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In-vitro antioxidant potential of conventional herbal decoction aiding diabetes metabolomics

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Diabetes mellitus (DM) is a risk factor of oxidative stress through the production of reactive oxygen species (ROS). ROS triggers cell damage which enhances the risk of several diabetes associated disorders such as neuropathy. There are many treatment options but no cure, and the quest for the natural, cost-effective treatment regimen is ongoing. Therefore, the present study was conducted to observe the *in-vitro* antioxidant and anti-cholinesterase activities of the indigenous natural herbs *Zingiber officinale* (Ginger), *Cinnamomum verum* (daarcheeni), *Piper nigrum* (Black pepper), and *Syzygium aromaticum* (clove). Herbal decoctions were prepared and *in-vitro* biochemical analysis were done of the antioxidant potential of herbs by inhibition of catalase (CAT), superoxide dismutase (SOD) enzymes with lowering of 2,2-diphenyl-1-picrylhydrazyl (DPPH) and Malondialdehyde (MDA) concentration. Moreover, spectrophotometric percentage inhibition of acetylcholine-esterase (AChE) and butyrylcholine-esterase (BChE) enzymes by the herbs was also measured. It was observed that herbal decoctions possess strong antioxidant activities especially *Z. officinale* decoction with significant percentage inhibition of enzymes with lowering of DPPH and MDA levels. Hence, herbal decoctions with potential antioxidant scavenging activities can be used to ameliorate progression of DM and associated psychoneurological disorders.

Key words: Antioxidants, oxidative stress, diabetes mellitus, decoction, neurological disorders.

INTRODUCTION

Diabetes mellitus (DM) is a major health concern that affects approximately 5% of the world's population. Recently it is reported that by 2025 the global cost for treating diabetes and its complications could reach US $ 1 trillion annually (Chakrabarti and Rajagopalan, 2002). If left untreated, DM leads to hyperglycemia and secondary pathophysiological amendments in various organs particularly in brain due to increased glucose auto-oxidation, which enhance accumulation of advanced glycation end (AGE) products (Starowicz and Zieliński, 2019). These AGE products generate ROS such as peroxides, superoxide, hydroxyl radical, singlet oxygen, and alpha-oxygen which causes cell damage and death (Starowicz and Zieliński, 2019). ROS further augments the risk of several diabetes associated disorders such as obesity (Kandimalla et al., 2017), hypertension, stroke, retinopathy, nephropathy, neuropathy (Chakrabarti and Rajagopalan, 2002)

coronary vascular disease, cardiomyopathy, and psyconeurological deficits (Kandimalla et al., 2017). In a biological system, ROS are constantly produced and countered by antioxidant enzymes such as catalase (CAT) and superoxide dismutase (SOD) (Evans et al., 2002; Allam et al., 2006). In addition to ROS, diabetes is related to abnormal brain neurochemical levels such as acetylcholine (ACh) and butyrylcholine (BCh) which also impairs learning and memory (Sharma et al., 2012; Almeida and Araújo, 2019). This alteration might be a consequence of increased AChE and BChE activity on account of metabolic dysfunction, which are reported to increase in diabetes (Dizdar et al., 2018; Chudoba et al., 2019).

Research shows that the augmented production of ROS during excessive oxidative stress can cause risk to cells by instigating lipid peroxidation, protein oxidation, nucleic acids damage, inhibition of enzyme and initiation of planned cell death (PCD) pathway, eventually leading to death of the neurons. These products if not controlled can be detrimental, affecting cell viability and leading to apoptosis that causes DM and associated neuropsychological disorders. This susceptibility of human brain is due to the presence of abundant amounts of polyunsaturated fatty acids (Starowicz and Zieliński, 2019) and high expression of insulin receptors (IR) on neurons and glia (Ghasemi et al., 2019). The highly expressed regions of brain for IR are documented to be in the hypothalamus, hippocampus, cerebral cortex, and olfactory bulb (Radünz et al., 2019). The interaction of insulin and Insulin-like-growth-factor (IGF) are important for cognitive function to ameliorate dementia (Tumminia et al., 2018). Hence, uncontrolled DM can further aggravate neuronal damage through ROS.

