AJPS

e-ISSN: 2980-0463
Volume 4 / Issue 1

Anatolian Journal of Pharmaceutical Sciences

Inonu University
Faculty of Pharmacy
Turkey

İNÖNÜ ÜNİVERSİTESİ 50. yıl

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e-ISSN: 2980-0463
Anat. J. Pharm. Sci., 2025:4(1)
AJPS
Review Article

Current Developments in Alzheimer's Disease and Treatment

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ABSTRACT: Alzheimer's Disease is a progressive and irreversible disease with a high incidence in older people both in our country and the world, and its pathophysiology is not fully understood. There is no definitive treatment for the disease, but some hypotheses that are thought to be effective in treatment have been determined; the most effective of these is the cholinergic hypothesis, which suggests that acetylcholine levels are low in the brains of patients. Other hypotheses are the tau and amyloid hypothesis, oxidative stress and neuroinflammation. Current treatments are mostly symptomatic, and the first drugs approved for use are the acetylcholinesterase inhibitor rivastigmine, galantamine, donepezil, and the N-methyl-D-aspartate receptor antagonist memantine. In developing new treatments, pathological causes have been targeted, new methods have been tried to reduce amyloid accumulation and tau phosphorylation, and effective drugs have been found. Still, they have not been put into clinical use. Antioxidant compounds have been studied to suppress oxidative stress. Other treatment methods include stem cell therapy, vaccination and the use of estrogen.

Keywords: *Alzheimer's disease, current treatment, pathophysiology.*

1 INTRODUCTION

Dementia is known as a chronic, progressive, and irreversible disease that is common after the age of 65. A diagnosis of dementia requires problems with at least two cognitive functions, such as memory impairment, speech abnormalities, and orientation problems. Additionally, patients have difficulty fulfilling their ordinary daily needs [1,2]. There is no definitive treatment for Alzheimer's Disease (AD) and the treatments applied are aimed at relieving the symptoms [3]. Several hypotheses have been proposed regarding the pathology of AD, including decreased cholinergic activity, amyloid deposition, excessive tau protein phosphorylation, and increased levels of inflammation and oxidative stress in brain tissue. Drugs commonly used in treatment include acetylcholinesterase (AChE) inhibitors (donepezil, rivastigmine, galantamine) and memantine, an N-methyl-D-aspartate (NMDA) receptor antagonist [4]. In current treatment studies, in addition to pathology-oriented studies, anti-inflammatories, antioxidants, and stem cell therapy have been

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Submitted: 31.10.2024 Accepted: 13.02.2025
doi.org/10.71133/anatphar.1577117

tried. Studies have been conducted on compounds aimed at reducing tau protein and amyloid accumulation, but suitable methods for clinical use have not been found [5]. Since the classical methods used in treatment today are inadequate and are symptom-oriented, this review study investigates whether there are new developments in treatment and at what stage these developments are [5].

Dementia is a chronic and progressive disease characterized by problems in at least two of the cognitive functions such as speech, memory loss, judgment, and problem solving. Dementia can develop due to trauma, inflammation, infection, vascular, brain degeneration, and toxic causes. It is classified according to its etiology, findings, and brain regions, and is divided into two groups according to its etiology [1].

A-Primary dementia; AD, Lewy body dementia, pediatric dementias, and rare dementias [1],

B-Secondary dementia; vascular dementia, toxic metabolic dementia, and normal pressure hydrocephalus [1].

AD is a neurodegenerative disease and is usually seen in advanced ages. The brain's behavioral and cognitive functions are impaired and it is a progressive disease [2]. When the brain tissue is examined microscopically, tangles and plaques are seen in the neurons. The first symptom is memory loss, and the patient has problems in fulfilling their daily needs (shopping, personal hygiene, eating, and using devices), in addition, the patient also has psychiatric disorders [3]. Pathological causes include senile plaque (SP) formation, neurofibrillary tangle (NFT) formation containing phosphorylated tau proteins in the hippocampus, and a decrease in acetylcholine (ACh) levels [4].

1.1 History

Dr. Alois Alzheimer is the first person to describe the clinical and anatomopathological findings of the disease. The first symptom of a 51 year-old patient named Auguste Deter, who caught Dr. Alzheimer's attention in 1901 at the institution where he worked in Frankfurt, was paranoid jealousy of her husband, which was soon followed by memory impairment, disorientation in time and space, paranoia, and auditory hallucinations. Later, the patient became bedridden with incontinence and died within 4.5 years. After the death of his patient, Dr. Alzheimer presented the histopathological findings that he had identified in the cerebral cortex at a scientific meeting in Tübingen, Germany, in 1906. However, since he did not receive the expected recognition, he published his

findings in 1907 as an article in the journal "*Allgemeine Zeitschrift für Psychiatrie und Psychisch-Gerichtliche Medicin*", titled "A Peculiar Disease of the Cerebral Cortex" [3].

1.2 Epidemiology

The number of elderly individuals who died from AD was 13,859 (4.6%) in 2018 and 11,880 (3.2%) in 2022. When deaths from AD are analyzed by gender, the rate for men was 2.3%, and the rate for women was 4.1% in 2022 [6]. The incidence of AD by age is 0.4% in the 65-69 age group and increases to 10% by the age of 90, while the prevalence is 2% in the 65-69 age group and exceeds 25% in individuals over 90. The prevalence doubles every 5 years over the age of 65. In developed countries, it is reported that AD symptoms occur in one in every 10 people aged 65 and over, and in one in three cases aged 85 and above [7].

1.3 Risk factors

1.3.1 Genetics

Three gene disorders responsible for Early Onset Alzheimer's Disease have been proven. These genes are Amyloid precursor protein (APP) on chromosome 21, Presenilin 1 (PSEN1) on chromosome 14, and Presenilin 2 (PSEN2) on chromosome 1. The only gene known to have an effect on Late Onset Alzheimer's Disease is Apolipoprotein E4 (ApoE4) [8].

APP gene: Although its exact function is not fully understood, it is thought to be involved in synaptic formation and neuron migration. The pathology of the disease is caused by the accumulation of amyloid β (Aβ) resulting from the cleavage by β and γ secretase enzymes. Similar findings to AD are also observed in patients with Down syndrome after the age of 40. Because Down syndrome and AD are associated with overexpression of APP on the q arm of chromosome 21 and Aβ accumulation [9].
PSEN1 gene: It is the gene with the highest risk in the development of the disease. It is a part of γ secretase, which is responsible for the degradation of APP. It is responsible for the catalytic activity of the enzyme. When PSEN1 is mutated, changes occur in γ secretase. As a result of the change in the enzyme, the formation and accumulation of Aβ40 and Aβ42 increases [9]. The PSEN2 gene is highly similar to the PSEN1 gene. The age of onset of the disease is higher. Mutations in the PSEN2 gene cause different clinical pictures compared to the PSEN1 gene. The mutated PSEN2 gene binds to kinases that regulate signaling and increases β secretase activity. Mutations in the PSEN2 gene are not as frequent as mutations in the PSEN1 gene [9].

ApoE4 gene: It is a serum protein involved in functions such as the transport and

storage of cholesterol. It has three alleles, ApoE 2, 3 and 4, and the ApoE4 allele increases amyloid accumulation (8). The ApoE2 allele has a protective effect against the disease [3].

1.3.2 Age

After the age of 65, the risk of AD doubles every five years. Those under the age of 65 are classified as Early Onset Alzheimer's Disease, and those over the age of 65 are classified as Late Onset Alzheimer's Disease Early and late-onset AD differ in terms of clinical, pathological, and imaging as well as age of onset [10].

1.3.3 Gender

Since women have a longer lifespan than men, their risk of developing the disease is higher. Although the disease is associated with low education levels in women, it is more meaningful to associate it with estrogen levels that fall, especially after menopause, because estrogen has a regulatory effect in the brain and increases the formation of new synapses in hippocampal cells [11]. Estrogen regulates brain functions by adjusting the levels of neurotransmitters, slows down the production of ApoE and increases its destruction, and reduces the formation of SP by reducing Aβ production. It also has an antioxidant effect by reducing the neurotoxic effects of Aβ and reduces inflammation by affecting interleukin-6 (IL-6), which is effective in the formation of SP. Although most epidemiological studies show that the risk of developing AD is significantly reduced in women who receive estrogen replacement therapy after menopause compared to women who do not, this result could not be confirmed in some studies, and it has been suggested that factors such as patient selection, the route of administration of the treatment, the dose, and the duration of the drug are different [12].

1.3.4 Depression

It has been suggested that depression doubles the risk of AD, but another hypothesis is that depression may actually be an early symptom of AD. Depression increases the risk of developing vascular dementia and AD in older individuals [13, 14]. Long-term use of selective serotonin reuptake inhibitors (SSRIs) has been found to delay the progression from mild cognitive impairment to AD, and it is thought that it shows its effect by reducing Aβ production [15].

