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Acetylcholinesterase Activity and Brain Histology of Albino Rats Treated with Lemongrass (*Cymbopogon citratus*) and Kaffir Lime (*Citrus hystrix* DC.) Teas

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ABSTRACT

Lemongrass (*Cymbopogon citratus*) and kaffir lime (*Citrus hystrix* DC.) teas are popularly consumed in many countries around the world to promote health including enhancing memory. However, the effects of these two plants on acetylcholinesterase (AChE), a key enzyme involved in the development of Alzheimer' disease, as well as the effects on brain structure, were still limited. Therefore, this research was done to investigate the possible effects of lemongrass and kaffir lime teas on AChE activity and brain histology of male rats. Eight-week-old male albino rats were randomly divided into five groups of six rats each. The first group was the normal control group, and they were orally administered dechlorinated water. The second and the third groups were received lemongrass tea (33 mg/ml) for 12 and 24 hours per day while the fourth and the fifth groups were received kaffir lime tea (33 mg/ml) for 12 and 24 hours per day. The animals in the treatment groups were given unrestricted access to the teas. After 30 days of the treatment period, all rats were euthanized by diethyl ether and their brains were collected to determine AChE activity by enzymatic spectrophotometric method and histology. The results demonstrated the significant elevation (p<0.05) of hippocampal AChE activities in all teas-treated groups when compared to the normal control group. Nevertheless, the significant increases (p<0.05) in cerebral AChE activities were found only in kaffir lime teas-treated rats when compared to the normal control rats. Histological investigation showed no alterations in the structures of hippocampus and cerebral cortex of rats treated with lemongrass and kaffir lime teas. The arrangement of neurons and neuronal morphology were normal and similar to the nervous tissues seen in the normal control group. It can be concluded that both lemongrass and kaffir lime teas were found to affect brain function by elevating AChE activities in the brain.

INTRODUCTION

Dementia is a neurodegenerative disease commonly found in the global elderly population [1]. Alzheimer's disease or AD is the most common type of dementia and it affects the quality of life by causing patients to lose their ability to learn and remember. Although the actual causes of AD remain largely unidentified, a reduction in acetylcholine (ACh) activity in the brain is one key factor leading to the development of AD. ACh is an important neurotransmitter in the cholinergic system that is related to memory formation. This neurotransmitter is hydrolyzed by acetylcholinesterase or AChE into acetic acid and choline at central and peripheral cholinergic synapses [2]. A previous report has revealed that AD patients have a reduction of neurons in their hippocampus and cerebral cortex, resulting in a low level of ACh, while the level of AChE remained constant [3]. Therefore, inhibition of AChE activity elevates cholinergic neurotransmitter activity in AD patients. Although synthetic AChE inhibitors are effective in the treatment of AD, they produce serious side effects and are costly [4-5]. In recent years, there has been a growing interest in the use of medicinal plants to develop AChE inhibitors and nootropic agents. Several studies have been conducted to investigate the AChE inhibitory activity of various plant species, such as *Gingko biloba*, Ginseng, *Salvia officinalis*, *Rosmarinus officinalis* and *Hypericum perforatum* [6]. Scientific reports have shown that these plant species contain bioactive compounds, including flavonones, rutin, quercetin, quercitrin, kaempferol, and terpenes, which act as neuroprotective and nootropic agents [7-8]. However, no plant species has yet been identified as a complete replacement for synthetic AD drugs in terms of therapeutic efficacy. Furthermore, prolonged consumption of medicinal plants or taking high doses can have negative effects on the nervous system. For example, rats treated with *Gelsemium elegans* at a dose of 70 mg/kg for 21 days exhibited an imbalance between neuronal gamma-aminobutyric acid (GABA) and glutamate in their brains [9]. In addition, Voodoo, a mixture of herbs and spices, caused neurotoxicity in rats by decreasing various neurotransmitters (adrenaline, noradrenaline, serotonin, and dopamine) and inducing histopathological changes in the rat brain, such as shrunken neurons and vacuole accumulation in neuronal cytoplasm [10]. Therefore, many researchers continue to study the toxic effects of medicinal plants in animal models to ensure their safety.