The current management of DM constitutes lifestyle modification and pharmacotherapy. Several FDA approved antidiabetic drugs (Mallick et al., 2016) are available; however, they possess various side effects such as weight gain, edema and anemia (Tilburt and Kaptchuk, 2008). The global epidemic emergence of DM has encouraged concerted efforts in drug discovery from natural plant sources for efficient, cost-effective treatment modalities with minimum side effects (Mallick et al., 2016). Previous studies have shown the medicinal reputation of herbs which is long-established through their utility in treatment of various ailments (Mallick et al., 2016; Ghasemi et al., 2019). Many of these herbs are natural edibles and in south Asian households they are consumed daily in diet to enhance aroma, color, and flavor of the food (Uhm et al., 2011).

Many herbs with ethno-medicinal importance are used for aromatherapy to establish efficient alternative treatment against psychotic disorders (Ghasemi et al., 2019; Alakbarov, 2003). However, this is not a strong mode of treatment as the active constituents in aerosol can be lost through dispersion in the ambient environment (Luo et al., 2012). On the other hand, 75% of the active ingredients in herbal decoctions are known to enter circulation and hence can effectively reach different parts of the body especially brain. Most of the herbal supplements are known to produce good glycemic control which plays a role in delaying the progression of diabetic complications (Luo et al., 2012). *Z. officinale* (ginger) is commonly known to produce immense antioxidative effects in many *in vitro* and *in vivo* studies due to the presence of gingerols and shogaol (Mashhadi et al., 2013; Bekkouch et al., 2019). *C. verum* (Cinnamon), *P. nigrum* (Black pepper) and *S. aromaticum* (Clove) due to the proven activities of their active substance 6-gingerol, cinnamaldehyde, piperine and eugenol, respectively (Shalaby and Saifan, 2014; Radünz et al., 2019) are taken in the present study to examine *in-vitro* antioxidant effects of these herbs through scavenging DPPH, SOD, CAT, and modulation of AChE, BChE and MDA concentrations to ameliorate progression of DM and associated disorders.

METHODOLOGY

Preparation of herbal decoction

In-vitro study was conducted on four most commonly and easily available herbs including *Z. officinale* (Ginger), *C. verum* (daarcheeni), *P. nigrum* (Black pepper), and *S. aromaticum* (clove). These herbs were collected from local markets of Karachi Pakistan. The herbal decoction was prepared by taking 10 mg of each herb in distilled water, boiled for 20 min, then cooled down individually. The decoction was then filtered, and the filtrate was used as herbal extract in preparation for biochemical analysis to evaluate the inhibitory capability of herbs on cholinesterase activity of AChE, BChE and oxidative stress markers Catalase (CAT), SOD, DPPH and MDA content.

Biochemical analysis

The determination of CAT activity

This was measured according to the method of Beutler (1984). The breakdown rate of H_2O_2 to H_2O and O_2 was estimated by spectrophotometer at 230 nm. The experimental medium comprises 1 mol/L Tris HCl-5 mmol/L disodium ethylene diamine tetra acetic acid (EDTA) buffer solution (pH 8.0), 1.0 mol/L, phosphate buffer solution (pH 7.0), and 10 mmol/L H_2O_2. Herbal decoction with Dilutions T1 = Original solution, T2 = 1:1 dilution and T3 = 1:10 dilution was prepared. CAT activity was expressed as U/mg protein. One unit of CAT activity is defined as the amount of enzyme causing about 90% destruction of the substrate in 1 min in a volume of one milliliter.

Estimation of SOD activity

This was determined as described by Beyer and Fridovich (1987). This method employs xanthine and xanthine oxidase to generate superoxide radicals, which react with 2-(4-iodophenyl) -3- (4-nitro phenol-s-phenyl tetrazolium chloride) to form a red formazon dye. SOD activity was then measured by the degree of inhibition of this reaction. Three test tubes were taken T1, T2, T3, where T1: 1 ml decoction, T2: 500 µl decoction + 500 µl distilled water, and T3: 100 µl decoction + 900 µl distilled water. Only 0.1 ml of samples

from all 3 test tubes was used for the experiment. To each of the test tubes T1, T2 and T3, 0.75 ml of ethanol was added followed by 0.15 ml of ice-chilled chloroform. All three test tubes were then centrifuged at 3000 rpm for 15 min. 0.5 ml of supernatant was collected from each of the test tubes and the rest was discarded. This supernatant was then treated with 0.5 ml of EDTA followed by 1ml of buffer. 0.5 ml of epinephrine was then added to each of the test tubes and the absorbance was taken at 480 nm using spectrophotometer.

