control, as expected. Because, almost all nitrocatechols are known that they behave uncompetitively with respect to SAM and tight binding inhibitors of COMT [5]. From these kinetic data, the K_m value was calculated as 6.83 ± 0.77 μM and V_{max} was found as 1.07 ± 0.02 nmol/min. mg protein. Dissociation constant (αK_i) for 3,5-DNC which was an uncompetitive inhibitor was obtained as 9.17 ± 0.46 ng/ml (i.e., 45.60 ± 2.29 nM). Also, IC₅₀ was calculated as 44.18 ± 0.78 nM from dose response curve of 3,5-DNC which was very similar to that value reported in Kurkela et al.'s study as 35 nM [9].

Moreover, the nonlinear fit of data obtained for alkaloid extract was given in Fig. 3A and reciprocals of velocities were given in Fig. 3B. Dissociation constant, αK_i was found as 2.24 ± 0.75 μg/ml where α was equal to 18.72 ± 2.28. Based on the criteria given in Section 2.4, the inhibition mechanism for alkaloid extract was determined as mixed type inhibition with respect to SAM unlike the uncompetitive mechanism found for positive control, 3,5-
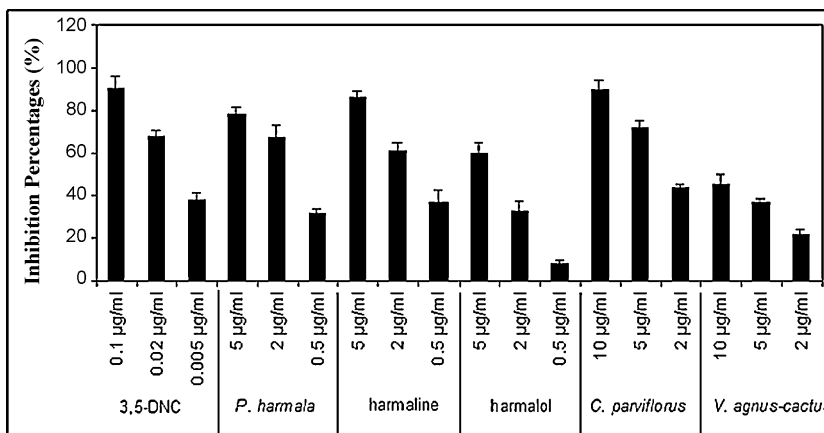
Fig. 1. Inhibition percentages of crude extracts and alkaloid standards with respect to 3,5-DNC. (SAM concentration was 100 μM.)
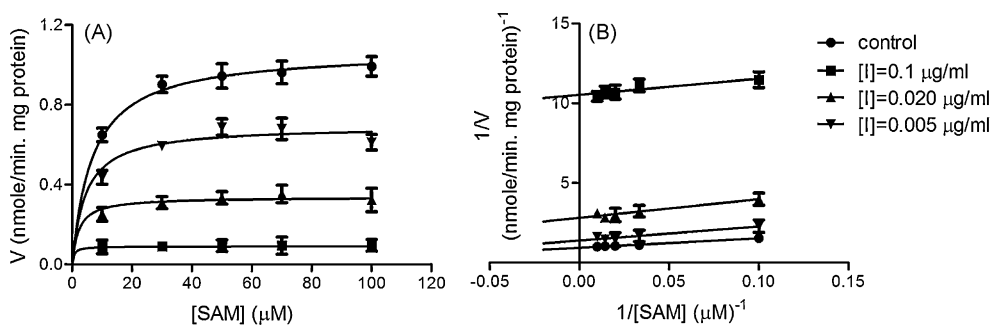

Fig. 2. (A) Plot of formation rate of scopoletin; (B) double reciprocal plot of formation rate against various SAM concentrations in presence of 3 different 3,5-DNC concentrations ($P < 0.01$, means of inhibited ones are significantly different from control).

DNC. Besides, the inhibitory effect of this natural alkaloid extract was found significantly lower than that of 3,5-DNC. From the kinetic data, the K_m and V_{max} values were also determined as 6.05 ± 0.69 μM and 1.06 ± 0.02 nmol/min. mg protein, respectively. IC$_{50}$ of alkaloid extracts was calculated as 1.09 ± 0.33 μg/ml.

