

Cholinesterase, α -Glucosidase, α -Amylase, and Tyrosinase Inhibitory Effects and Antioxidant Activity of *Veronica officinalis* Extracts

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Abstract: In this study, inhibition ability of *Veronica officinalis* extracts against Alzheimer's disease-related enzymes acetylcholinesterase (AChE) and butyrylcholinesterase (BuChE), diabetes mellitus related enzymes α -glucosidase and α -amylase and antioxidant of *Veronica officinalis* were investigated. To the best of our knowledge, there are no previous studies on the enzyme inhibition activities of the *V. officinalis* extracts. For this aim, *V. officinalis* extracted with methanol and water by maceration method and their antioxidant activities were evaluated by DPPH (2,2-Diphenyl-1-picrylhydrazyl), ABTS [2,2'-Azino-bis-(3-ethylbenzothiazoline-6-sulphonic acid)] radical scavenging assays, total phenol, total flavonoid content, iron chelating and ferric reducing power assays. The enzyme inhibition activity was performed with 96-well plate method. According to the obtained results; the plant extracts have good antioxidant effects. In the same time, the water extract have stronger inhibition activity against AChE, BuChE and α -glucosidase, α -amylase, and tyrosinase than methanol extract. The experimental findings indicated that the water extract exerted the higher α -glucosidase, α -amylase and tyrosinase (82.07 ± 2.91 , 63.61 ± 3.93 and 47.47 ± 0.53 at 2 mg mL^{-1} , respectively) inhibition than reference drugs. The obtained results demonstrate that this plant has a significant potential for improving pharmaceutical formulations.

Keywords: Antioxidant, alzheimer's, diabetes, tyrosinase, *Veronica officinalis*

1. Introduction

Veronica L. genus belongs to *Plantaginaceae* family, and is characterized by 450 species. These species are annual and perennial plants and located in Europe and Turkey. The aerial parts of *Veronica* species are used for the treatment of cough, rheumatism and have wound-healing properties (Albach and Meudt, 2010; Beara et al., 2015; Mocan et al., 2015; Raclariu et al., 2017). According to the previous data on phytochemical investigations, *Veronica* species have different constituents such as iridoid glucosides, especially benzoic and cinnamic acid esters of catalpol, some phenylethanoid and flavonoid glycosides (Taskova et al., 2002; Taskova et al., 2006; Harput et al., 2011).

The oxygen radical derivatives and peroxides-like reactive species cause oxidative stress and

reactive free radicals can oxidize proteins, lipids, and nucleic acids. As a result, various diseases such as neurodegenerative, cardiovascular, inflammation are emerging as a result of oxidative stress. (Halliwell, 1987; Emerit et al., 2004; Junqueira et al., 2004; Kiss et al., 2009). Medicinal plants that are rich in terms of polyphenols, can retard the oxidative degradation of lipids. Flavonoids are phenolic components and can prevent some cardiovascular and inflammatory diseases (Havsteen, 2002; Rice-Evans, 2004).

Tyrosinase is a copper-containing proteolytic enzyme that allows o-hydroxylation of monophenols and then oxidation of the o-quinones from the o-diphenols (Zhou et al., 2017). This enzyme is playing an important role in melanin synthesis in mammalian cells and browning in plants and microorganisms. However, excessive production and accumulation of melanin in the

human skin lead to the formation of hyperpigmentation. Therefore, control the overproduction of melanin by using tyrosinase inhibitory agents is a therapeutical strategy for hyperpigmentation in the skin.

Alzheimer's disease (AD) is a neurodegenerative chronic disease related to decreasing of brain transmitter choline. Acetylcholinesterase and butyrylcholinesterase are involved in the hydrolysis of acetylcholine to choline and acetic acid. Therefore, cholinesterase inhibitors have been used in the treatment of AD (Ertaş et al., 2014). However, the side effects of cholinesterase inhibitors make it necessary to search for new, potential and alternative cholinesterase inhibitors from natural sources.

Diabetes mellitus is a multifactorial disease leading to severe complications. Therefore, multiple therapeutic approaches are used in the treatment. In diabetic patients, postprandial hyperglycemia occurs after a meal due to glucose absorption from the gastrointestinal tract. Preventing glucose uptake in the intestines and promoting glucose uptake in tissues can control the level of blood glucose in the case of postprandial hyperglycemia, which is common for people with diabetes (Thilagam et al., 2013). When the literature were evaluated, there was a little study of biological and enzyme activity on *V. officinalis*. For this reason, we planned to evaluate the *V. officinalis* for potential inhibitory activities against α -glucosidase, α -amylase, cholinesterase, and tyrosinase as well as its *in vitro* antioxidant activities.