Lemongrass (*Cymbopogon citratus*) and kaffir lime (*Citrus hystrix* DC.) are known as Takrai and Makrut in Thai, respectively. They have long been used by traditional folk around Southeast Asia to prevent

various ailments. Lemongrass contains various bioactive constituents including phenolic acids, stilbenes, flavonoids, alkaloids and terpenoids, while kaffir lime leaves consist of saponins, tannins, reducing sugars, flavonoids and phenolics [11-12]. The reported medicinal properties of lemongrass include analgesic, spasmolytic, antipyretic, anti-inflammation and diuretic effects [13]. Kaffir lime possesses antioxidant properties, immunomodulation, cardioprotection and hepatoprotection [14]. Temitayo et al. [2020] found that essential oil from lemongrass was an effective agent for neuroprotection in rats, attenuating brain damage induced by aluminum chloride by modulating the antioxidant system to inhibit oxidative stress [15]. Lemongrass tea also improved memory function in scopolamine-induced amnesia in experimental mice by inhibiting oxidative stress and AChE [16]. Additionally, kaffir lime peel extract reduced oxidative stress in dementia induced-mice by reducing malondialdehyde level in their brain [17]. Given their broad spectrum of reported therapeutic efficacies, there are limited scientific reports on the effects of these plants on AChE activity and brain histology. Active compounds derived from various plants, such as phenolics, flavonoids and alkaloids have been reported to have neuroprotective efficacy by inhibiting AChE activity [18-19]. However, continuous administration of alkaloids from *Alstonia scholaris* (L.) R. Br. at doses of 50, 100 and 300 mg/kg for 3 weeks altered hematological and biochemical indices in rats and mice [20]. Therefore, we aimed to investigate whether teas prepared from the stems of lemongrass and the leaves of kaffir lime could maintain brain function and structures in experimental rats. The results obtained from our study might provide baseline information regarding the consumption of these teas as the substitute for drinking water to improve cognitive deficits.

METHODOLOGY

Chemicals

Acetylthiocholine iodide (ACThI), 5,5'-dithiobis 2-nitrobenzoic acid (DTNB), eosin, hematoxylin, paraplast, permount and quercetin were purchased from Sigma-Aldrich (St. Louis, MO, USA). Folin & Ciocalteu's Phenol Reagent was supplied by Loba Chemie, Pvt, Ltd. (Mumbi, India). 95 % ethyl alcohol, absolute alcohol and xylene and all other chemicals and reagents used in this study were of analytical grade and obtained from Union Science Co., Ltd., Chiang Mai, Thailand.

Tea preparation

The powder from the leaves of kaffir lime and the stems of lemongrass was purchased from an organic local commerce in Ratchaburi Province, Thailand. Both teas were prepared freshly on the day of animal experimentation following the manufacturer's method. The tea solution was extracted from 15 g of the fine powder in 450 ml of hot water (33 g/ml) for 4 minutes. The concentration of tea used in this study was equivalent to the recommended amount for human tea consumption. They were then cooled to room temperature before administration to animals.

Phytochemical analysis

A phytochemical screening was conducted to detect the presence of bio-constituents in kaffir lime and lemongrass teas, such as phenols, flavonoids, saponins, tannins, terpenoids, alkaloids, phlobatannins, anthraquinones, cardiac glycosides, and reducing sugars, following the standard methods described by [21] and [22].

Experimental Animals

Eight-week-old male Wistar rats (*Rattus norvegicus*) weighing between 150-180 g were purchased from Nomura Siam International, Co. Ltd., Thailand. They were acclimatized for one week in the animal care facility. Room temperature was controlled at 24-26 °C, and there was a 12/12-hours light/dark cycle. During the adjustment period, the animals had access to a standard diet and water *ad libitum*. All animal procedures used in this research were reviewed and approved by the Institutional Animal Care and Use Committee of the Biology Department, Faculty of Science, Chiang Mai University (ID: Re. 003/19).

Animal treatments

The experimental rats were randomly assigned to five groups (six rats per group). The first group was normal control and was given distilled water all day. The second group received lemongrass tea as a substitute for drinking water for 12 hours during the day and was replaced with distilled water for 12 hours during the night (LGW), while the third group received lemongrass tea all day (LG). The fourth group was given kaffir lime tea as a substitute for drinking water during the day and replaced with distilled water at night (KLW). Rats in the last group received kaffir lime tea all day (KL). The animals had free access to teas or distilled water for 30 consecutive days.