Estimation of DPPH activity

The DPPH activity was estimated by Shimada et al. (1992) method. This method was based on the principle of scavenging the DPPH radical. To assess the antioxidant activity, 0.5 mM of solution in ethanol was prepared. After preparing herbal decoction with water, assay dilutions were made and assigned as T1 pure decoction, T2, and T3 in ratio of 1:1 and 1:10 with decoction and distilled water, respectively. 0.2 ml of the sample was taken and 2 ml of DPPH solution was added to prepare samples T1', T2' and T3', respectively. Spectrometry was performed at 0 min to measure the absorbance. Absorbance was measured again at 517 nm for 30 min. Lower absorbance of the reaction mixture indicated higher free radical scavenging activity.

Estimation of percent inhibition of MDA

Ohkawa et al. (1979) determined the percentage inhibition of MDA content. To estimate the MDA in herbal water decoction, this was characterized by the formation of light pink colored complex between MDA and thiobarbituric acid (TBA). TBA is prepared by mixing 75 ml of thiobarbituric acid, 15 g of Trichloroacetic acid, and 2.08 ml of 0.2 N HCl. All the reagents were mixed together to 100 ml volume with distilled water (D/W). All the reagents used were of 'analar' grade. Three dilutions were prepared and labeled as T1, T2 and T3 of 1 ml each; T1 is the pure decoction, T2 is water and decoction in 1:1 ratio, and T3 is water and decoction in 1:10 ratio. 2 ml of 1:1 TCA and TBA was added to each dilution, boiled for 20 min, and then cooled at 4°C. Each dilution was centrifuged at 2000×g for 10 min after which the supernatant was collected. Absorbance of the light pink supernatant was recorded at 535 nm using a spectrophotometer.

Estimation of AChE activity

The estimation of AChE activity was done by using 20 μl of AChE with 4 ml of phosphate buffer and 0.016 g/4 ml substrate Acetyl Thiocholine Iodide. Herbal decoction with Dilutions T1 = Original solution, T2 = 1:1 dilution and T3 = 1:10 dilution was prepared. The reagents were added in a sequence; 150 μl phosphate buffer, 10 μl of each herbal decoction, 20 μl AChE enzyme solution, 10 μl DTNB and 10 μl substrate, then incubated for 10-15 min at a temperature of 25°C. Analysis was done using a spectrophotometer at 412 and 415 nm for 10 min at a temperature of 25°C.

Estimation of BChE activity

BChE activity was analyzed by preparing 200 μl of BChE and mixed with 2 ml of phosphate buffer. The 0.016 g Butyryl Thiocholine Iodide (substrate) was prepared in 4 ml deionized water. The decoction of substrate having dilutions: T1 = Original Solution, T2 = 1:1, T3 = 1:10 were prepared and then the reagents added as 150 μl phosphate buffer, 10 μl of each herbal decoction, 20 μl AChE enzyme solution, 10 μl DTNB and 10 μl substrate, followed by

incubation for 10-15 min at a temperature of 25°C. The absorbance was recorded using a spectrophotometer at 412 and 415 nm for 10 min at a temperature of 25°C. The values of sample absorbance were used in calculating the percentage inhibition according to the following formula:

Percentage Inhibition = 100 - (Absorbance of test / Absorbance of Control) × 100

Statistical analysis

Data is expressed as mean ± standard deviation and analyzed by two-way ANOVA using SPSS version 21. Tukey's test followed by post-hoc comparison was used for analysis. Values were considered statistically significant at $p < 0.05$. *Z. officinale* was taken as control herb and activities of other herbs studied were compared against it.