1.3.5 Diabetes

Patients with diabetes are at risk for cognitive impairment and dementia. Toxicity and increased oxidative stress caused by the accumulation of glycation products damage the structure of the vessels, resulting in increased neuronal loss,

and as a result, long-term hyperglycemia leads to worsening of cognitive impairment. Hypoglycemia is also effective in cognitive impairment. In severe hypoglycemia, cognitive impairment accelerates with neuronal loss and fibrinogen formation. Hyperinsulinemia reduces the number of insulin receptors, disrupts insulin response, and thus inhibits insulin passage to the cerebrospinal fluid (CSF) and brain tissues, resulting in problems in learning and memory formation. Insulin sensitivity in the brain is impaired in Alzheimer's patients, and the enzymes responsible for the destruction of insulin also destroy amyloid. When insulin levels are high, enzymes compete to destroy insulin, and Aβ destruction decreases and amyloid accumulation increases in the brain [16]. Diabetes is also effective in tau protein accumulation; antidiabetics used in the treatment of diabetes have been found to reduce Aβ and tau protein accumulation and provide cognitive improvement [17]. The most effective are metformin, dipeptidyl peptidase-4 inhibitors, glucagon-like peptide-1 analogs, and sodium glucose transporter-2 inhibitors [18].

1.3.6 Hypertension

High blood pressure is considered a risk factor for AD, but the relationship between them has not been fully determined. Hypertension is thought to cause poor cognitive performance and cognitive decline [19]. A postmortem study in humans found that hypertension in middle age causes a decrease in brain volume, increased Aβ accumulation, and NFT formation [20].

1.3.7 Dyslipidemia

Hypercholesterolemia seen in middle age is a risk factor for AD, and the information supporting this is the decrease in Aβ40 and Aβ42 levels in patients using statins [8]. Increased cholesterol levels in the brain increase oxidative stress, neuronal loss and phosphorylation of tau proteins. Another negative result of an increase in cholesterol level is that it affects APP degradation, leading to an imbalance and amyloid accumulation. [21]. In some studies, there is no relationship between hypercholesterolemia and AD [8]. The reason for the different results may be due to the subjects used in the studies or changes in the protocol [21].

1.3.8 Family History

Family history is a more important factor in Early Onset Alzheimer's Disease, and most cases that start before the age of 60 have a family history. The risk of developing the disease in children of people with AD is 6 times higher than in healthy people [22].

1.3.9 Smoking and alcohol

Smoking increases the risk of AD. Smoking and alcohol disrupt the balance between the formation and reduction of oxidants and free radicals, and due to the disrupted balance, the level of oxidants and free radicals in the body increases, ultimately causing oxidative stress. Increased oxidative stress is effective in the accumulation of SP and NFTs and plays a role in the pathology of the disease with this mechanism of action [23]. Long-term alcohol use negatively affects the motor and cognitive functions of the brain, causes cholinergic neuron loss and atrophy in brain tissue. In a study conducted in mice, it was observed that it caused an increase in the phosphorylation of tau protein and memory impairment. Acetaldehyde, which is formed as a result of alcohol metabolism, has a neurotoxic effect on the brain, thus causing permanent damage to the brain. Although alcohol has an immunomodulatory effect depending on the dose and frequency of consumption, the effect of alcohol on AD is not fully understood [24].

1.3.10 Nutrition and Obesity

Obesity in middle age is a risk for AD, but there is no definitive data on obesity in advanced age. In fact, some studies have shown that obesity is protective against AD [25]. Obese elderly individuals have less Aβ accumulation and larger hippocampus volumes [26]. There is no specific diet for AD, but the Mediterranean diet is recommended. This diet includes more vegetable and fruit consumption. It limits the consumption of saturated fat, meat, and dairy products. It reduces oxidative stress in AD due to its antioxidant content. Many studies have shown that microbiota is important in brain functions. Disruption of the microbiota can cause degeneration in the brain, and exercise and probiotic use reduce inflammation, oxidative stress and Aβ accumulation. Aluminum accumulation in the brain has been observed in AD, and long-term exposure is risky because aluminum causes neuropathy, oxidative stress, and inflammation. High copper intake leads to learning deficits, lead increases neurodegeneration, and cobalt accumulation negatively affects neurotransmission. Mercury accumulates in neurons and increases the accumulation of Aβ and tau protein. Even small amounts of arsenic can cause neurological disorders. High calcium levels can affect AD pathology by increasing Aβ accumulation and phosphorylation of tau protein [27].

Vitamin B_{12}: Neurological problems are experienced in vitamin B_{12} deficiency. In vitamin B_{12} deficiency, the homocysteine cycle is disrupted, and its level in the blood increases [28]. High homocysteine levels

increase neurotoxicity and hydrogen peroxide formation [8]. Homocysteine shows its neurotoxic effect by activating the NMDA receptor or by converting to homocysteine acid. Another negative result of high homocysteine levels is that it causes Aβ accumulation and phosphorylation of tau protein. Methylation reaction is also disrupted in vitamin B_{12} deficiency. Methylation reaction is important in neurotransmitter, phospholipid, and nucleotide production in the brain. Therefore, vitamin B_{12} deficiency is a risk factor for AD [29].

Vitamin D: In vitamin D deficiency, individuals may experience decreased cognitive functions, intermittent memory loss, and executive dysfunction [22]. Vitamin D is important in neurotransmission, protection, and synaptic plasticity in the brain. Its active form, 1.25 $(OH)_2D$, is effective in the phagocytosis of amyloids. In this case, $A\beta_{42}$ accumulation decreases and plays a role in the differentiation and maturation of neurons. Since it regulates the genetic expression of neurotransmitters in the hippocampus, vitamin D intake may be beneficial for Alzheimer's patients [30].

Vitamin A: Vitamin A plays a role in the differentiation and protection of neurons in the brain and in the release of neurotransmitters. Vitamin A and beta-carotene levels are decreased in AD. In a study conducted in mice, it was observed that low vitamin A levels resulted in Aβ accumulation, and Aβ inhibits retinoic acid synthesis, which worsens the pathology of AD. The importance of vitamin A is explained by the retinoid-dependent transcriptional regulation of genes such as APP, $PSEN_1$, and $PSEN_2$, which are effective at the amyloid level. All-trans-retinoic acid regulates the transcription and activation of these genes in an antiamyloidogenic manner. Giving vitamin A supplementation to patients improves cognitive impairment [30].

Vitamin E: The most abundant form of vitamin E in human tissue is α-tocopherol. As a result of low levels of vitamin E in the body, $ApoE_4$ transport in the brain increases and causes cognitive damage. In addition, vitamin E has antioxidant properties for the brain [22].

Vitamin C: It is found in very small amounts in neurons. It cleans superoxide, a product of mitochondria in neurons. Vitamin C protects synaptic activity and helps detoxification. It suppresses genes that cause inflammation and prevents Aβ accumulation. Taking vitamin C supplements improves memory performance and memory problems [30].

Vitamin K: Cognitive performance disorders are observed in vitamin K deficiency. It protects brain cells from inflammation and oxidative stress. Therefore, the use of vitamin K may be beneficial in AD [30].

1.4 Pathology and physiopathology

Despite extensive studies, the pathology and physiopathology of AD are not fully understood. Macroscopic findings show atrophy in the cortex and hippocampus regions, while histological findings show NFT, amyloid plaques, synaptic loss, and cholinergic neuron loss [31].

1.4.1 Amyloid hypothesis

The enzymes responsible for APP metabolism are α, β, and γ secretase. In the first step, it is metabolized by being cleaved by α and β secretase enzymes. When cleaved by the α-secretase enzyme, SAPPα and C_{83} fragments are formed. These fragments are not toxic to the brain. Then, the SAPPα fragment is secreted out of the cell, while the C_{83} fragment remains in the cell membrane. As a result of the cleavage by β secretase, sAPPβ and C_{99} fragments are formed. The sAPPβ fragment is secreted out of the cell, while the C_{99} fragment remains in the cell membrane. In the second step, the C_{99} fragment is cleaved by γ-secretase and Aβ and APP intracellular domain fragments are formed. The fragments formed as a result of the cleavage are neurotoxic. This cleavage is heterogeneous, and $A\beta_{40}$ formation is more than $A\beta_{42}$ formation. Amyloid plaque accumulation is caused by $A\beta_{42}$. Because it is more prone to turning into fibrillar form and has a hydrophobic structure [9]. Amyloid accumulation is seen in the meningeal vessels of the brain. This accumulation leads to cerebral hemorrhages and cognitive impairments [32].

1.4.2 Tau hypothesis

Tau protein is mostly found in mature neurons but can also be found in the nucleus, mitochondria, dendrites, synapses, and membrane. Tau protein maintains the stability of microtubules, which are important for the transport of products in neurons and cell structure [33]. Therefore, tau protein is responsible for maintaining cell shape and axonal transport. After tau protein is synthesized, it differentiates with reactions such as phosphorylation and nitration, and with the increase in phosphorylation reaction, the structure of tau protein is disrupted, and it cannot bind to microtubules. When tau protein cannot bind to microtubules, it binds to each other and forms double or single helices. The mechanism that causes increased phosphorylation is not fully known. However, cyclin-dependent kinase 5 (CDK5), a serine-threonine kinase,

phosphorylates cytoskeletal proteins, synaptic proteins, and transcription factors, including tau protein. It is suggested that phosphorylation of tau protein increases as a result of high activation of CDK5, and the increased activity of CDK5 is caused by Aβ accumulation [34]. The resulting helical structures lead to the formation of NFTs [35]. NFTs facilitate the aggregation of tau proteins and disrupt cell integrity by preventing the stability of microtubules, causing neuronal death [36].