On the last day of the experimental period, all rats were deprived of food for 12 hours and sacrificed under anesthesia, and their brains were quickly collected. Cerebral cortex and hippocampus of each rat were dissected and divided into two parts (left and right). The left part was frozen for the analysis of AChE activity, while the right part was fixed in Bouin's solution to observe histopathological alterations.

Determination of acetylcholinesterase activity

The AChE activities in cerebral cortex and hippocampus of all groups were determined by following the method described by [23]. Twenty milligrams of cerebral cortex or hippocampus from each rat was homogenized in 1 mL of 0.1 M phosphate buffer solution, pH 7.4. Then, the homogenate was centrifuged at 3,500 rpm for 15 minutes to collect the supernatant.

The AChE activity was determined by following the elevation of yellow color produced from the reaction between ACThI, the substrate, and DTNB. Briefly, the brain supernatant from each rat was mixed with DTNB solution (0.2 mM) in 96-well plate. Then, ACThI (0.75 mM) was added and mixed well. The kinetic reaction of the mixture was measured at 405 nm using an ELISA reader (GDV, DV 990BV4, Italy) every 2 minutes for 10 minutes. The protein content in the brain of each rat was determined by using the method of Bradford (1967) [24]. The rate of AChE activity in cerebral cortex or hippocampus of each rat was calculated and expressed as µmole/min/mg protein.

Histological investigation

The brain tissues were fixed in Bouin's solution for 24 hours before being dehydrated in ascending grades of ethyl alcohol (70-100% v/v) and having the dehydrating agents removed from the tissues with xylene. They were then infiltrated and embedded in paraffin wax. Tissue ribbons were prepared by sectioning at 6 micrometers thickness and mounted on glass slides. The tissue slides were stained with hematoxylin and eosin (H&E) and permanently mounted [25]. Histopathological alterations in the cerebral cortex and the dentate gyrus (DG), as well as the Cornu Ammonis (CA) regions of hippocampus or CA1, CA2, and CA3, were observed. Micrographs of brain tissues were taken using a light microscope (Olympus BX41) coupled with a camera.

Statistical Analysis

The results of AChE activities were expressed as means ± standard deviation (SD) and were analyzed by one-way analysis of variance (one-way ANOVA) followed by Duncan's post hoc test for multiple comparisons using SPSS version 26. The values of $p < 0.05$ were considered as statistically significant differences.

RESULTS

Phytochemical analysis

Preliminary phytochemical screening of lemongrass and kaffir lime teas revealed the presence of phenols, flavonoids, saponins, tannins, terpenoids and reducing sugars. However, alkaloids, cardiac glycosides, phlobatannins, and anthraquinones were not detected in these teas (Table 1).

Table 1. Phytochemical constituents from lemongrass and kaffir lime teas.

phytochemical constituents	The presence of phytochemicals	
	lemongrass tea	kaffir lime tea
phenols	+	+
flavonoids	+	+
saponins	+	+
tannins	+	+
terpenoids	+	+
alkaloids	w-	-
cardiac glycosides	-	-
anthraquinones	-	-
phlobatannins	-	-
reducing sugars	+	+

+ present, - absent

Determination of AChE activitiesw

The results of hippocampal and cerebral AChE activities of rats treated with drinking teas from lemongrass and kaffir lime for 30 consecutive days are represented in Figure 1 and 2. The values of hippocampal AChE activities were significantly elevated (p <0.05) in rats in the LGW, LG, KL and KLW groups when compared to the control group. Additionally, rats in KLW and KL groups had significantly higher levels of AChE activities in their cerebral cortex (p <0.05) than rats in the control group. However, the intake of lemongrass teas for 12 hours/day or all day did not significantly alter (p>0.05) the cerebral AChE activities in rats when compared to the control group (Figure 1).