RESULTS

Effects on catalase

The two-way ANOVA showed a significant inhibition of the catalase enzyme by all the four herbal decoctions (F_{23}=5.343E4; $p < 0.05$) and the same was observed in the different concentrations of these decoctions in the treatment × doses effect (F_{23}=2.724E4; $p < 0.05$). Post hoc Tukey's test showed that all the concentrations of herbal decoctions had a significant increased ($p < 0.05$) inhibition of catalase enzyme except for the T3 concentration of *C. verum* and *P. nigrum* when compared with *Z. officinale* (Figure 1).

Effects on SOD

The effects of treatment of all herbs on SOD showed a significant reduction in the enzyme level (F_{23}=2.335E3; $p < 0.05$) which was consistent with the treatment × doses of all herbal decoctions (F_{23}=673.051; $p < 0.05$) when compared and analyzed by two way ANOVA. The post hoc Tukey's test also showed a significant decreased ($p < 0.05$) SOD inhibition at all three concentrations of herbal decoctions when compared with *Z. officinale*. However, T2 and T3 concentrations of *P. nigrum* showed more efficient inhibition than *Z. officinale* (Figure 2).

Effects on DPPH

The effects of all herbs by two-way ANOVA on DPPH showed a significant decrease (F_{23}=3.723E3; $p < 0.05$). The same significance was observed in the treatment × doses on the DPPH concentration (F_{23}=264.948; $p < 0.05$). Post hoc by Tukey's test showed a significant decreased $p < 0.05$ inhibition in DPPH in all groups except for the *C. verum* group at concentration of T2 comparing it with *Z. officinale*. However, all concentrations of *S. aromaticum* showed significant increased inhibition $p < 0.05$ than *Z. officinale* (Figure 3).
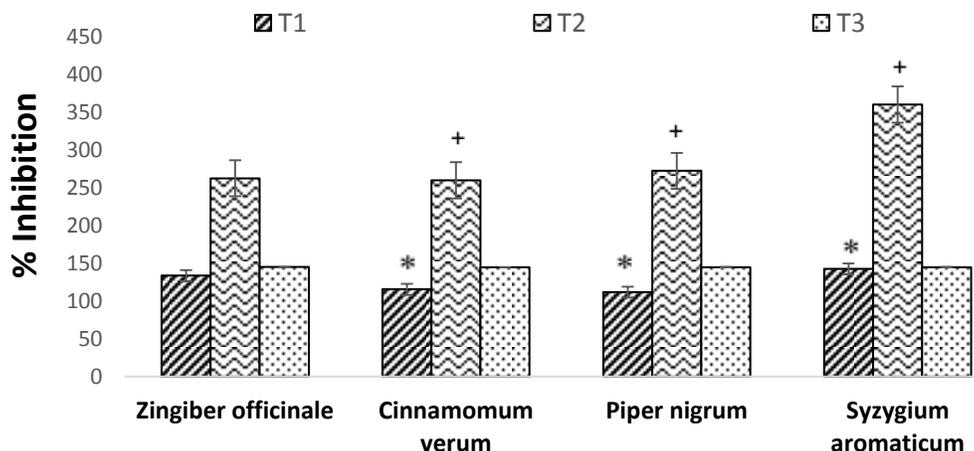
Figure 1. *In vitro* effects on catalase.
Values are presented as means ± SD. Significant differences by Tukey test:* p<0.05 from *Zingiber officinale* T1, +p<0.05 from *Z. officinale* T2.
Source: Author