2. Materials and Methods

2.1. Plant material

This study was conducted in the Laboratories of Faculty of Pharmacy, Cumhuriyet University in Sivas in the 2018 year. Leaves of *V. officinalis* were collected from Cumhuriyet University Campus.

2.2. Preparation of extracts

The plant material was powdered with a grinder after dried in shade. 10 gr of the leaves were soaked in 50 mL of methanol (Sigma) and water for 24h with intermittent shaking. At the end of extraction, it was filtrated by No. 1 Whatman filter paper. The filtrate was concentrated to dryness under reduced pressure with a rotary evaporator at 40°C and this process was repeated for three times. The residue of plant material then subjected to maceration with water as solvent and after filtering dried under vacou to yield water extract. The obtained extracts were analyzed by GC-MS (Gas Chromatography-Mass Spectrometry). The obtained methanol and

water extracts were used for evaluation of the antioxidant potential and enzyme inhibition activity as well as chemical composition through GC-MS.

2.3. *In-vitro* antioxidant activity

The experiments were performed in completely randomized design with three replications.

2.3.1. DPPH radical scavenging activity

The free radical scavenging activity of the extracts was conducted according to the method reported previously (Miser-Salihoglu et al., 2013). The 150 μ L of methanol extract was mixed with 50 μ L of 1.0×10^{-3} M DPPH solution freshly prepared in methanol. Methanol was used as a control of the experiment. After incubation for 30 min at room temperature (25°C), the reduction of the DPPH free radical was measured by spectrophotometer at 517 nm. Trolox was used as positive control. The antioxidant activity as a percentage (%) of inhibition was calculated using the formula given below (Equality 1):

$$\% \text{ Inhibition} = \left[\frac{\text{Absorbance of control} - \text{Absorbance of test sample}}{\text{Absorbance of control}} \right] \times 100 \quad (1)$$

2.3.2. ABTS radical scavenging activity

For determination of ABTS radical scavenging activity of the extracts, followed by the method of Re et al. (1999) with slight modification. The stock solution of ABTS was generated by reacting 7 mM ABTS solution with 2.4 mM of potassium persulfate solution in equal volume for 16h. Working solution was then prepared by diluting the stock ABTS solution with methanol to give an absorbance of 0.7 ± 0.02 units at 734 nm using a spectrophotometer. In each assay, the ABTS solution was prepared freshly. 50 μ L of extract was mixed with 150 μ L ABTS working solution and left for 10 min in darkness. All the analyses were performed in triplicate and the results expressed as a mean \pm standard deviation. Appropriate blanks (methanol) and standard [BHT (Butyl hydroxy tolüen) in methanol] were run simultaneously. The absorbance was measured at 734 nm in a microplate reader (Epoch, USA).

2.3.3. Determination of total phenolics (TPC, total phenolics content)

The spectrophotometric Folin-Ciocalteu (F-C) method was used to measure the TPC of the extracts, as previously described by Clarke et al. (2013). Briefly, 20 μ L of extract diluted appropriately in DMSO (Dimethyl sulfoxide) was mixed with 100 μ L F-C reagent freshly diluted 1/10 with distilled water. After five minutes, the solution was mixed with 80 μ L 7.5% Na_2CO_3 solution, and

incubated for 30 min at 25°C. The absorbance was measured at 650 nm with a microplate reader (Epoch, USA). All the analyses were performed in triplicate and the results expressed as a mean \pm standard deviation. Appropriate blanks (DMSO) and standard (gallic acid in DMSO) were run simultaneously, after which the TPC was calculated as milligrams gallic acid equivalents per gram of dry extract.

2.3.4. Estimation of total flavonoids (TFC, total flavonoid concentration)

The aluminum chloride colorimetric method was used for the determination of TFC, as previously described by Molan and Mahdy (2014), using catechin as the reference standard. Briefly, 25 μ L of test sample solution with a concentration of 1 mg/mL, 100 μ L of doubled distilled H₂O and 7 μ L of NaNO₂ 5% were mixed together in 96-well plates. After 15 min of incubation at room temperature, 7 μ L of 10% (w/v) AlCl₃ was added. 5 min later, 50 μ L of 1 M NaOH and 60 μ L of distilled water were added to each well. Then the absorbance was measured at 490 nm with a spectrophotometer. All determinations were carried out in triplicates. The content of total flavonoids was expressed as mg of catechin equivalent per g of dry weight of the extract.