Histological investigation

Hippocampal histology

The histological features of the hippocampus of rats treated with lemongrass and kaffir lime teas for 30 days are displayed in Figure 2 and 3. The hippocampal tissues of normal rats in the control group showed normal histological features. The hippocampal formation of control rats consisted of hippocampal proper including Cornu Ammonis CA1, CA2, CA3 and dentate gyrus, as shown in figure 2. The CA1 and CA2 contained small pyramidal cells, while CA3 contained large pyramidal neurons. The dentate gyrus was characterized by small granule cells. The arrangements of pyramidal cells in Cornu Ammonis as well as in the granular cells in Dentate area of all tea-treated rats were similar to the normal rats. Treatment with both lemongrass and kaffir lime teas did not alter the normal histoarchitectures of hippocampus. Moreover, there are no characteristic of neuronal cell death, such as necrotic cell death and apoptotic cell death, cell shrinkage, plasma membrane breakage and swelling of organelles observed in hippocampal tissues of all control and tea-treated rats, as shown in Figure 3.

Light microscopic investigation of the cerebral cortex of rats-treated with lemongrass and kaffir lime teas revealed healthy neurons similar to the rats in control group. A regular six layers of the cortex consisted of molecular layer, external granular layer, external pyramidal layer, internal granular layer, internal pyramidal layer and multiform layer. Normal neuron was characterized by dark-stained nucleus and clear cytoplasm. Neuroglia, blood vessels and myelinated axon were regularly observed in the cerebral cortex of all rats (Figure 4).

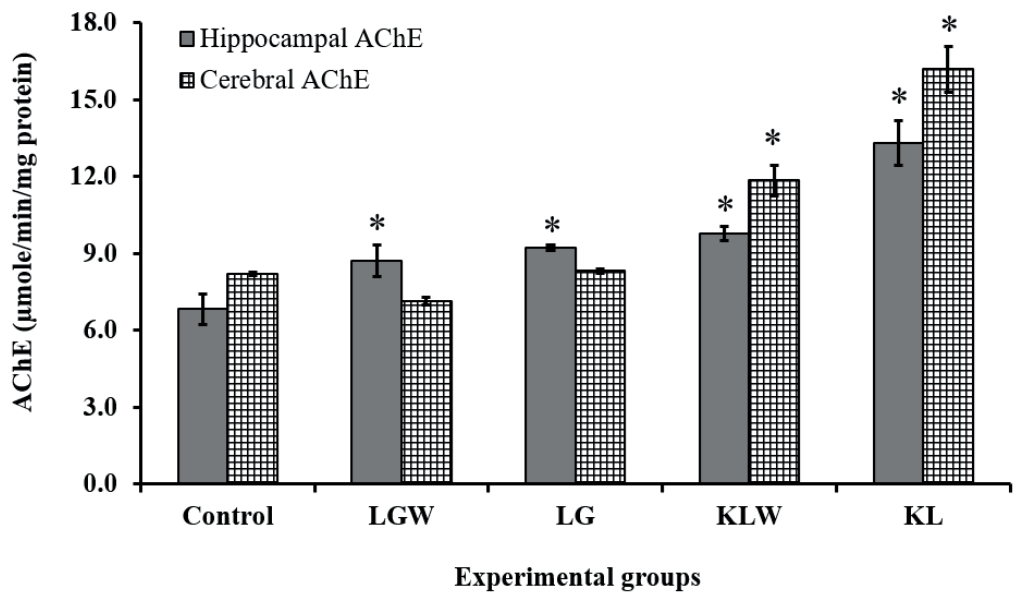

Figure 1. Hippocampal and cerebral AChE activities of rats treated with kaffir lime and lemongrass teas for 30 consecutive days as compared with normal control rats. Values are represented as mean ± SD of six rats. * p < 0.05 as compared with control group (One-way ANOVA followed by Duncan's multiple comparison test).