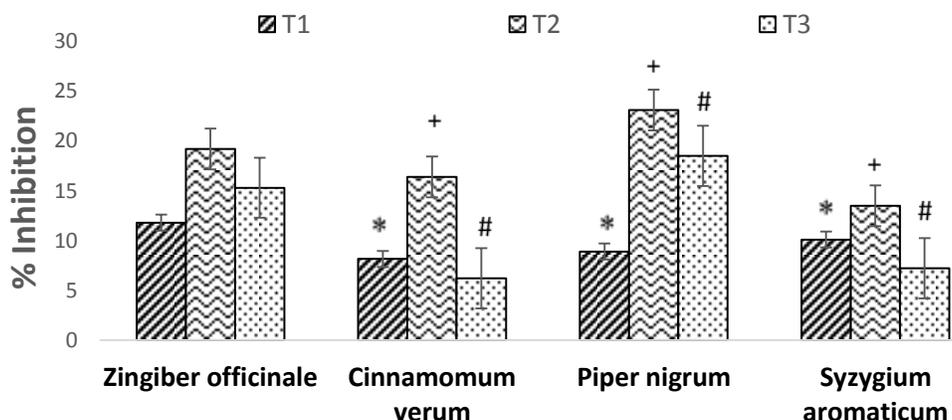**Figure 2.** *In vitro* effects on SOD.
Values are presented as means ± SD. Significant differences by Tukey test:* p<0.05 from *Z. officinale* T1, +p<0.05 from *Z. officinale* T2, #p<0.05 from *Z. officinale* T3.
Source: Author

Effects on MDA

The two-way ANOVA analysis revealed a significant effect of all herbs on MDA content (F_{23}=2.188E4; p<0.05). The effects of herbs in treatment × doses showed significant decrease in MDA concentration (F_{23}=3.740E3; p<0.05). Moreover, post hoc Tukey's test analysis also showed a significant decreased (p<0.05) inhibition of MDA by all herbs when compared with *Z. officinale* except T3 concentration of *C. verum* which showed significant increased inhibition of MDA content (p<0.05) compared to *Z. officinale* (Figure 4).

Effects on AChE activity

The effects of herbal decoction on AChE through two-way ANOVA showed significant inhibition of AChE activity (F_{23}=8.088E3; p<0.05). Significant effects were seen in treatment × doses of the herbs on AChE activity (F_{23}=5.128E3; p<0.05). The post hoc analysis by Tukey's test of herbal decoctions upon comparison with *Z. officinale* showed a significant increased p<0.05 AChE inhibition, to all groups except *P. nigrum* at T3 concentration. However, T1 and T3 of *S. aromaticum* and T3 of *C. verum* showed increased inhibition of AChE enzyme when compared with *Z. officinale* (Figure 5).

Effects of herbs on BChE levels

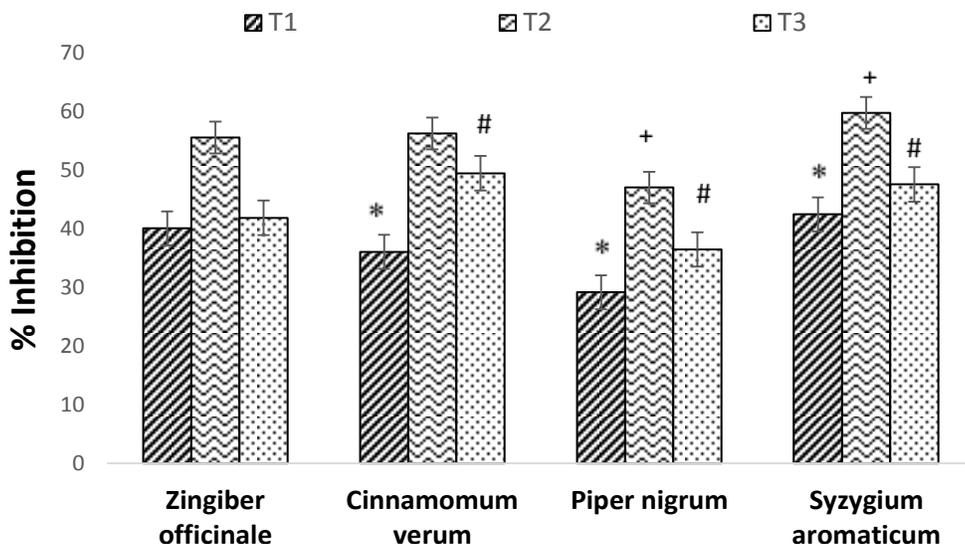Two-way ANOVA showed a significant inhibition of BChE enzyme when treated with the herbal decoctions