1.4.3 Cholinergic hypothesis

ACh has activity throughout the cortex, basal ganglia, and basal forebrain. It is effective in physiological processes such as memory, attention, sleep, and sleep disorders. ACh is synthesized with the help of choline and acetyl coenzyme A by choline acetyltransferase (ChAT). After synthesis, it is released into the synaptic cleft and shows its effect by binding to the postsynaptic receptor. Unbound excess ACh is broken down into choline and acetic acid by AChE. These are taken back to the presynaptic neuron by a mechanism for recycling to acetyl coenzyme A. According to the hypothesis, ACh level is reduced in AD pathology, and the reasons for this decrease are decreased ChAT level, increased AChE activation, and insufficient choline reuptake. In short, insufficient ACh production leads to cognitive impairment [37].

1.4.4 Oxidative stress

Free radicals can form in both physiological and pathological conditions. Reactive oxygen species (ROS) play a role in cellular metabolism and signal transduction pathways. Due to excessive production, lipids, intracellular proteins, and DNA are damaged. ROS are retained or transformed by antioxidants. Aβ causes neuronal loss by inducing oxidative stress in the brain. As an indicator of this, 4-hydroxynonenal and malondialdehyde levels, which are markers of lipid damage, are increased. When the antioxidant system becomes inadequate, mitochondrial function is impaired and cell death occurs [38]. As a result of lipoperoxidation, phospholipids decrease, and plaque formation can be observed in the brain due to lipid formation and antioxidant depletion. Oxidative stress markers have been detected in brain tissue and CSF in AD [39].

1.4.5 Neuroinflammation

Pro-inflammatory mediators are found in high amounts in the brain tissue and CSF of Alzheimer's patients. Neuroinflammation occurs due to the excessive secretion of pro-inflammatory cytokines and chemokines, which activates macrophages in the brain and increases the levels of tumor necrosis factor-alpha,

interleukin-8, transforming growth factor-β, and macrophage inflammatory protein-1α and Aβ plaques [32].

1.5 Clinical findings

The disease usually starts at the age of 40 in Early Onset Alzheimer's Disease, and after the age of 65 in Late Onset Alzheimer's Disease. The first complaint of most patients is memory problems, and the symptoms can be divided into cognitive disorders and non-cognitive, i.e., psychiatric symptoms [40].

Cognitive disorders;

• Dysphasia: Speech disorder,

• Anomia: Forgetting the names of objects or people,

• Inability to do calculations,

• Difficulty solving problems,

• Disorientation: Decreased sense of direction and time perception, inability to recognize people,

• Agnosia: Inability to process sensory information,

• Difficulty in remembering,

• Dyspraxia: Inability to perform tasks that require skill [40].

Non-cognitive (psychiatric) symptoms;

• Depression,

• Psychotic behaviors: Seeing hallucinations and paranoia,

• Non-psychotic behaviors: anxiety, exhibiting repetitive behaviors, and hyperactivity [40].

Disease stages; Early stage: Patients begin to experience memory problems. They have difficulty learning new information, repeating their questions and conversations. They forget names and the location of objects. They have difficulty using devices and doing their hobbies. Although they have problems with reasoning skills in the first stage, they do not have much trouble in terms of behavior [41].

Middle stage: Patients now have difficulty performing their daily activities. Despite receiving help, they are unable to recognize people, have difficulty eating, and have decreased motor functions such as incontinence and walking. They can go out with their relatives, but they cannot find their way when they are alone, i.e., behavioral problems begin to emerge [42].

Advanced stage: The patient's personality traits have completely changed. They have difficulty speaking and understanding what is said, and have difficulty chewing and swallowing. They cannot express their

feelings [43]. They can no longer take care of themselves and need an assistant. They cannot perform behaviors such as eating, cleaning and dressing on their own. The disease progresses and results in death, and the cause of death is due to bed sore infections, lung infections, and nutritional deficiencies [44].

1.6 Diagnosis

For the definitive diagnosis of AD, a biopsy or an autopsy can be performed to examine brain tissue. In the clinic, a diagnosis can be made with high accuracy using patient history, laboratory results, imaging techniques, psychological tests, and neurological examination. There are criteria used in the diagnosis of the disease. These are established by NINCDS-ADRA and DSM-V [45].

Alzheimer's type dementia according to DSM-V criteria is diagnosed as follows [45].

A. Diagnostic criteria for severe or mild neurocognitive impairment must be met.

B. There is a slowly progressive, insidious, and silent deterioration in one or more cognitive domains.

C. Diagnostic criteria for probable or possible AD are met as follows:

• C1. For severe neurocognitive impairment:

If 1 of the following is present, the diagnosis of possible/probable AD should be made; if not, possible AD should be made.

1. Evidence of a causative AD heritable mutation (genetic mutation) from family history or genetic measurements

2. All three of the following:

a. Clear evidence of decline in memory and learning and at least one other cognitive domain (by detailed history or a battery of neuropsychological measurements)

b. Steady, progressive decline in cognitive function without long-term cessation.

c. No evidence of other confounding factors that could cause cognitive impairment. (Other neurogenerative or cerebrovascular disease or other neurological, mental or systemic disease or condition that may contribute to cognitive decline)

• C2. For mild neurocognitive disorder:

A diagnosis of probable AD is made if there is evidence from family history or genetic measurements of a causative AD hereditary change (genetic mutation). In the absence of this evidence, a diagnosis of probable AD is made if all three of the following are present:

1. Clear evidence of memory and learning decline

2. Steady, progressive decline in cognitive functions without long-term cessation.

3. No evidence of other confounding factors that could cause cognitive impairment. (Another neurodegenerative or cerebrovascular disease, or another neurological, mental, or systemic disease, or a condition that may contribute to cognitive decline)

D. The disorder is not explained by the effects of cerebrovascular disease, another neurodegenerative disease, a substance, or another mental, nervous, or systemic disorder [45].

1.6.1 Taking patient history

Since sufficient information cannot be obtained from the patient if the disease is advanced while taking the history, information should be obtained from the patient as well as from the relative. The patient's relative can see the differences between the patient's past and current state. The patient should be asked about the functions that have been affected, and questions such as eating, remembering recent events, driving, calculating, finding one's way, repeating the same conversations and questions, solving problems and changes in personality traits can be used to get an idea about the stage of the disease. The patient's general medical, psychological, toxicological and neurological history should be asked, and family history should be taken and the presence of people with AD in their relatives should be questioned [46].

1.6.2 Neuropsychological evaluation

An examination should be performed initially to understand the changes in functions such as memory impairment, language, attention, visual skills, problem solving and perception in the patient. It also helps to understand the cause of the patient's cognitive impairment [47].

1.6.3 Imaging methods

Magnetic resonance imaging (MRI) of the patient's brain is used for diagnosis. The findings seen are regional or general atrophy and white matter lesions [47]. Distinguishing AD from other types of dementia becomes easier with computerized tomography or MRI, and atrophy in the temporal lobe and hippocampal formation in volumetric studies strengthens the diagnosis. Among other methods, cerebral blood flow with single photon beam computed tomography and decreased glucose metabolism in the temporo-parietal region with positron emission tomography (PET) are helpful in diagnosis [31]. Fluorodeoxyglucose-positron emission tomography is used to differentiate AD from frontotemporal dementia [47].

1.6.4 Laboratory findings

There is no laboratory test with proven validity for AD. There are recommended tests for the differential diagnosis of the disease. These tests help in the diagnosis of metabolic and systemic diseases that may lead to AD [31].

1.6.5 Recommended Laboratory Parameters

Complete blood count, sedimentation, serum electrolytes, glucose, BUN, creatinine, B12 and folate levels, liver, kidney and thyroid function tests [47].

1.7 Treatment

Although there is no drug that completely stops AD, there are FDA-approved AChE inhibitors (Rivastigmine, Galantamine and Donepezil) and Memantine, an NMDA receptor antagonist [48]. Treatments available for AD are used to slow the progression of cognitive, behavioral and psychological symptoms. The treatment applied is symptomatic and improves the quality of life for patients and their relatives. If the drugs are started before degeneration begins, i.e., before symptoms appear, their effectiveness may increase, in other words, early diagnosis provides significant benefits in treatment. Theeffectiveness of the drugs is also affected by their degree of penetration into the brain [49]. The primary treatment of the disease aims to improve cognitive symptoms, while the secondary treatment aims to improve psychological symptoms [31].

1.7.1 Classical treatment approaches

AChE inhibitors: According to the cholinergic hypothesis, the source of cognitive functions, especially memory impairment, is seen as a decrease in cholinergic activity. The fact that AChE activity is low in the disease and that neuronal loss is mostly seen in cholinergic neurons supports the hypothesis [50]. These drugs inhibit the AChE enzyme, reducing ACh destruction in the synaptic cleft, and after the destruction decreases, ACh levels increase in the synaptic cleft, which results in ACh binding to muscarinic and nicotinic receptors and increased stimulation intensity [51]. AChE inhibitors increase ACh levels, provide neuroprotective effects via nicotinic receptors, and regulate APP and Aβ formation by enabling neuron regeneration via muscarinic receptors [52].

AChE inhibitors:

a-Pseudo-reversible; Carbamates (Rivastigmine) [51].

Rivastigmine: It is approved by the FDA for use in the early and middle stages of AD. In the MR and PET results of patients who used rivastigmine for 6 months, great improvements were seen in the hippocampal metabolism of the brain. Side

effects include diarrhea, vomiting, anorexia, and weight loss [36]. Like ACh, it binds to the esteratic part of the enzyme, but its dissociation from the enzyme is slow. Although its plasma half-life is short due to its pseudo-reversible inhibition, the enzyme inhibition in the brain lasts 10 hours. It is highly selective for the hippocampus and cortex regions, and high doses are more effective in treatment [52]. It does not have a toxic effect on the liver, and since it binds to plasma proteins less and liver enzymes do not take part in its metabolism, it has very few interactions with other drugs [50].

b-Reversible; Tacrine, Donepezil, Galantamine [51].