2.3.5. Iron chelating activity

The iron chelating activity of the extracts was determined according to their interaction with the formation of the ferrozine-Fe²⁺ complex. A previously described procedures were used (Chai et al., 2014). Briefly, a mixture of 0.2 mL of 0.1 mM FeSO₄ [Iron (II) sulfate], 0.2 mL of extract, and 0.4 mL of 0.2 mM ferrozine was allowed to react at room temperature. Absorbance was read after 10 min of incubation at 562 nm.

2.3.6. Ferric reducing antioxidant power (FRAP) assay

FRAP assay uses antioxidants as reductants in a redox-linked colorimetric method. The FRAP assay was conducted according to the previously reported method with slight modification (Wojdylo et al., 2007; Yang et al., 2011). Each extract was dissolved in DMSO to prepare a stock solution. The working FRAP reagent was prepared by freshly mixing 0.3 M acetate buffer (pH 3.6), a solution of 10 mM TPTZ (2,4,6-tripyridyl-s-triazine) in 40 mM HCl and 20 mM FeCl₃ solution at a ratio of 10:1:1. 30 μ L of the sample solution and 270 μ L of FRAP working solution were mixed together in 96-well plates and warmed at 37°C for 4 min. All determinations were performed in triplicate. The absorbance was measured at 593 nm. A standard

calibration curve was prepared using different concentrations of FeSO₄ solution. All solutions were prepared freshly. Trolox was used as reference. The results were expressed as FRAP value.

2.4. Enzyme inhibitory activity

2.4.1. Acetylcholinesterase (AChE)/ Butyrylcholinesterase (BChE) inhibition assay

A 96-well plate colorimetric method described by Ellman et al. (1961) was used for determining of the AChE inhibitory activity of the plant extracts. The experiments were carried out with three replications. Galanthamine was used a positive control, while the buffer was used as a blank. Briefly, 140 μ L of 0.1 mM phosphate buffer (pH 6.8), 20 μ L of extracts prepared in the buffer from 50 mg mL⁻¹ stock sample solution in DMSO, 20 μ L AChE from Electric eel (5 \times 10⁻³M)/BuChE from equine serum (5 \times 10⁻³M) was added in each well of microtiter plate. After 10 min of incubation, 10 μ L of 3 mM DTNB was added. Then the reaction was initiated with adding of 10 μ L of 0.71 mM acetylcholine iodide (AChI)/0.2mM Butyrylcholine iodide (BChI), absorbance was measured at 412 nm with a microplate reader (Epoch, USA).

2.4.2. Alpha-glucosidase inhibition activity

Alpha-glucosidase inhibition method was followed by Kumar et al. (2012). Acarbose was used as a positive control, while phosphate buffer was used as negative control in place of the sample. Each concentration was carried out in triplicate. 25 μ L of sample solution was mixed with 25 μ L of α -glucosidase (0.5 U mL⁻¹), then incubated for about 10 min at 25°C. After incubation 25 μ L of 0.5 mM PNPG was added to each well as substrate and incubated further 30 min at 37°C. After the incubation period, 100 μ L of 0.2 M sodium carbonate was added to terminating the reaction and the absorbance was read at 405 nm.

2.4.3. Alpha-amylase inhibition activity

Alpha-amylase inhibition method was followed by Kumar et al. (2013). Acarbose was used as a positive control, while phosphate buffer (0.02 M PBS, pH 6.9) was used as negative control in place of sample. Each sample was carried out in triplicate with different concentrations. The reaction mixture containing 50 μ L of sample solution, 25 μ L of α -amylase (0.5 mg/mL) incubated for about 10 min at 25°C. Then 50 μ L of 0.5% starch solution (w/v) prepared freshly was added to each well as substrate and incubated further 10 min at 25°C. After the incubation period, 100 μ L of 1% DNS (3,5-dinitrosalicylic acid), the color reagent was added

as a color reagent and heated at water a bath for 10 min. The absorbance was read at 540 nm.

2.4.4. Tyrosinase enzyme inhibition activity

Tyrosinase enzyme inhibition method was followed by Jeong et al. (2009). In 96 well plate, 20 μ L of sample solution diluted with buffer, and 100 μ L of phosphate buffer were mixed with 20 μ L of tyrosinase (250 U mL⁻¹) in each well and incubated for about 10 min at 25°C. Then 20 μ L of 3 mM L-tyrosine was added as a substrate and incubated further 30 min at 25°C. After the incubation period, the absorbance was read at 492 nm. Kojic acid was used as a positive control, while phosphate buffer (100 mM PBS, pH= 6.8) was used as a negative control in place of sample. Each sample was carried out in triplicate with different concentrations.