Figure 3. *In vitro* effects on DPPH.
Values are presented as means ± SD. Significant differences by Tukey test:* $p<0.05$ from *Z. officinale* T1 +$p<0.05$ from *Z. officinale* T2, #$p<0.05$ from *Z. officinale* T3.
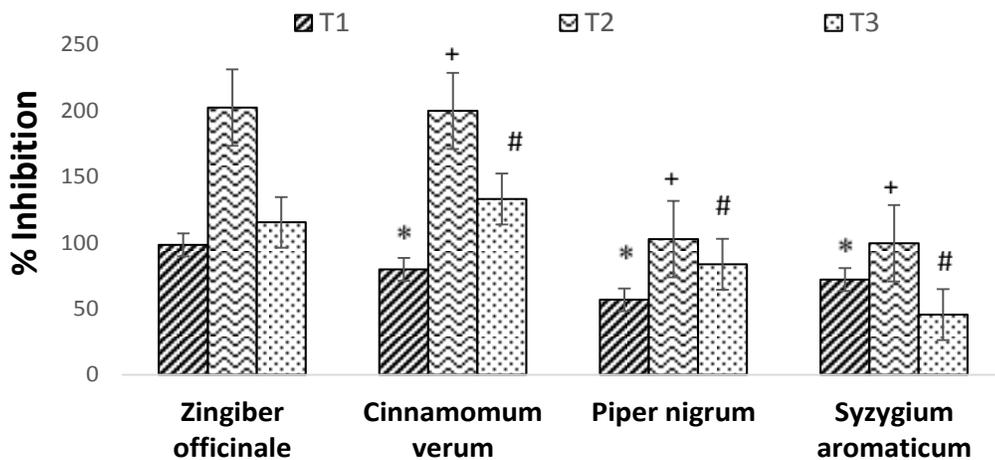Source: Author

Figure 4. *In vitro* effects on MDA.
Values are presented as means ± SD. Significant differences by Tukey test:* $p<0.05$ from *Z. officinale* T1 +$p<0.05$ from *Z. officinale* T2, #$p<0.05$ from *Z. officinale* T3.
Source: Author

(F_{23}=5.964E3; $p<0.05$). In addition, the effects of treatment × doses on BChE inhibition (F_{23}=3.637E3; $p<0.05$) was also found to be significant in this study. The post hoc Tukey's test showed a significant increased $p<0.05$ inhibition of BChE by all herbs excluding *C. verum* and *S. aromaticum* at T2 concentration when compared with *Z. officinale.* However, T1 concentration of both *C. verum* and *S. aromaticum* showed decreased inhibition than *Z. officinale* (Figure 6).