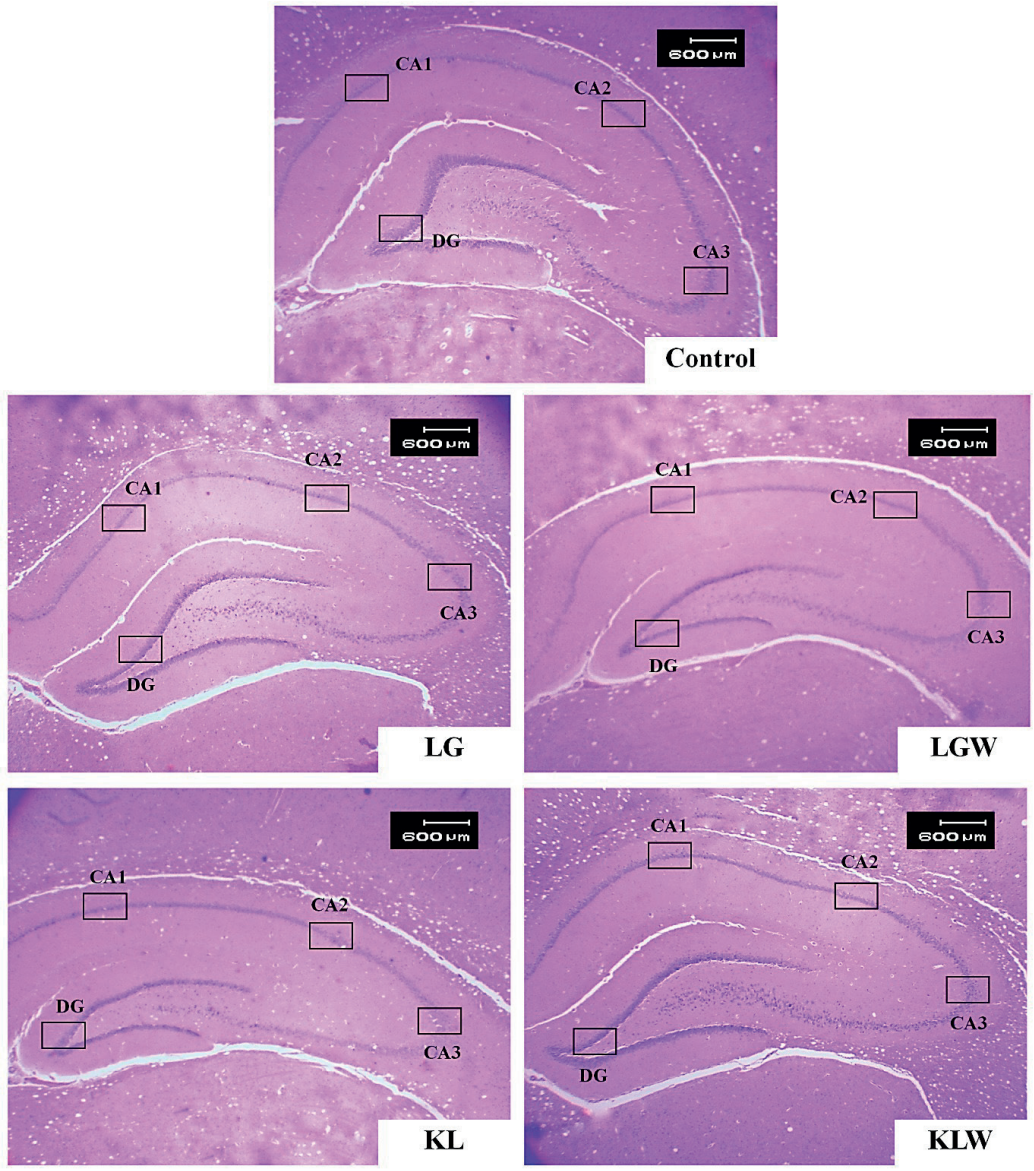

Figure 2. Histological features of the hippocampus of rats treated with lemongrass and kaffir lime teas for 12 and 24 hours/day. H&E stain, 2x magnifications.

DISCUSSION

Nowadays, although modern pharmaceutical agents are available and highly effective in treating neurodegenerative diseases and promoting health, many people throughout the globe still prefer to use plant materials due to their low cost and perceived fewer adverse effects. In many countries, including Thailand, people have used several plants to prevent memory deficits or promote memory, and have avoided synthetic drugs, which are expensive and have side effects. Lemongrass and kaffir lime leaves have been used as traditional medicine to treat various ailments. Lemongrass tea is consumed to remedy hypertension, diabetes mellitus, obesity, hyperlipidemia and hyperglycemia [26-29]. Kaffir lime leaves possesses various pharmacological properties, such as relieving headache, fever, diarrhea, rheumatoid arthritis, heart diseases, hypertension and diabetes mellitus, as well as boosting sexual performance [30-32]. However, there are also problems associated with overdose consumptions, and there has been no scientific evidence supporting their efficacy. Therefore, our attention in this study has focused on studying the effects of the consumption of lemongrass and kaffir lime teas as a substitute for drinking water on brain function and structures in rats.