2.5. Statistical analysis

Data obtained from *in vitro* antioxidant and enzyme inhibition activity were expressed as the mean \pm standard deviation (SD). The % inhibitory activity of the extract and reference compounds were calculated through extract dose-response curve by GraphPad Software (San Diego, CA, USA).

3. Results and Discussion

3.1. Chemical composition of the extracts

The chemical compositions of water and methanol extracts obtained from leaves of *V. officinalis* have been determined (Table 1). When methanol and water extracts were compared in terms of content, methanol extract was found to have a higher component content. While the most

Table 1. Chemical components of water and methanol extracts of *Veronica officinalis*

Components	Retention time (min)	Water (%)	Methanol (%)
2-Furanmethanol (CAS)	6.743		3.81
1,2-Cyclopentanedione	8.941		1.51
4-Hydroxy-6-methyl-2H-pyran-2-one	15.424		1.30
4H-Pyran-4-one, 2,3-dihydro-3,5-dihydroxy-6-methyl- (CAS)	16.865		1.28
1,4:3,6-Dianhydro-.alpha.-d-glucopyranose	18.565	6.24	
1,4-Benzenediol (CAS)	20.865	9.30	
2-Methoxy-4-vinylphenol	22.502		2.41
Phenol, 2,6-dimethoxy-	24.121	1.86	
Phenol, 2,6-dimethoxy-	24.184		5.77
Cyclopropane, nonyl-1-Tridecanol	28.241		4.72
Phenol, 2,4-bis(1,1-dimethylethyl)-	29.316		3.23
2,6-Dimethyl-3-(methoxymethyl)-p-benzoquinone	30.695		3.41
(-)-Caryophyllene oxide	31.205		1.32
1,5-Cycloundecadiene, 8,8-dimethyl-9-methylene-	33.196		3.82
Cyclododecane	33.448		4.70
Neophytadiene	36.206		1.62
2-Pentadecanone, 6,10,14-trimethyl	36.326		1.18
6-Octen-1-ol, 3,7-dimethyl-, propanoate	36.961		1.09
(4S,5R)-5-Hydroxycaryophyll-8(13)-ene-4,12-epoxide	37.333		0.99
Hexadecanoic acid, methyl ester (CAS)	37.705		0.85
(+) spathulenol	38.151		1.65
Hexadecanoic acid (CAS)	38.426		6.60
Aromadendrene oxide-(2)	38.603		0.93
3,4'-Difluoro-4-Methoxybiphenyl	39.215		1.43
(3E,5E,8E)-3,7,11-Trimethyl-1,3,5,8,10-dodecapentaene	39.381		0.56
2,2,6-Trimethyl-1-(2-methyl-cyclobut-2-enyl)-hepta-4,6-dien-3-one	40.383		3.74
1-(3'-methyl-2'-cyclobutenyl)-2,2,6-trimethyl-4,6-heptadien-3-one	40.726		3.10
Cyclododecane	41.167		1.27
Isoxeniolide-A	45.372		0.71
Isosteviol methyl ester	45.904		0.88
Lactic Acid Di-TMS	47.060		1.26
Silane, Trimethyl 5-Methyl-2- 1-Methylethyl Phenoxy -	55.168	0.64	
Total		18.04	65.14

abundant components are 1,4:3,6-dianhydro-.alpha.-d-glucopyranose (6.24%) and 1,4-benzenediol (9.30%) for water extract, are Hexadecanoic acid (6.60%) and Phenol, 2,6-dimethoxy- (5.77%) for methanol extract (Table 1).

3.2. In-vitro antioxidant activity

Natural antioxidants present in plants can be inhibited or prevent reactive oxygen species, by the way, protect the human body from oxidative stress. In this study, we evaluated the antioxidant activities

of water and methanol extracts of *V. officinalis* leaves using DPPH and ABTS radical scavenging assays. In both assays, the decrease in the absorbance of free radical causes by the natural antioxidants present in the extract which can be scavenged radical by hydrogen donation (Supriya and Radha, 2018). According to our results which were shown in Figure 1, the methanol extract of *V. officinalis* leaves showed a higher percentage of DPPH radical scavenging activity in lower concentration in comparison to water extract. However, in ABTS radical scavenging, both the methanol and water extract showed the same effect which was comparable with the positive control of BHT.