It was known that *P. harmala* contains several β-carbolines like harmaline, harmine, harmalol, harmane, vasicinone, vasicine, etc. [11,12]. It was also reported that among these alkaloids harmaline and harmine are known as monoamineoxidase (MAO-B) inhibitors and they have several types of activities such as antioxidant, antiviral and anticancer [12,13]. Our HPLC analysis has shown that the major alkaloid in this *P. harmala* seed extract was harmaline. Therefore, in this study harmaline and harmalol standards were used to determine which type of alkaloids could be responsible for the inhibitory effect. In Figs. 4 and 5, the results obtained for these two alkaloid standards were given. As expected, harmaline strongly inhibited COMT by mixed type manner and its performance was found higher than that of harmalol which inhibited COMT by uncompetitive manner like positive control, 3,5-DNC.

From the data given in Figs. 4 and 5, it was found that the obtained dissociation constant for harmaline were closer to that of crude alkaloid extract compared to those determined for harmalol. The αK_i value for harmaline were found as 1.36 ± 0.11 μg/ml having α value of 2.49 ± 0.78. The lower α value obtained for harmaline compared to that of found for *P. harmala* extract (18.72 ± 2.28) can be explained by shift in the dominant inhibition mechanism from competitive to noncompetitive. The total net inhibitory effect was reflected and explained by mixed type inhibition. The αK_i of harmalol was obtained as 3.21 ± 0.16 μg/ml. Besides, IC$_{50}$ values of harmaline and harmalol were calculated as 0.98 ± 0.12 μg/ml (4.57 ± 0.14 μM) and 3.59 ± 0.37 μg/ml (13.19 ± 0.31 μM), respectively.

It was reported by several researchers that polyphenolics mainly tea catechins, gallic acid and hydroxybenzoic acid derivatives have
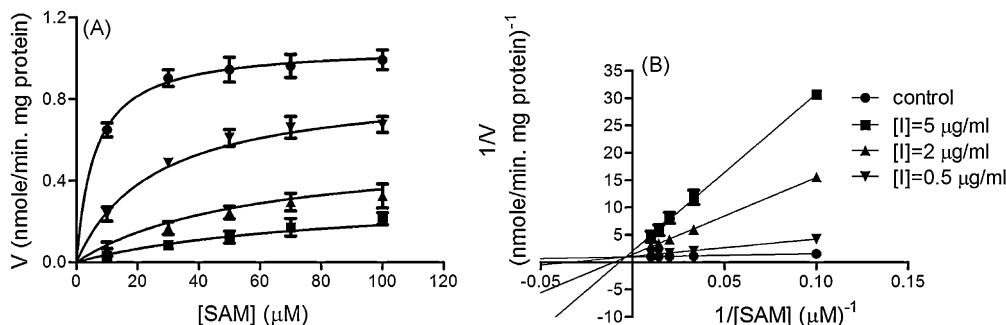

Fig. 3. (A) Plot of formation rate of scopoletin; (B) double reciprocal plot of formation rate against various SAM concentrations in presence of 3 different concentrations of crude alkaloid extracts obtained from *P. harmala* seed ($P < 0.01$, means of inhibited ones are significantly different from control).
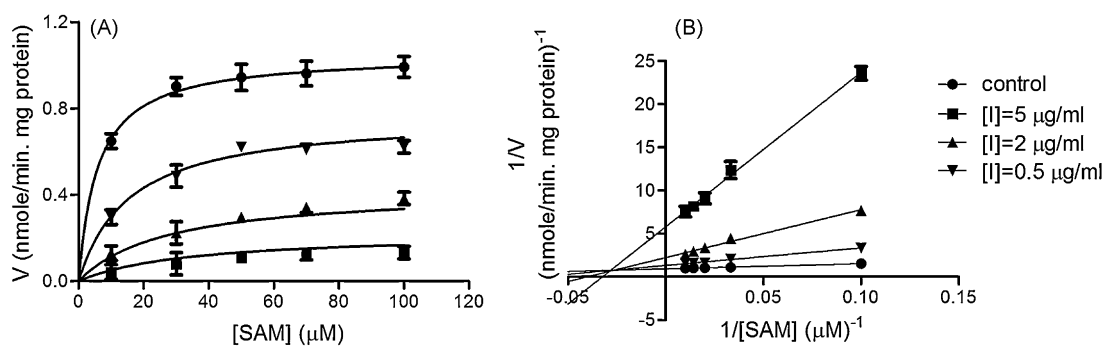
Fig. 4. (A) Plot of formation rate of scopoletin; (B) double reciprocal plot of formation rate against various SAM concentrations in presence of 3 different concentrations of harmaline ($P < 0.01$, means of inhibited ones are significantly different from control).
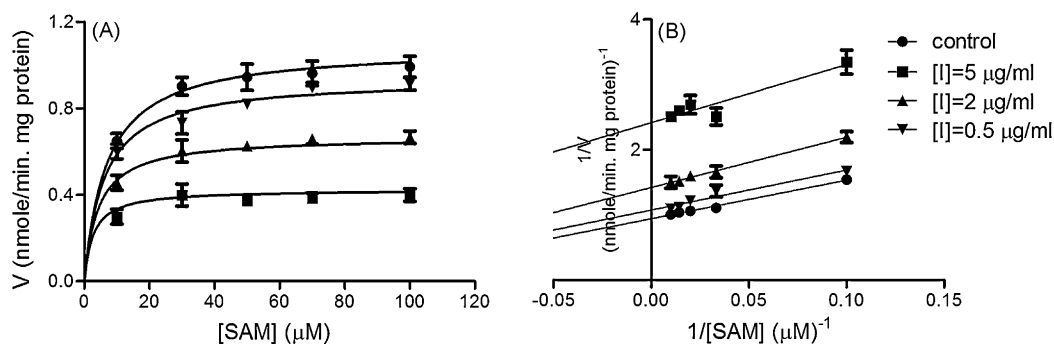