The study found that rats that received lemongrass tea for 12 or 24 hours/day for 30 consecutive days had elevated AChE activities in their hippocampus. Additionally, rats that received tea from kaffir lime leaves for 12 or 24 hours/day showed significant increases in AChE activities in both hippocampus and cerebral cortex when compared to normal rats. Therefore, intake of kaffir lime and lemongrass teas as a substitute for drinking water did not inhibit AChE, but instead stimulated the activities of AChE in both the hippocampus and cerebral cortex of rats. The increase in AChE activities in rat's brain may link to the accumulation of amyloid plaques and neurofibrillary tangles, as reported previously [33]. Thus, the consumption of kaffir lime tea for 12 or 24

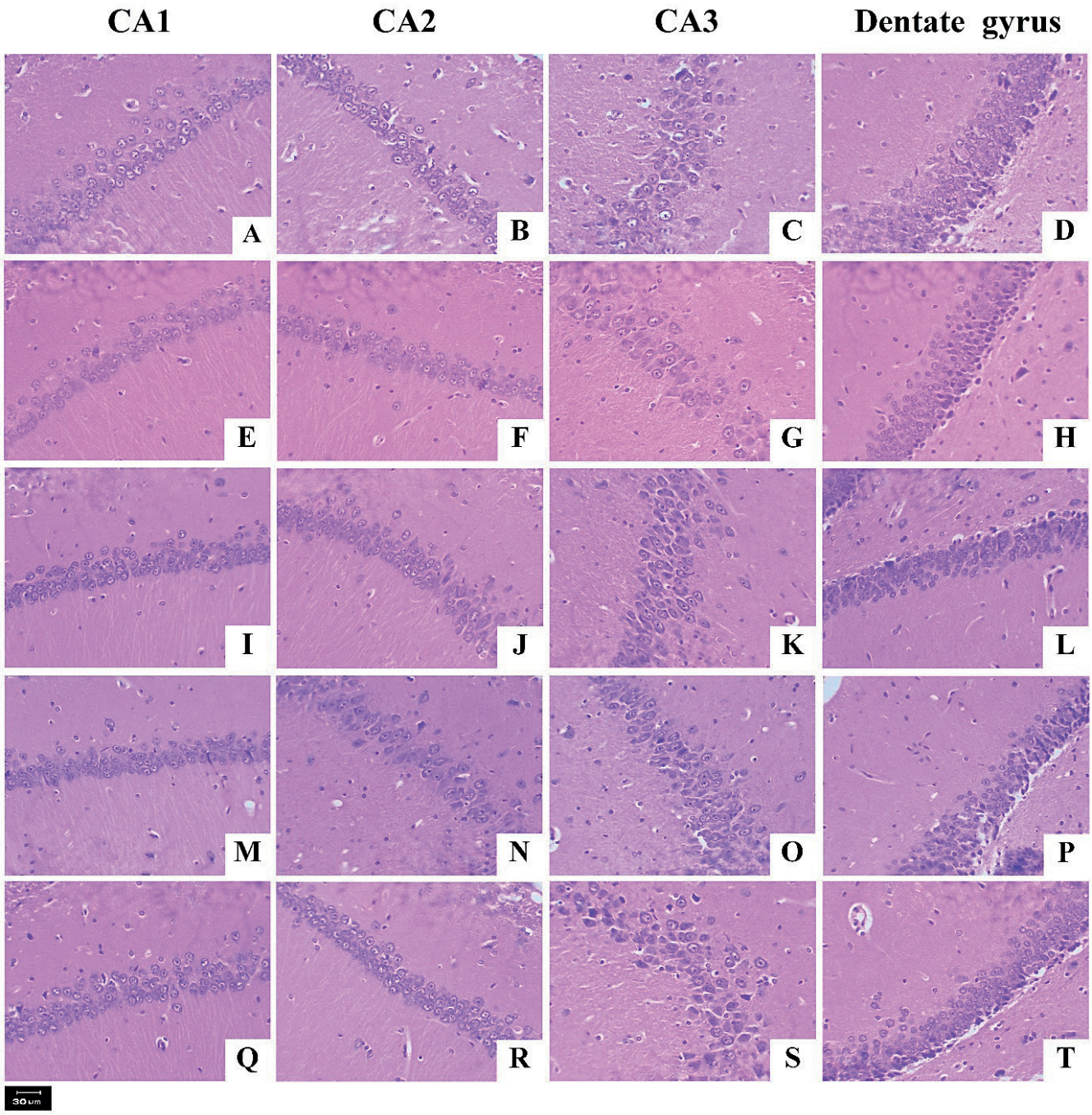

Figure 3. Histological features in different areas of hippocampus of rats treated with lemongrass tea for 12 (E-H) and 24 hours/days (I-L) and kaffir lime tea for 12 (M-P) and 24 hours/day (Q-T) compared to the control group (A-D). H&E stain, 20x magnifications.

hours/days or lemongrass tea instead of water may affect memory and cognition. However, the AChE result from our study is different from a previous report [34], which indicated that the extract from the leaves of kaffir lime at 50 and 100 mg/kg body weight effectively enhanced memory function and attenuated scopolamine-induced amnesia in mice. The results may have been influenced by different factors, such as variations in herb cultivations, condition of animals, treatment periods, and extraction methods. Additionally, it is possible that kaffir lime and lemongrass teas may inhibit only certain type of AChE. For example, studies by [35] and [36] demonstrated that the type of AChE found in the brain was globular monomer 1 (G1 AChE-S). Moreover, high AChE activities in the hippocampus and cerebral cortex of rats may be linked to the high production of ACh [37]. An increase in brain AChE activities may be a normal physiological response, rather than a pathological one, triggered by an increase in brain activity and a greater demand for ACh. Therefore, the brain may elevate AChE production to regulate ACh levels. However, determining only AChE activity is not enough to conclude the effects of lemongrass and kaffir lime teas on the cholinergic system in cognitive function. Further studies are needed to examine the activities of choline acetyltransferase, the enzyme responsible