Tacrine: It is the first drug approved for treatment. Its most obvious side effect is the increase in serum alanine transferase (ALT) enzyme levels. If the increase reaches three times the normal level, no change in treatment is made, and the use of the drug is stopped at higher values. After the drug is stopped, the ALT value returns to normal. In this case, the drug can be started again. However, when jaundice occurs, the drug is stopped and not restarted. Other side effects include headache, nausea and diarrhea [50]. It has central and peripheral effects and inhibits the enzyme reversibly. Its effect is not selective, meaning that it inhibits other choline esterases as well as the AChE enzyme. Drugs it interacts with include theophylline, warfarin and cimetidine [51].

Donepezil: Side effects are less common than other AChE inhibitors. It has no hepatotoxic effect [31]. It can be used in the early, middle and advanced stages of the disease. In addition to its cholinergic effect, it suppresses the production of inflammatory cytokines, oxidative stress and glutamate-induced toxicity. When taken in high doses, hypotension, respiratory distress, vomiting and muscle weakness are observed [36]. It has a more selective effect than other inhibitors. It binds to the AChE enzyme at a high rate. It has a lower affinity for butyrylcholinesterases and a higher central effect. It improves cognitive and behavioral symptoms. Sleep problems, anorexia, fatigue and cold symptoms are observed among its side effects [51].

Galantamine: In addition to enzyme inhibition, it is suggested that it stimulates nicotinic receptors and increases ACh release [50]. It is thought to provide improvement in cognitive disorders in Alzheimer's treatment. In mouse experiments, it is seen that it reduces inflammation and Aβ accumulation. Side effects include watery eyes, muscle weakness and nausea [36].

Metrifonate: It irreversibly inhibits the AChE enzyme. It has a higher affinity for the AChE enzyme than other enzymes. According to research, it has no hepatotoxic effects. Its side effects mostly affect the digestive system, and it is reported to have an effect on behavioral symptoms as well as cognitive symptoms [52].

c-Irreversible; Organo phosphorus compounds (metrifonate) [51].

NMDA receptor antagonists: This group includes memantine. Moderate improvement is observed when used alone or together with AChE inhibitors in the middle and advanced stages [53]. It has low selectivity for the NMDA receptor. It prevents toxicity in neurons, but there is no definitive information that it prevents degeneration [48]. It prevents toxicity because it reduces excessive glutaminergic activity in the nervous system [32].

1.7.2 Current treatment approaches

A-Muscarinic and nicotinic agonists: Another option to increase the activation of the cholinergic system is to stimulate the receptors. It has been observed that despite the decrease in cholinergic activity in AD, muscarinic receptors in the cortical and hippocampal regions remain intact. Therefore, muscarinic agonists are thought to be effective [31]. Muscarinic and nicotinic agonists are thought to be a good option for treatment because of the bradyarrhythmia, gastric acid secretion and bronchial secretion caused by AChE inhibitors, the increase in the effects of cholinergic drugs and the decrease in the effects of anticholinergic drugs. Their use is advantageous because they do not stimulate the inhibition of M_2 muscarinic autoreceptors. However, research on these drugs is ongoing [51]. Nicotinic receptor agonists play a role in memory and learning [50].

Xanomoline; It shows high selectivity for the M_1 muscarinic receptor. Aβ production decreases by stimulating the M_1 receptor. It has been tried in patients in the early and middle stages. According to the results, it has been found to be good for psychiatric symptoms such as hallucination, delusion and agitation. However, its use is limited due to fainting and digestive system side effects observed during the use of the drug. It is thought that the cause of its side effects may be due to the product formed as a result of its metabolism and its transdermal formulation is being developed [50].

ABT-418; It binds to the α4 and β_2 subtypes of nicotinic receptors with high selectivity. The structure of ABT-418 is similar to the structure of nicotine. It is seen to increase cognitive performance in studies conducted in animal models. It improves memory,

attention and executive function. Research is ongoing for its use in treatment [54].

B-Drugs based on the amyloid hypothesis:

The aim of this group of drugs is to prevent amyloid formation or reduce Aβ accumulation. Therefore, it is thought that their use in the early and middle stages of AD may be more beneficial [53].

BACE inhibitors; BACE enzymes cut APP and form the C99 fragment that causes Aβ to form. The cutting of APP by the BACE enzyme is the rate-limiting step. In the treatment, this enzyme is inhibited and the formation of Aβ is reduced. Therefore, it is important for the treatment [5]. The first examples were unsuccessful due to low bioavailability, inability to cross the brain barrier and liver toxicity [48].

γ secretase inhibitors; γ secretase enzyme does not only cut APP. The most important member of this enzyme family is the notch protein. Because it has important functions in the immune and digestive systems. In the treatment of Alzheimer's, selective inhibition is desired to prevent only APP from being cut by γ secretase. Non-selective inhibitors have serious side effects. For this reason, failure is observed in studies [32]. The most undesirable side effect among the observed side effects is learning disability. This situation is an undesirable side effect in the treatment [48].

α-secretase modulators; When the α-secretase enzyme cleaves APP, APPα and C_{83} fragments are formed. Since the cleavage is between the 16th and 17th amino acids of the Aβ sequence, Aβ production is prevented. The fragments formed are retained in the membrane and p3 is formed as a result of cleavage by γ-secretase. p3 is a non-amyloidogenic peptide [32]. Therefore, increasing the activation of the α-secretase enzyme reduces amyloid accumulation. There are many proteases that help in the activity of the enzyme. However, since it is not known which protease will increase the activity of α-secretase, this causes slow progress in drug development. Another problem is that there is no evidence as to whether it is indicated in AD [53].

RAGE inhibitors; By activating the receptor, it activates inflammatory responses and oxidative stress by increasing the activities of β and γ-secretase enzymes and helps Aβ formation [32]. RAGE is a receptor that binds many ligands. Among these ligands are Aβ peptides. Therefore, inhibitory agents may be useful. This inhibition reduces Aβ accumulation [48].

C-Drugs based on the tau hypothesis:

The aim of this group is to inhibit excessive phosphorylation and aggregation of tau protein. Another aim is to dissolve

previously found aggregates [5]. Prevention of tau hyperphosphorylation: Hyperphosphorylation prevents proteins from performing their functions. Hyperphosphorylation is a prerequisite for aggregation. The normal progression of phosphorylation depends on the balance between tau protein kinases and phosphatases. In Alzheimer's, the aim is to inhibit kinases and activate phosphatases. Therefore, kinase inhibitors are being developed. Glycogen synthase kinase 3 (GSK3β), CDK5 and extracellular signal-regulated kinase 1/2 are more focused on inhibition of these kinases. Tideglusib is a GSK3β inhibitor. Hyperphosphorylation is also observed by inhibiting enzymes such as protein phosphatase 1, protein phosphatase 2A (PP2A) and protein phosphatase 2B. PP2A is the enzyme most closely associated with the tau protein. Sodium selenate is an agonist that stabilizes the tau complex with PP2A [5].

Tau aggregation inhibitors; Tau aggregation leads to neuronal loss. For this reason, studies are being conducted to develop tau aggregate inhibitors. PE859, which is similar to the structure of curcumin, has been shown in experiments to prevent tau aggregation and prevent nerve dysfunction. Small molecules such as rhodanines, traquinones, and quinoxalines have also been found to prevent tau aggregation [5].

A study was conducted with methylthioninium and its oxidized form was found to be more stable. The chloride salt of the oxide form is called methylene blue. TRx0237 was developed based on methylene blue. However, studies have found that it is not effective in early and mid-stage Alzheimer's [48].

Microtubule stabilizing agents; As a result of increased phosphorylation or mutations of tau protein, tau protein cannot bind to microtubules. Thus, stability and axonal transport are impaired. Therefore, it has been thought that stabilizing agents can be used in treatment. Paclitaxel has been used and it has been observed that it increases the number of microtubules and axonal transport. This situation shows that it is suitable for treatment. However, it is unclear whether it helps treatment since it cannot pass the blood-brain barrier (BBB). Epitilons can pass the BBB. It has been observed that it improves cognitive functions by stabilizing microtubules in mice without causing a toxic effect. Davunetide has been reported to reduce tau pathology and cognitive function decline in mice and increase microtubule formation [5].

D-Antioxidant treatment:

Oxidative stress causes DNA fragmentation, cell membrane damage and

neuron death in the brain. When the activity of glutamine synthetase, which is sensitive to oxidation, decreases, free radicals are formed and antioxidant activity decreases. This pathological condition is seen in Alzheimer's patients. Antioxidants with neuroprotective effects are thought to be MAO inhibitors, vitamin E, ginkgo biloba and coenzyme Q10 [52].

Monoamine oxidase (MAO) inhibitors; The focus has been on developing MAO-B inhibitors for AD. Inhibition should be selective and reversible. Being reversible is important in preventing side effects. The amount and activation of MAO-B in the brain increases in advanced ages. This abnormal increase both reduces the level of neurotransmitter substances and increases the accumulation of radicals resulting from the oxidation of neurotransmitters. The increase in radicals causes an increase in oxidative stress. These can be prevented by using MAO-B inhibitors. Selegiline is in this group [55].