Fig. 5. (A) Plot of formation rate of scopoletin; (B) double reciprocal plot of formation rate against various SAM concentrations in presence of 3 different concentrations of harmalol ($P < 0.01$, means of inhibited ones are significantly different from control).

inhibitory effect on COMT [4,7,16]. Therefore, extracts of *V. agnus-cactus* and *C. parviflorus* leaves having high polyphenol content were used in inhibition study. As seen in Fig. 1, the crude alkaloid extract and its standards showed higher inhibition potency than polyphenolic extracts obtained from *V. agnus-cactus* and *C. parviflorus* leaves.

Scopoletin formation rate was also decreased in presence of *V. agnus-cactus* extract. Mechanism was determined as mixed type with respect to SAM from nonlinear fit of data given in Fig. 6A supported with double-reciprocal plot of formation rate that was given in Fig. 6B. Dissociation constant, αK_i was found as $9.48 \pm 0.58\ \mu g/ml$ where α was 2.14 ± 0.23 that indicates inhibition was obviously closer to noncompetitive when the criteria given in Section 2.4 was taken into consideration. The apparent K_m and V_{max} values were calculated as $5.84 \pm 0.90\ \mu M$ and $1.03 \pm 0.02\ nmol/min.$ mg protein. From dose-response curve, IC_{50} value for *V. agnus-cactus* was determined as $12.96 \pm 0.29\ \mu g/ml$. Inhibition performance of *V. agnus-cactus* extract was relatively lower than that of *P. harmala*

seed extract. This was an expected result, since the main polyphenol content of *V. agnus-cactus* extract was gallic acid, p-hydroxybenzoic acid, rutin and ferulic acid which were known the examples of first generation COMT inhibitors. It was also known that the inhibition performances of them much lower than that of nitrocatechols [5,16]. Beside that, this plant contains some estrogenic compounds like linoleic acid and also halimane and labdane type diterpenoids which were also thought having possibility of inhibitory effect [14].

Similar observations were obtained for the crude extracts of *C. parviflorus* leaves. Changes in the formation rate against different SAM concentrations in absence and presence of inhibitors were given in Fig. 7. From the reciprocal plot (Fig. 7B) of the nonlinearly fitted curves (Fig. 7A), it was obtained that *C. parviflorus* leaf extract inhibited COMT also in mixed type manner with respect to SAM like it was found for the other plant extracts used in this study. The αK_i value for *C. parviflorus* leaf extracts was found as $1.99 \pm 0.35\ \mu g/ml$ and α was calculated as 0.79 ± 0.32. The closest α value to 1.0 supported inhibition was more similar to noncompetitive profile
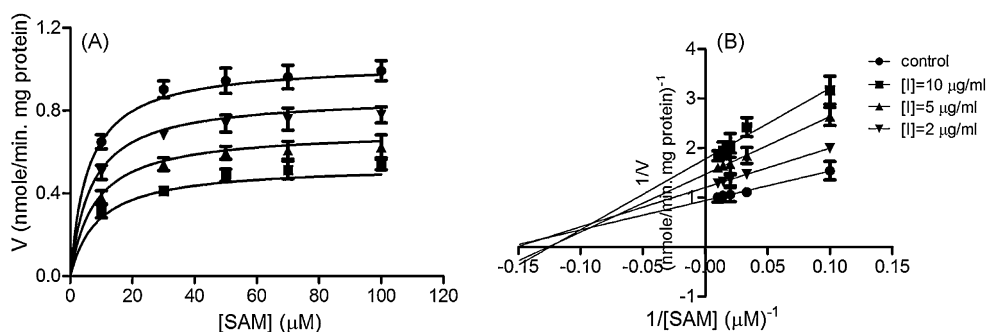