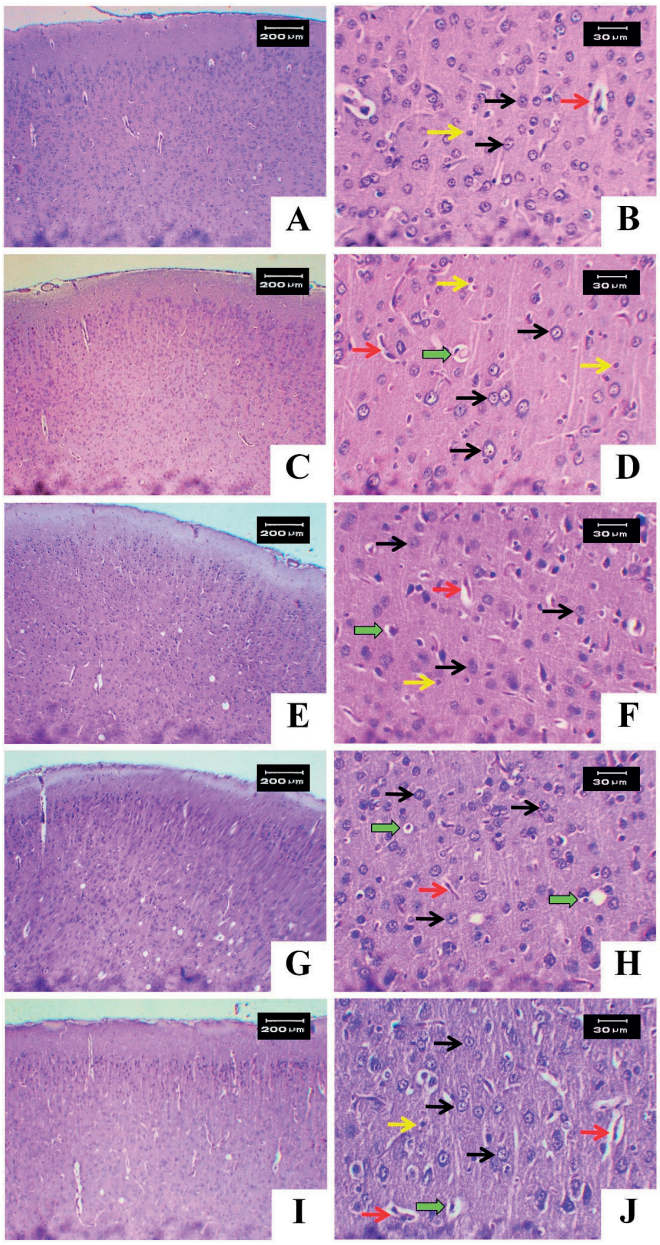

Figure 4. Histological features of the cerebral cortex of rats treated with lemongrass tea for 12 (C-D) and 24 hours/days (E-F) and kaffir lime tea for 12 (G-H) and 24 hours/day (I-J) compared to the control group (A-B). H&E stain, 4x and 20x magnifications. Neuron (dark arrow), neuroglia (yellow arrow), myelinated axon (green arrow), blood vessel (red arrow).

for synthesizing ACh, to support the level of ACh production. This would provide valuable insights into the regulation of acetylcholine in the rat's brain after drinking lemongrass and kaffir lime teas. The alteration of AChE activities produced from lemongrass and kaffir lime teas may come from the bioactive elements contained in the plants. It is currently reported that several plants species contained flavonoids, such as tiliroside, 3-methoxy quercetin, quercitrin and quercetin and those flavonoids exhibited AChE inhibitory activity [38]. Furthermore, alkaloids, which are biologically active compounds found in different species, have been reported to strongly inhibit AChE [39-40]. From our preliminary phytochemical screening, both lemongrass and kaffir lime teas were found to contain the same phytochemicals, including phenols, flavonoids, tannins, saponins, terpenoids, and reducing sugars. These phytochemicals may support brain function in various ways. Polyphenols derived from plant materials have been reported to have neuroprotective benefits through several processes, such as anti-amyloidosis, anti-inflammation and antioxidant effects [41]. The intake of flavonoids, such as (-)-epigallocatechin-3-gallate, has been found to stimulate cerebral blood flow in frontal cortex [42]. Tannins may enhance against learning and memory by reducing oxidative stress and elevating sodium/potassium-ATPase activation in streptozotocin-induced dementia models [43-44]. However, the mechanisms underlying the neuroprotective and cognition-enhancing effects of kaffir lime and lemongrass on memory have not been elucidated in our research. Moreover, there were limitations and variations across different rat models and experimental setups in our study. Therefore, further research using dementia-induced rat models or other appropriate models can help fill the gaps and provide a more comprehensive understanding of the specific effects of lemongrass and kaffir lime teas and the mechanisms related to dementia.

The results of the histological study showed that both lemongrass and kaffir lime teas did not produce any toxicity in the brain. Histological investigations of rat brains did not reveal any significant alterations in their cerebral cortex and hippocampus after receiving lemongrass and kaffir lime teas for 30 consecutive days. The hippocampus of all rats showed the normal pattern of cell body layers in both the Cornu Ammonis and dentate areas. The cell body layer consisted of normal pyramidal cells without degenerative cells. The cerebral cortex of rats in the tea-treated and control groups showed normal histological features, composed of a normal arrangement of neurons. Normal neurocytes, such as neurons and neuroglia, were characterized by dark-stained nuclei and clear cytoplasm. However, some phytochemical compounds presented in both kaffir lime and lemongrass teas can cross blood-brain barrier and may have negative or positive effects on brain tissues [45-46]. Therefore, further research is needed to correctly determine the risk of tea consumption as a substitute for drinking water.

CONCLUSION

The present study provided basic information on the effects of consuming kaffir lime and lemongrass teas as a substitute for drinking water. Continuous consumption of these teas for 30 consecutive days affected brain function by elevating AChE activity in rat's brain. However, these teas showed no effect on brain histology of the experimental rats.

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