Vitamin E; There is evidence that free radicals may contribute to cognitive impairment in AD. Therefore, it has been thought that vitamin E can be used as an antioxidant. However, there is no evidence that vitamin E improves cognitive functions [56].

Coenzyme Q10; It is found in the membranes of many cells in the body and is fat-soluble. It is found in reduced form near unsaturated lipids to collect free radicals. The amount of coenzyme Q10 in the body decreases with age. Studies have found that its use may be beneficial because it reduces amyloid accumulation, degeneration and neuron loss in AD [58].

Ginkgo Biloba: It has an antioxidant effect due to the flavonoids it contains and an anti-inflammatory effect due to the terpenoids. It is also effective on memory and cognition [58]. Ginkgo biloba reduces the level of intracellular reactive oxygen species by inhibiting vanilloid type 1 channels. It also reduces the death of neurons. In this way, it is thought to have a protective effect on AD [59].

E-Anti-inflammatory treatment:

In epidemiological studies, anti-inflammatory drugs have been tried because AD is less common in patients with rheumatoid arthritis and inflammation is effective in Alzheimer's pathology. Indomethacin has been observed to slow the progression of AD [31]. There are active microglial cells and cytokines around the SP and astrocytes. Interleukin-1 and IL-6 cytokines contribute to the formation of Aβ by increasing APP production. Therefore, it has been thought that anti-inflammatory

drugs may be effective [51]. It is thought that they show their effects by inhibiting the cyclooxygenase enzyme or by affecting the γ-secretase enzyme. Based on the studies, it is understood that anti-inflammatory drugs are effective when taken in middle age and have no effect after the disease occurs [58].

F-Estrogen treatment: Estrogen receptors are found in the hippocampus and cholinergic neurons. Estrogen stimulates the release of nerve growth factor (NGF) and has an antioxidant effect. It increases Ach formation by increasing ChAT activity. In the study, it was found that estrogen is beneficial for verbal memory. Estrogen studies are ongoing in Alzheimer's. However, it is not recommended to use it as a primary treatment method [50].

G-Vitamin B_{12} and Folate; Vitamin B_{12} deficiency causes homocysteine balance to be disrupted and homocysteine levels in the blood increase. Therefore, it is thought that vitamin B12 and folate supplements may be useful to reduce homocysteine levels. However, in the study, no significant effect of vitamin B_{12} supplementation was found against the deterioration seen in cognitive functions [53].

H-Statins; Statin use disrupts the production of Aβ proteins and ApoE4. It reduces the hyperphosphorylation of tau proteins with its anti-inflammatory effect. In other words, tau aggregation also decreases. Thus, it is thought that they have a positive effect in AD. Studies have shown that statin use reduces the risk of developing AD [60].

I-Rosiglitazone; Insulin is effective on memory. Since the enzyme responsible for Aβ destruction is from the same group as the enzyme that breaks down insulin, it is thought that insulin may be effective in the formation of APP and Aβ. Therefore, eliminating insulin resistance has the potential to be beneficial for AD. Studies on the potential of rosiglitazone as a treatment for AD are ongoing. [53].

J-Stem cell therapy: The aim of the treatment is to use stem cells to replace neurons lost in Alzheimer's. There are four types of stem cells that can be produced from the body. These are neural stem cells (NSCs), mesenchymal stem cells (MSCs), embryonic stem cells and induced pluripotent stem cells. MSCs differentiate into neural cell types, increase ACh levels and improve cognitive functions. They reduce Aβ formation. They prevent tau phosphorylation and Aβ-related cell death. Studies have been conducted on MSCs and no serious side effects have been observed. However, studies are still ongoing. NSCs differentiate into mature cell types in the

brain and improve learning and memory [5]. In the study, NKH was injected into the brains of mice, and the stem cells in the injection area differentiated into brain cells, 3resulting in improvements in brain functions. In this way, damaged cells were repaired, memory loss was prevented, and positive results were obtained in learning [61].

K-Treatment with vaccination; It was aimed to benefit from the immune system to reduce Aβ accumulation in the brains of Alzheimer's patients, and for this purpose, vaccines containing different fragments of Aβ were administered to the patients subcutaneously or intravenously. However, since infection developed in the brains of the patients after the vaccination, studies on vaccination are continuing [62].

L-Treatment with neurotrophic factors; Studies have been conducted on neurotrophic factors such as NGF, neurotrophin-3 and insulin-like growth factor (IGF-1). NGF receptors have been found in cholinergic neurons in the basal forebrain, and it has been shown that the life span of cholinergic neurons is extended with NGF treatment. It has been found to be effective in cognitive recovery in animal experiments. Since it cannot pass to the central nervous system when given systemically, different administration methods have been tried. When given intrathecally, it caused meningeal thickening, and when used nasally, it caused extremity pain. Gene therapies are being studied for continuous NGF release. As another way, agents that will increase the effect of NGF are being tried. These agents include AIT-082, idebenone and propentofilin. Another neurotrophic factor is IGF-1. Its effect is to protect hippocampal neurons from Aβ-induced toxicity. Experimental studies on IGF-1 are ongoing [50].

2 CONCLUSION

AD is a neurodegenerative disease seen in older individuals and its incidence is increasing worldwide due to the increasing human lifespan. The lack of a definitive treatment method is a major problem. Existing drugs improve the patient's quality of life by reducing symptoms and slowing down the progression of the disease. The basis of the disease is targeted in the development of new treatment methods. AD does not occur due to a single cause. In other words, its development is influenced by multiple factors, making it difficult to find an appropriate treatment. Another reason for the failure of the studies conducted is that it is difficult for drugs to pass through the blood-brain barrier. The lack of clinical application for hypothesis-based drugs raises questions about their

accuracy. Therefore, understanding the causes of the disease is essential for developing definitive treatments. Because for a definitive treatment, those problems must be eliminated. Therefore, the development of new treatments will be facilitated by increasing the research on the causes and mechanisms of the disease.

3 CONFLICT OF INTEREST

Authors declare that there is no conflict of interest.

4 AUTHOR CONTRIBUTIONS

Literature review: S.K., İ.Y.; Manuscript writing: S.K., İ.Y.

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e-ISSN: 2980-0463
Anat. J. Pharm. Sci., 2025:4(1)
AJPS
Research Article

Development of a Sensitive Electrochemical Sensor for the Simultaneous Detection and Quantification of Acetaminophen Traces Using a Carbon Paste Electrode Modified with Black Carbon and $La_{0.6}Sr_{0.4}Co_{0.8}Fe_{0.2}O_3$ Nanoparticles

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ABSTRACT: Recently, the development of an advanced electrochemical sensor has received significant attention in the field of drug monitoring. The $La_{0.6}Sr_{0.4}Co_{0.8}Fe_{0.2}O_3$ (LSCF) nano-sized oxides were synthesized by a simple sol-gel citrate method, modified with black carbon (BC) and prepared as a carbon paste electrode (CPE) for the simultaneous determination of paracetamol (PCM) traces in PBS electrolyte through cyclic voltammetry (CV), differential pulse voltammetry (DPV) and electrochemical impedance spectroscopy (EIS) as sensing techniques. X-ray diffraction (XRD), scanning electron microscopy (SEM), and Ferrocyanide tests were used as characterization techniques. The calculated crystallite size *d*, was found to be 208.317 nm by XRD and 65.05 nm by SEM analysis. In conclusion, the modified prepared sensor LSCF/BC/CPE demonstrates a very satisfactory response, sensitivity, and selectivity towards PCM molecules compared with literature with a very low detection limit of 36 nM, high sensitivity of 75 $\mu A.\mu M^{-1}.cm^{-2}$, with a wide linear range from 0.1 µM to 180 µM by DPV technique. The modified LSCF/BC/CPE sensor demonstrated excellent results in the real pharmaceutical samples with a very good recovery of 94.56 % and a satisfactory relative standard deviation of 3.26 %.

Keywords: *Sensor, Nanoparticles, Black carbon, Paracetamol, Cyclic voltammetry, Differential pulse voltammetry.*

1 INTRODUCTION

Drugs traces determination in biofluids (blood, urine, serum, sweat, etc.), using newly developed sensors made of porous highly sensitive nanoparticles, especially with their application in advanced electrochemical techniques, is a crucial step in the field of drug quality control [1-3]. Paracetamol, also known as PCM, acetaminophen, or N-acetyl-p-aminophenol (as chemically illustrated in Scheme 1), is the key active ingredient of many analgesics and antipyretic medicinal formulations, commonly used for the alleviation of different symptoms like fever, headache, cough, cold, migraine and various types of chronic pain. PCM acts as an analgesic by inhibiting the synthesis of prostaglandins in the central nervous system and relieves fever through the hypothalamic heat regulation