Phenolic compounds are primarily responsible for the antioxidant activity of plants. In our study, the total phenolic contents of the water and methanol extracts of *V. officinalis* are shown in Figure 1, which were found as 14.26 and 40.93 mg GAE/g (GAE: Gallic acid equivalent) dry weight of

extract, respectively. Total phenolic contents of both of the extracts showed higher values than those reported in three *Veronica* species that was presented TPC values expressed as gallic acid equivalent ranging 17.71-34.40 mg g⁻¹ (Mocan et al., 2015). The obtained results showed that the difference in the amount of phenolic compounds may be dependent on the geographical environment, collection time, drying and extraction method, as well as other genetic factors.

In our study, the total flavonoid content of water and methanol extracts of *V. officinalis* was detected as 9.98 and 12.57 mg CE g⁻¹, respectively. Our results revealed that higher concentrations of TFC as compared to those reported in tree *Veronica* species that their TFC were expressed as quercetin equivalents ranged from 2.52 to 6.60 mg g⁻¹ (Mocan et al., 2015). The variations in the content of flavonoids may be due to the growing habitat, investigated plant parts and extraction solvent differences.

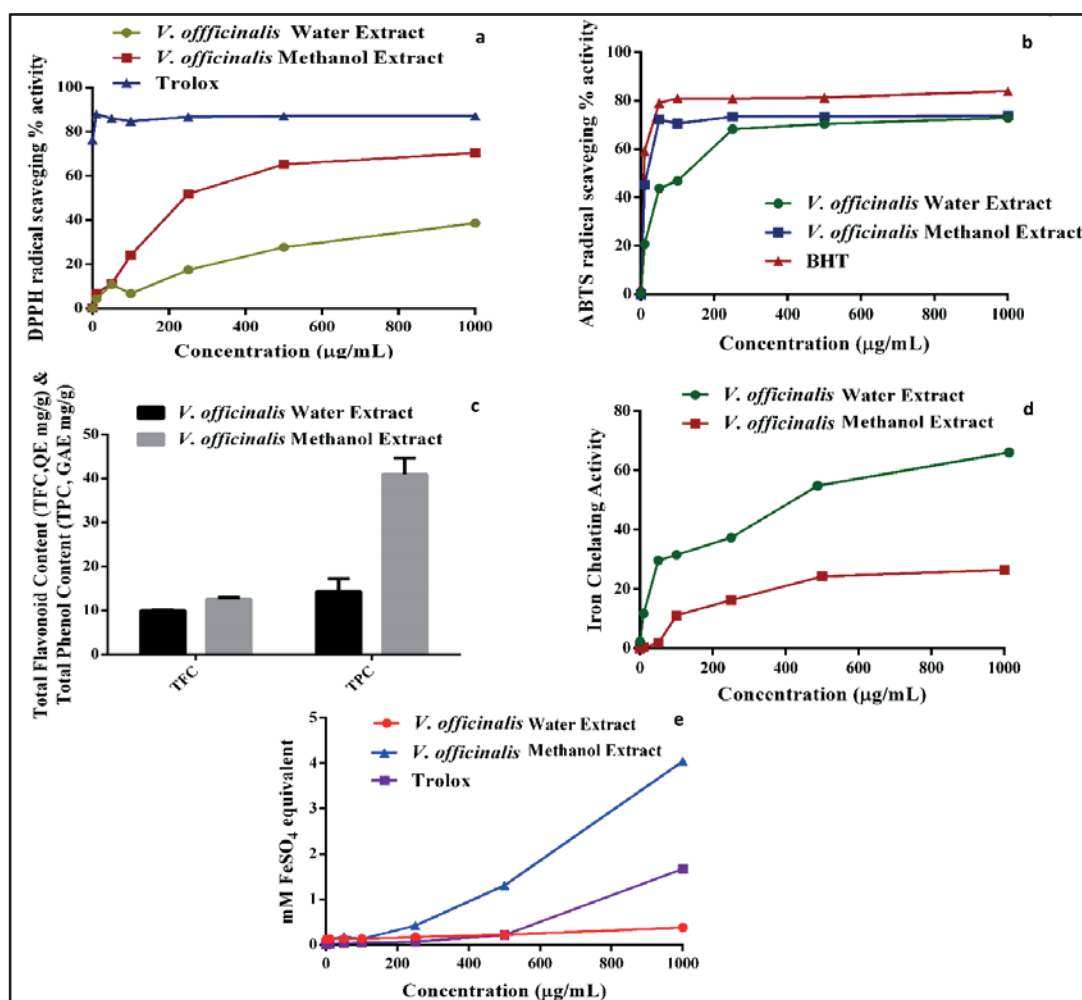


Figure 1. The *in-vitro* antioxidant activity of methanol and water extracts of *V. Officinalis*. a) DPPH radical scavenging activity, b) ABTS radical scavenging activity, c) total phenol and total flavonoid contents, d) iron chelating % activity, e) ferric reducing antioxidant power as FeSO₄ equivalent