DISCUSSION

DM is a quintessential example of oxidative stress (Kandimalla et al., 2017) which induces metabolic alterations (Kedare and Singh, 2011). The present study was conducted to investigate the *in vitro* antioxidative effects of conventional herbs such as *C. verum, P. nigrum* and *S. aromaticum* on oxidative stress enzymes and markers CAT, SOD, DPPH, and MDA, respectively
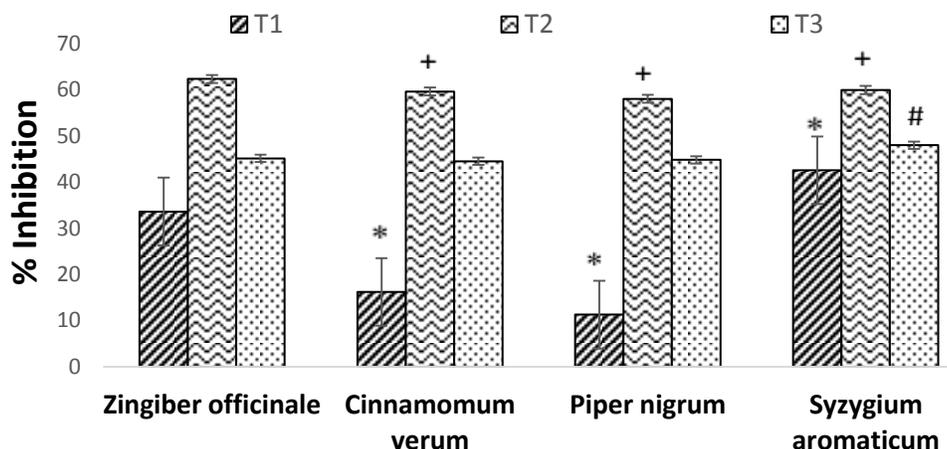
Figure 5. *In vitro* effects on AChE
Values are presented as means ± SD. Significant differences by Tukey test:* $p<0.05$ from *Z. officinale* T1 +$p<0.05$ from *Z. officinale* T2, #$p<0.05$ from *Z. officinale* T3.
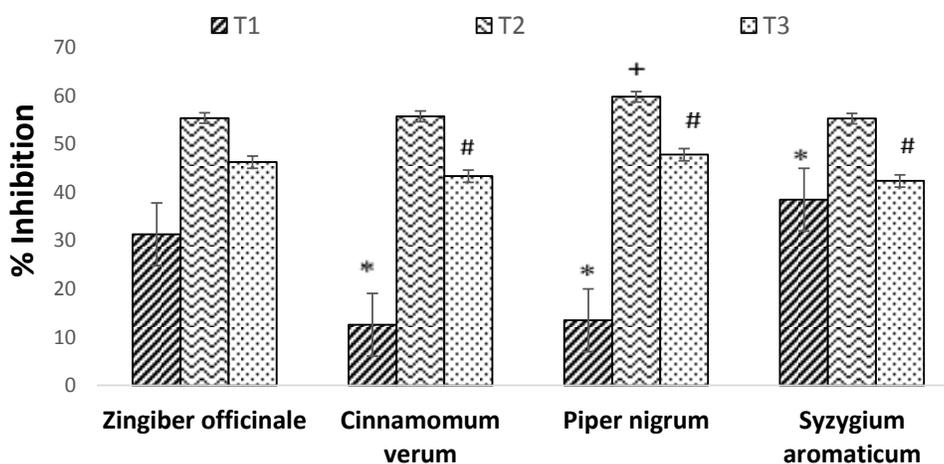Source: Author

Figure 6. *In vitro* effects on BChE.
Values are presented as means ± SD. Significant differences by Tukey test:* $p<0.05$ from *Z. officinale* T1 +$p<0.05$ from *Z. officinale* T2, #$p<0.05$ from *Z. officinale* T3.
Source: Author

as well as effects on cholinesterase enzymes AChE and BChE. It was observed that these herbs produce promising antioxidant effects with potent anti-cholinesterase. The bioactive components of the aqueous extract of *Z. officinale* are recognized as zingerone, 6-shogaol, 6-gingerol paradols, and zingerone, which are responsible for immense antioxidant, antidiabetic and anti-inflammatory properties (Al Hroob et al., 2018; Bekkouch et al., 2019). The protective anti-inflammatory effects of *Z. officinale* are established through its action against ROS generation and lipid peroxidation by increasing the mRNA expression of antioxidant enzymes SOD and CAT (Al Hroob et al.,

2018). A study reported that treatment with *Z. officinale* decreased MDA content and elevated antioxidant defenses (Al Hroob et al., 2018). Furthermore, evidence based *Z. officinale* inhibition of AChE has assisted its utilization for treatment of age-related cognitive decline and Alzheimer's disease (Tung et al., 2017), due to the potential effects on oxidative stress; *Z. officinale* was used as control in present study.

Another herb used in the present study was *C. verum* that was previously studied by a group of scientists Shalaby and Saifan (2014), who showed enhancement in insulin signaling pathway, thereby augmenting insulin regulated glucose utilization in the body and exerting anti-