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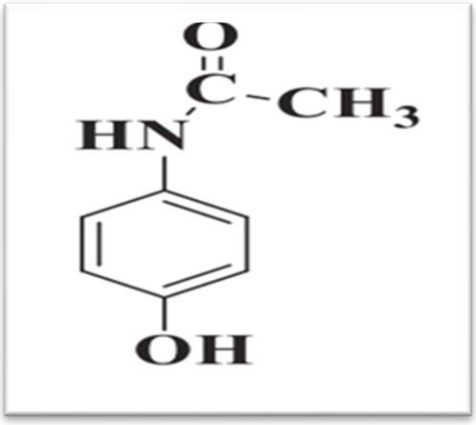
Submitted: 30.11.2024 Accepted: 18.02.2025
doi.org/10.71133/anatpharm.1594005

center [4]. Generally, acetaminophen does not have any harmful side effects, but its hypersensitivity or chronic use, also PCM overdoses can lead in some cases to the formation of certain hepatic and nephrotoxic metabolites which can result in kidney and liver failure [5]. Therefore, it is crucial to develop a new low-cost, highly effective, sensitive, and consistent sensor for the simultaneous sensing of paracetamol drug. Sensors made with metal nanoparticles are known as one of the most effective catalysts in many organic and electrochemical applications due to their excellent performance back to the high reactivity induced by their high active surface area and the small porous nano-sizes [6-8]. So far, several studies have reported on the study of PCM drugs with various analytical techniques mainly including spectrophotometry [9], flow injection chemiluminometry [10], thin layer chromatography [11], and high-performance chromatography [12]. Unfortunately, most of these conventional methods have several disadvantages related to low reproducibility, high instrument cost, complicated, and long time-consuming nature [13]. Developed electrochemical techniques especially cyclic voltammetry [13], differential pulse voltammetry [14], and electrochemical impedance spectroscopy have recently drawn a very useful approach overall these methods for PCM and other compound detection [15, 16]. Because they offer cheaper, highly sensitive and selective, low detection limits, and safe and simple analysis. The use of excellent new modified electrodes like carbon nanotubes (CNTs) or CPEs with good electrical conductivity, high electro-catalytic activity, and low detection limits is a very important step in the fabrication of high-performance electrochemical sensors [17-20]. Recently, different researchers studies have been reported the electrochemical detection of PCM molecules with carbon paste electrodes, such as: $MnFe_2O_4$ and $CoFe_2O_4$ nanoparticles modified graphite paste electrode GPE [21], Stevensite-modified carbon paste electrode (Stv-MCPE) [22], zirconium oxide ZrO_2 nanoparticles on modified CPE [23], imidazolium ionic liquid crystal on carbon paste composite (ILC/CPE) [24], magneto gold nanoparticles on CPE (Au NPs /Fe_3O_4/CPE) [25], La_2NiO_4 and Pr_2NiO_4 nano particles on CPE [26], polymerized L-phenylalanine modified carbon paste electrode (PLPAM/CPE) [27], poly(L-Leucine) layered carbon nanotube paste electrode [28], and 2,2 -[1,2 butanediylbis(nitriloethylidyne)]-bis-hydroquinone and TiO_2 nanoparticles CPE [20].

Currently, no report is available on the simultaneous electrochemical detection of paracetamol drug with $La_{0.6}Sr_{0.4}Co_{0.8}Fe_{0.2}O_3$/BC/CPE modified sensor. In this context, cheaper, highly

selective, sensitive, and very low detection limit than literature was reported.



Scheme 1. Chemical structure of Paracetamol drug.

2 MATERIAL AND METHOD

2.1 Chemicals and reagents

$La(NO_3)_3.6H_2O$(≥99%),$Sr(NO_3)_2$(≥98 %), $Co(NO_3)_3.6H_2O$(≥99%), $Fe(NO_3)_3.9H_2O$ (≥98%), citric acid ($C_6H_8O_7/H_2O$)(≥99%) were purchased from Sigma-Aldrich for the synthesis of LSCF nanomaterials using the citrate method. Paracetamol (PCM) was purchased from the BIOPHARM laboratory (Algeria), and the pharmaceutical product was purchased from PHYSIOPHARM laboratories (Algeria). Black Carbon (BC) (≥99%), Ferricyanide $K_3[Fe(CN)_6]$, and Ferrocyanide $K_4[Fe(CN)_6]$(≥98%), Ethylene Glycol (99%) were obtained from Biochem Chemopharma and Sigma Aldrich. All the reagents were of analytical grade, stored at room temperature (25°C), and all the solutions were prepared by dissolving the specified quantities of each product in 0.1 mM phosphate buffer solution (PBS).

2.2 Preparation of the sensor

LSCF nanoparticles were synthesized by a simple citrate method; all the details are illustrated in [29, 30]. The modified LSCF/BC/CPE sensor was prepared by mixing an appropriate quantity of the $La_{0.6}Sr_{0.4}Co_{0.8}Fe_{0.2}O_3$ synthesized nanoparticles and black carbon powder at ¼ and ¾ (w/w) ratios. LSCF NPs and BC powders were grounded well to achieve a smooth blending. After that, the resulting powder was mixed with 2 to 3 drops of the viscus ethylene glycol gel until a black dough (paste) was formed [31]. The formed paste was transferred into a clean capillary glass tube (d = 0.9 mm; S = 0.073 cm^2) by pressing it with a metal rod at the bottom of the glass tube with attaching a platinum wire for electrical contact. Finally, the LSCF/BC/CPE was air-dried for 2-3 minutes approximately, and then polished using 5-micron alumina, rinsed with methanol, and dried well before experiments.

2.3 Electrochemical instrıments and measurements

The XRD diagrams were obtained with an XPERT-PRO diffractometer using the CuKα radiation (1.5406 Å) (France). The morphology and microstructure of the LSCF synthesized nanoparticles were studied using a scanning electron microscope with LEO EVO-40xVPtype (Turkey). All voltammetric analyses were performed in a Voltalab Potentiostat-Galvanostat PGZ-301, (Algeria),

Gamry Interface 1010B (Gamry, USA) electrochemical analyzer, (Malatya, Turkey). Electroanalytical measurements were performed in a cell (BASi C3 Cell Stand) at room temperature with conventional three electrodes consisting of an LSCF-modified CPE (0.9 mm in diameter) as a working electrode, Platinum (Pt) as a counter electrode, and a saturated silver chloride (Ag/AgCl) as a reference electrode. The voltammetric measurements were performed in a potential scanning range between -200 mV to +900 mV (vs. Ag/AgCl). Cyclic voltammetry and DPV measurements for PCM monitoring were performed in a 0.1 mmol.L^{-1} phosphate buffer solution (PBS) pH 7.4 (3µM to 180 µM in CV, and 0.1 µM to 180 µM in DPV). The electrochemical impedance spectroscopy (EIS) spectra were recorded in the frequency range of 10^5 to 10^{-2} Hz. The operating conditions for DPV studies were: a step potential of 2.0 mV, a pulse size of 50 mV (vs. Ag/AgCl), a sample period of 0.2 s, a pulse time of 0.1 s, and an equilibrium time of 2 s.

3 RESULT AND DISCUSSION

3.1 X-Ray diffraction analysis

The purity, structural characteristics, and fundamental crystallographic parameters of the prepared NPs were investigated using X-ray diffraction analysis, indicating the presence of a single-phase for LSCF nano-oxide with perovskite-type [32-40]. The diffraction lines of the XRD patterns presented in Figure 1 (a) for our $La_{0.6}Sr_{0.4}Co_{0.8}Fe_{0.2}O_3$ synthesized nanoparticles were in great agreement compared with LSCF perovskite prepared in previous work [41] with a rhombohedral symmetry with space group of (R3CH). This indicates that LSCF NPs have a single pure phase and are composed only of the desired metals (La, Sr, Co, Fe). The structural parameters and refinement of LSCF perovskite were obtained using the Jana 2006 software. The main parameters are presented in (Table 1). The comparisons of the resulting XRD patterns of LSCF nanoparticles and the characteristic peaks (2θ) with hkl values are at 2θ = 24.07°, 32.91°, 40.53°, 47.34°, 53.29°, 58.61°, 69.13°, and 78.90° corresponding to hkl values (100), (110), (111), (200), (210), (211), (220), and (310), the obtained average size *d* was equal to 208.317 nm. LSCF NPs characterized by a pure phase, contain Co and La transition metals which are considered very good catalysts, and have a small and narrow particle diameter, these main characteristics increase their surface area and lead to a very weak concentration detection limit and improve their best performance in this work.

3.2 Scanning Electron Microscopy Analysis

Scanning electron microscopy (SEM) analysis is an excellent characterization tool that is used to describe the shape, morphology, structure, and micro and nanosize of materials [29, 30]. The LSCF NPs powder was examined

Table 1. Crystallographic main parameters of the synthesized LSCF nanoparticles.