Antioxidant activity of herbal extracts resulting from various compounds by different kinds of antioxidant mechanisms such as radical scavenging, delaying peroxidation, iron chelating etc. Therefore, it was suggested that the antioxidant capacity was not evaluated by using a single test. In our study, total phenol and flavonoid content, iron chelating and ferric reducing antioxidant power assays which were widely used were conducted for determining the antioxidant potential of water and methanol extracts of *V. officinalis*. When compared each other, the *V. officinalis* water extract exhibited relatively low levels of DPPH scavenging activity, however in ABTS radical scavenging assay, there is not much difference between the water and methanol extract. Both scavenged DPPH and ABTS radical in a concentration-dependent manner (Figure 1).

3.3. *In-vitro* enzyme inhibition activity

The water and methanol extracts of *V. officinalis* were tested to evaluate their tyrosinase, AChE and BuChE enzyme inhibition effects as well as starch breakdown enzyme inhibition abilities against α -amylase and α -glucosidase. Prevention of the absorption of glucose and control of blood sugar by using these two enzyme inhibitors in reducing the postprandial blood glucose level in diabetic patients is an effective treatment approach in the treatment of diabetes nowadays. According to our results, the water extract showed stronger α -amylase, α -glucosidase, AChE BuChE and tyrosinase inhibition activities than the methanol extract at the concentration of 2 mg mL⁻¹ (Table 2). From the results it can be say that the enzyme inhibition activity of water extract may be attributed to the water-soluble polar compounds which are perhaps

Table 2. Enzyme inhibitory activities (%) of the water and methanol extracts of *Veronica officinalis* (in 2 mg mL⁻¹ concentrations)

Extracts	Anticholinesterase activity		Antidiabetic activity		Tyrosinase
	AChE	BChE	α -glucosidase	α -amylase	
Water extract	70.94 ± 2.37	66.89 ± 2.35	82.07 ± 2.91	63.61 ± 3.93	47.47 ± 0.53
Methanol extract	69.65 ± 1.69	65.66 ± 1.94	72.60 ± 1.73	57.45 ± 0.84	26.40 ± 0.57
Galanthamine HBr	93.87 ± 0.56	89.89 ± 0.01			
Acarbose			57.56 ± 0.52	58.40 ± 0.63	
Kojic acid					45.55 ± 1.65

playing effective role against enzymes than non-polar compounds dissolved in methanol extract. When the results compared with standard compounds, both water and methanol extract exhibited lower AChE and BuChE inhibitory activity than galanthamine, however, at the α -glucosidase inhibition assay, both of the water and methanol extract demonstrated higher inhibitory activity than reference drug acarbose.

The antityrosinase activity of plant extracts is important in terms of their usage in depigmenting agents for cosmetic formulations. Otherwise, the water extract (47.47%) also demonstrated higher tyrosinase inhibitory activity than the methanol extract (26.40%) at the concentration of 2 mg mL⁻¹. Moreover, it was found that the water extract had higher inhibition effect than the positive control (45.55%) at the same concentration (Table 2).

4. Conclusions

In conclusion, water and methanol extract of *V. officinalis* were evaluated for *in-vitro* antioxidant activity and were tested for their inhibition activity against five different enzymes which are AChE, BuChE, α -glucosidase, α -amylase, and tyrosinase. From the obtained results, it was observed that the

methanol extract showed higher total phenolic content and possess a higher antioxidant activity than water extract. Otherwise, the water extract exhibits higher inhibition effect on all the tested enzymes except for α -glucosidase than methanol extract. However the water extract demonstrated lower inhibitory activity than Galanthamine in AchE and BuchE and higher than the acarbose in α -glucosidase and α -amylase. It could be emphasize that the water extract is possessing potential tyrosinase inhibition activity even stronger than the reference compound- kojic acid. The results demonstrated that the plant may be a good source for developing herbal formulations for the Alzheimer's disease and diabetes patients.

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