diabetic effects. The same study also reported the hepatoprotective, antioxidant, anti-obesity and anti-hyperlipidimic activity of the herb (Shalaby and Saifan, 2014). Further research on *C. verum* showed its effectiveness in the enhancement of SOD and CAT enzymes, and the modulation of MDA and DPPH levels which is due to the presence of flavonoid compound phytoestrogens (Khaki et al., 2013). Published research suggested that the reducing property of DPPH is generally associated with the presence of reductones, which have shown to exert antioxidant action by breaking the free radical chain by donating a hydrogen atom (Mallick et al., 2016). Another study revealed that MDA as a by-product of lipid peroxidation is found to be elevated in metabolic syndromes such as obesity, cancer atherosclerosis pathogenesis aging cancer, and AD (Tangvarasittichai, 2015). MDA has been documented as a primary biomarker of free radical mediated lipid damage and oxidative stress (Dizdar et al., 2018). It has been reported that significant changes in lipid metabolism and structure is involved in the progression of diabetes (Evans et al., 2002). Recent studies demonstrated that the aqueous extract of Cinnamon contain eucalyptol, benzene propanol, cinnamyl acetate, coumarin, and eugenol, known to possess antioxidant potential through DPPH scavenging activity (Błaszczyk et al., 2021).

The additional herbs studied in this report were *P. nigrum* and *S. aromaticum.* The reason for including *P. nigrum* in the study was to determine its *in vitro* efficiency as antioxidant because it has been previously identified as a decreasing liver MDA content with improvement in the levels of oxidative stress enzymes SOD and CAT (Takooree et al., 2019). The major active entities of *P. nigrum* are piperine, and phenolic contents present in aqueous extract produce antioxidative activities (Akbar et al., 2014). A large number of data have also demonstrated that the anti-inflammatory and antioxidative response of aqueous extract of *S. aromaticum* by lowering oxidative stress supports the formation of lipid through the accumulation of SOD and CAT protein (Marmouzi et al., 2019). The aqueous extract of *S. aromaticum,* known to contain a large range of total phenolic and tannin contents, produce the various pharmacological activities (Nikousaleh and Prakash, 2016).

The damage in integral parts of the cell leading to cell death and inhibition of cell integrity are maintained through herbal products (Tangvarasitthai, 2015). Luo et al. (2012) reported that the antioxidant effects of plant products by inhibiting SOD and CAT is mainly due to their redox properties, and further suggested that this can play an important role in absorbing and neutralizing free radicals, quenching singlet and triplet oxygen, or decomposing peroxides and radical scavenging activity of phenolic compounds found in herbs such as flavonoids, polyphenols, tannins, and phenolic terpenes. Our results showed that all the herbs possessed antioxidant activity by inhibiting CAT and SOD at significant concentrations. These results may have great relevance in the prevention and treatment of diseases associated with oxidants or free radicals such as DM, hyperlipidemia and neuropsychological deficits (Tangvarasitthai, 2015).

Evidence shows that reduction of brain acetylcholine (ACh) and butyrylcholine (BCh) concentration plays a vital role in development of neuropsychological deficits and this may be due to excessive action of cholinesterase enzymes AChE and BChE (Dizdar et al., 2018). Therefore, one of the rational targeted mechanisms to treat AD may be the inhibition of ACh and BCh degradation enzyme acetylcholineesterase (AChE) and Butylcholineesterase (BChE) (Evans et al., 2002).

Currently, several synthetic formulations of AChE inhibitors are available for treatment of neurological disorders such as Alzheimer disease (AD), but these are expensive, provide timely relief, and pose serious side effects. Therefore, there is a quest for the identification of natural products that are safe, cost effective with better efficacy, and possess least or no side effects. The herbs used in the present work showed promising results in the treatment of AD and dementia through significant inhibition of AChE and BChE levels, and may serve as a viable replacement in the treatment of dementia additionally.

Thus, the present study showed the effectiveness of these natural herbs as potent antioxidants with strong ability to inhibit AChE and BChE enzymes levels that become a hallmark of DM related cognitive decline and age-related AD. The present *in-vitro* study also endorsed the benefits of the traditional way of preparing herbal decoction that is easy, cost-effective and with no side effects, to alleviate DM and associated neuropsychological disorders.

Conclusion

The present *in-vitro* study showed the potential antioxidant scavenging activity with modulation of AChE, BChE, Catalase, DPPH, SOD and MDA levels, contributing towards comparatively inexpensive and easy way to treat DM and associated neuropsychological disorders than the synthetic treatment formulations.

CONFLICT OF INTERESTS

The authors have not declared any conflicts of interests.

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