Material	Lattice parameters (Å)				
	S.G	a = b	c	α=β=γ	Volume (Å^3)
LSCF	**R3$^-$CH**	**5.428**	**13.225**	**120°**	**60.799**

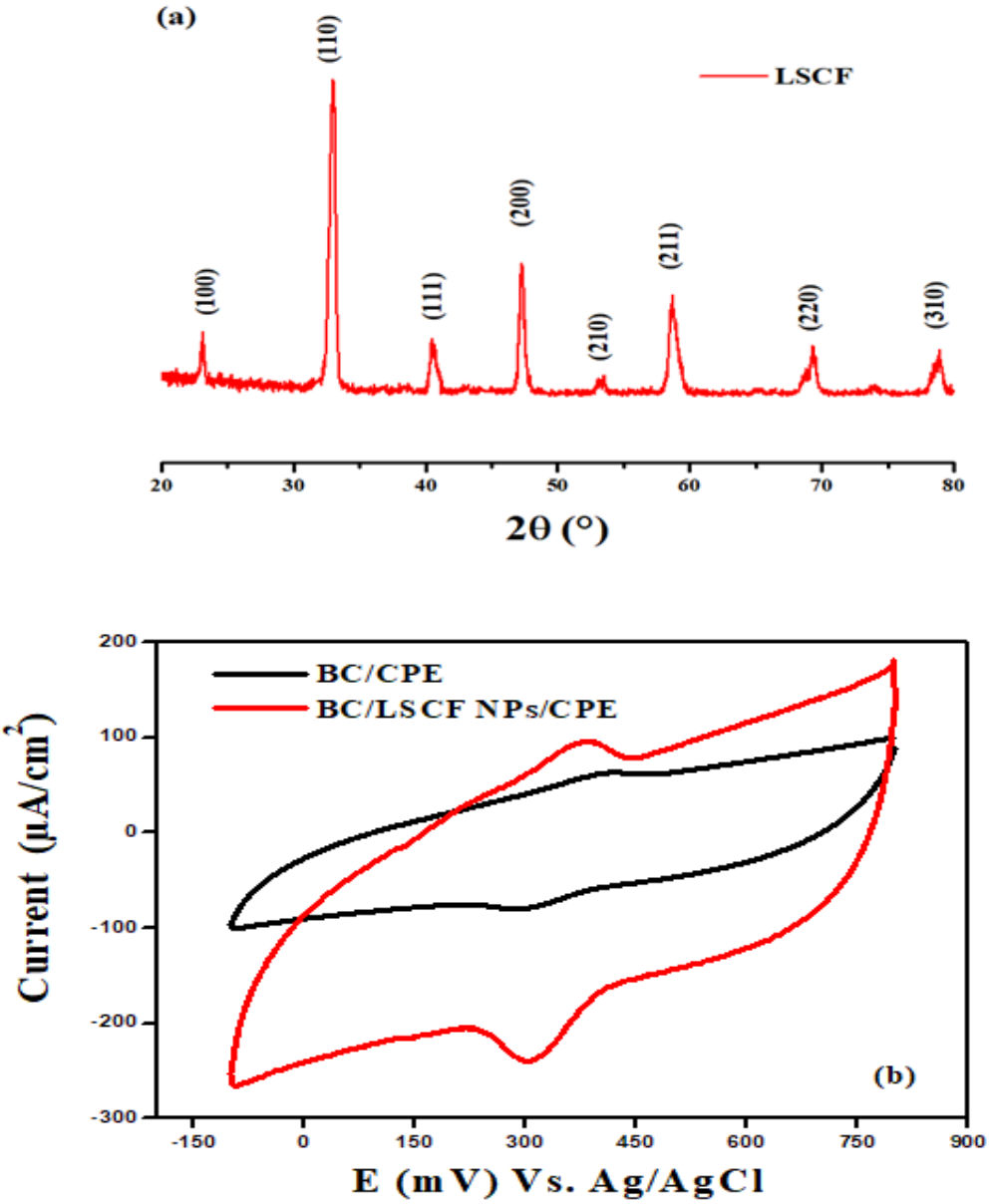


Figure 1. X-ray diffraction patterns recorded for $La_{0.6}Sr_{0.4}Co_{0.8}Fe_{0.2}O_3$ nano-sized particles synthesized by the citrate method, (b) CVs obtained for 3 mM $[Fe(CN)_6]^{4-}/[Fe(CN)_6]^{3-}$ system mixture as electrolyte analyzed with BC+LSCF NPs and BC only two paste electrodes at 50 mV.s^{-1} scan rate in 0.1 mM phosphate buffer solution pH 7.4.

using SEM, all the obtained images are provided with a 1 µm scale bar. As depicted in Figure 2, the grains of LSCF nanomaterials exhibit homogeneity, primarily possessing a spheroid shape and uniform size distribution. Also, the micrographs reveal a significant presence of pores across various scales, enhancing their catalytic properties by increasing the specific surface area. This porous structure facilitates the diffusion and adsorption of drug molecules into both small and large pores of the modified CPE. The average grain size *d* of $La_{0.6}Sr_{0.4}Co_{0.8}Fe_{0.2}O_3$ synthesized nanoparticles was obtained from the histograms at 20x magnification, with the smallest values of 65.05 nm.

3.3 Ferro-cyanide test

Before any electrochemical detection study, it's of big importance to check out the kinetic process of the prepared CPE towards the ferrocyanide Red/Ox couple $[Fe(CN)_6]^{4-}/[Fe(CN)_6]^{3-}$ with 3 mM concentration in 0.1 mM PBS pH 7.0 [29, 30]. For this test, two pastes were prepared: one

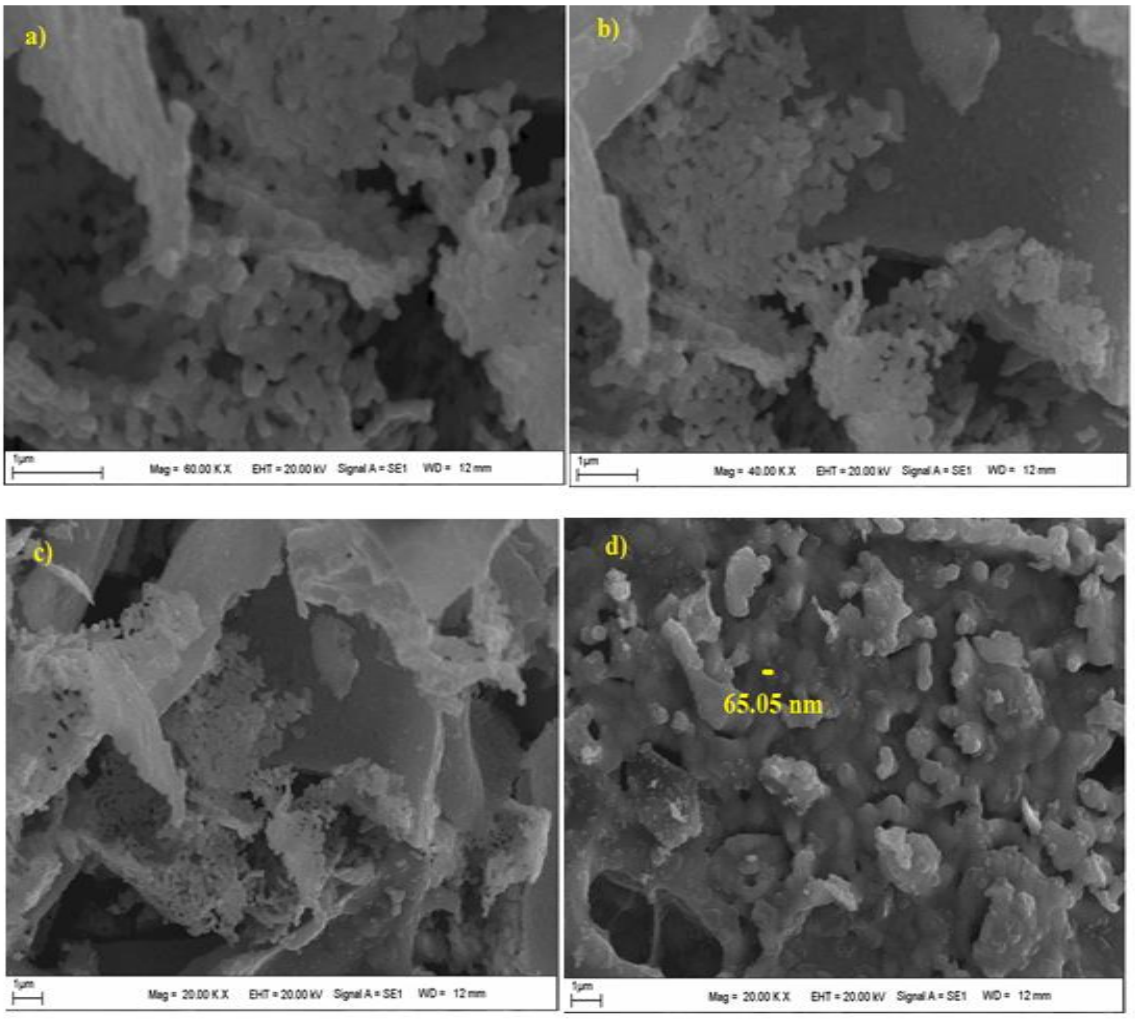


Figure 2. SEM surface images of $La_{0.6}Sr_{0.4}Co_{0.8}Fe_{0.2}O_3$ nanomaterials at different magnifications (a) 60, (b) 40, (c) and (d) 20 (scale bar=1 µm).

paste composed of BC and ethylene glycol only, and the second paste consisted of BC and LSCF NPs grouped with ethylene glycol. Each paste was filled into a clean capillary glass tube and attached with a Pt wire for electrical contact. After that, the two pastes were studied as working electrodes for detecting the ferrocyanide system using the CV technique at 50 $mV.s^{-1}$ scan rate. The comparative cyclic voltammograms of the two pastes are represented in Figure 1 (b). It was noted that the anodic electro-oxidation peak potentials (E_a) at the BC/CPE and the BC+CLFN NPs modified CPE were 450.068 mV (vs. Ag/AgCl) and 353.56 mV (vs. Ag/AgCl) respectively. Similarly, the cathodic electro-reduction potentials (E_c) were 300.068 mV and 313.27 mV, with the following potential differences (ΔE_P) of 150 mV/Ag/AgCl and 40.29 mV/Ag/AgCl, respectively. The observed low ΔE_P of BC+CLFN NPs modified CPE indicates the highest and fastest electron transfer between the BC+CLFN NPs/CPE surface area and ferro-cyanide couple. It is evident that electrodes modified with LSCF nanoparticles are more efficient and exhibit a superior electrocatalytic effect for both electron and mass transfer compared to those composed of BC only, and characterized with significantly higher current peaks, low potential values, and prominent peaks.