Fig. 6. (A) Plot of formation rate of scopoletin; (B) double reciprocal plot of formation rate against various SAM concentrations in presence of 3 different concentrations of crude extracts obtained from *V. agnus-cactus* leaves (means obtained for different inhibitor concentrations are significantly different from each other and from control ($P < 0.01$)).
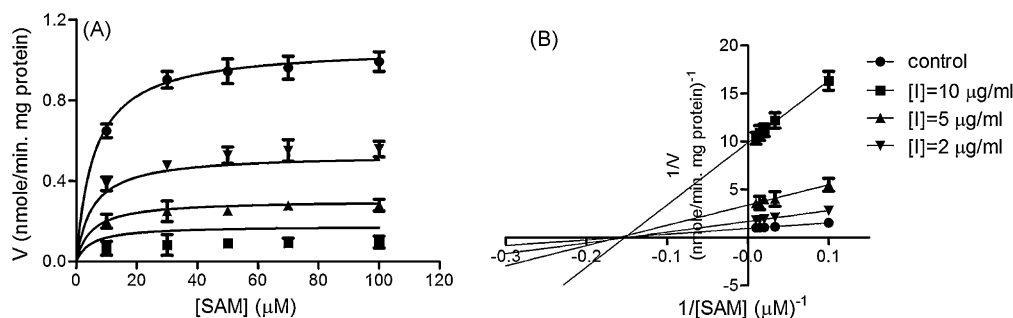
Fig. 7. (A) Plot of formation rate of scopoletin; (B) double reciprocal plot of formation rate against various SAM concentrations in presence of 3 different concentrations of crude extracts obtained from *C. parviflorus* leaves (means obtained for different inhibitor concentrations are significantly different from each other and from control ($P < 0.01$)).

for *C. parviflorus* leaf extracts as seen from double-reciprocal plot given in Fig. 7B. The apparent K_m and V_{max} values were obtained $6.02 \pm 1.29\,\mu M$ and 1.06 ± 0.04 nmol/min mg protein, respectively. IC_{50} value for this plant extract was found as $2.44 \pm 0.09\,\mu g/ml$ which revealed that this plant extract showed 5 times stronger inhibitory activity than that of *V. agnus-cactus,* however it was about 2 times lower when it was compared to the extracts of *P. harmala.*

The known high terpenoid and flavonoid content of leaves of *C. parviflorus* were thought to be responsible for high inhibitory effect which could be caused by the relaxation activity reported by other researchers [17]. Also, it was known that this plant composed of numerous terpenoid essential oils like carvacrol, thymol, manoyl oxides and also esters like sabinene hydrate acetate, benzyl benzoate [15]. However, in order to determine the found inhibitory effect of this polyphenolic extract on COMT, further investigations are still needed.

4. Conclusion

The alkaloid extracts obtained from *P. harmala* seed were found as the most potent inhibitors among the three different plant species. However, the inhibition performance of *P. harmala* seed extract was significantly less than the performance of 3,5-DNC. The relatively high harmaline content of crude extracts obtained from *P. harmala* seed was thought to be responsible for the observed inhibitory effect. The inhibition mechanisms for both extract and harmaline were explained by mixed type inhibition model while it was determined as uncompetitive for 3,5-DNC.

On the other hand, the polyphenolic extracts of *C. parviflorus* and *V. agnus-cactus* leaves were found as less effective COMT inhibitors than alkaloids. Among these polyphenolics, the extract of *C. parviflorus* showed higher inhibitory effect than that of *V. agnus-cactus*. Both polyphenolic extracts inhibited COMT in mixed type manner with respect to SAM having different α values.

As a result of this study, it was observed that natural compounds obtained from *P. harmala* and *C. parviflorus* can be an alternative source of medicine in treatment of Parkinson's disease.

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