3.4 Electrochemical behavior of LSCF/BC/CPE modified electrode for PCM detection

3.4.1. PCM behavior with cyclic voltammetry

The effect of scan rate variation for the electro-catalytic mechanism of PCM drug and its stability towards our prepared CPE was investigated by varying the scan rates from (10 $mV.s^{-1}$ to 400 $mV.s^{-1}$) with 100 µM of PCM concentration in 0.1 mM PBS at pH 7.0. It was noted that, with increasing the value of the scan rate, there was a notable proportional linear increase in the peak current I_P. Figure 3 (a) indicates that the oxidation and reduction peaks of the reversible system paracetamol were well controlled with a stable diffusion of PCM molecules towards the LSCF/BC/CPE surface area with a perfect correlation regression of 0.9984. From the cyclic voltammetric plots shown in Figure 3 (b), we note a reversible system of PCM drug at LSCF/BC/CPE modified sensor according to reaction (1) below. It is observed that increasing PCM concentration, results in a proportional increment in peak current with a fixed potential of 435 mV/Ag/AgCl approximately, this leads to the establishment of two linear regression equations: a) $I_1 = -18\ C + 44.20$, $R^2 = 0.902$ for the high concentrations of PCM, and b) $I_2 = -464.51\ C - 839.47$, $R^2 = 0.9923$ for low concentrations of PCM, with low detection and quantification limits of LOD = 0.38 µM and LOQ = 1.14 µM,

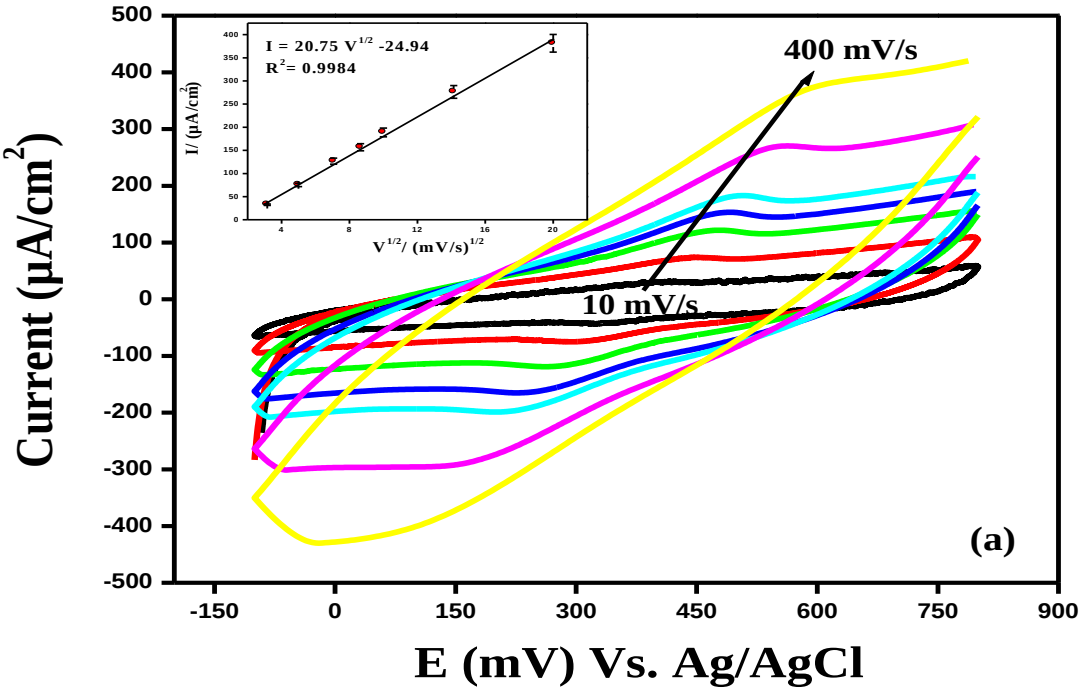
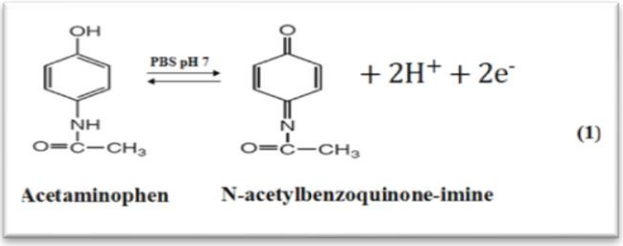


Figure 3. Scan rate variation from 10, 25, 50, 75, 100, 200 to 400 mV.s^{-1} of 100 µM PCM at (a) LSCF/BC/CPE, (b) Cyclic voltammograms plots for varied concentrations of PCM solutions (3 µM, 40, 80, 100, 120, 140, 160 to 180 µM), at 25 mV.s^{-1} scan rate in 0.1 mM PBS pH 7.4.

respectively, and high sensitivity of S = 464.51 µA.µM^{-1}.cm^{-2}. According to the reaction number (1), the oxidation mechanism of paracetamol in PBS pH 7.0 is a process of two electrons and two protons transfer [26]. As a result, the working electrode shows very high currents, well-pulsed, and prominent peaks, demonstrating its high sensitivity and selectivity towards paracetamol drug using the CV technique.

The mechanism of LSCF/BC/CPE towards PCM detection is as follows [21]:



Scheme 2. Suggested oxidation mechanisms for paracetamol drug.

The LOD was calculated using the following equation:

$$LOD = 3 \times \frac{SD}{S} \qquad (1)$$

Where *SD* is the standard deviation of the blank and *S* is the slope or the sensitivity of the first linear range.

3.4.2. PCM determination with differential pulse voltammetry

The behavior of PCM at LSCF/BC/CPE was investigated using the DPV technique [30] in 0.1 mM PBS pH 7.0 with a wide linear range of 0.1 µM to 180 µM. For DPV measurement represented in Figure 4 (a), the electro-oxidation potential of PCM was approximately noted at +450mV/Ag/AgCl. Notably, well-defined, single, and prominent pulsed peaks were observed, also with increasing PCM concentration, the peak current exhibits a direct increment, indicating a proportional relationship between the current and diffused quantities of drug molecules, with the extracted regression equation from Figure 4 (b): $I = 0.75\ C - 0.835$, $R^2 = 0.9812$ with a very low detection and quantification limits of 0.036 µM and 0.11 µM and a high sensitivity of 75 $\mu A.\mu M^{-1}.cm^{-2}$ respectively. In conclusion, DPV is the best electrochemical method for PCM monitoring because it is characterized by high sensitivity and selectivity, able to detect and follow very low concentration values until 36 nM which could not be achieved using the CV technique.

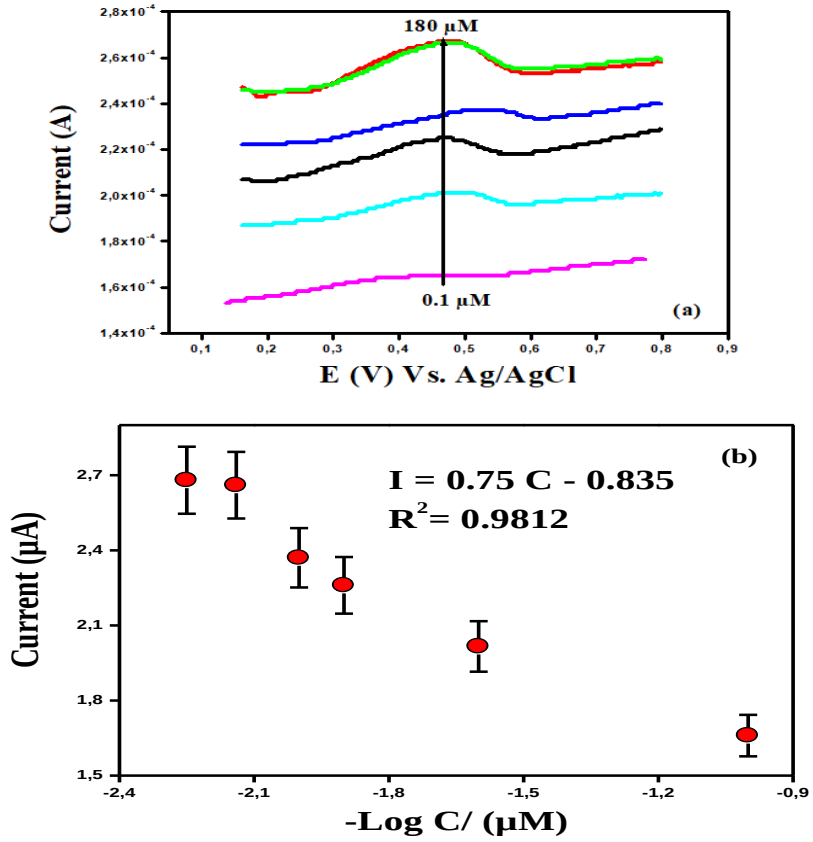


Figure 4. Differential pulse voltammetric plots for several concentrations of paracetamol (0.1 µM, 40, 80, 120, 160 to 180 µM), varied at LSCF NPs/BC/CPE with 50 mV pulse size, sample period of 0.2 s, pulse time of 0.1 s, and a step potential of 2.0 mV in 0.1 mM PBS pH 7.4.

3.4.3. PCM behavior with electrochemical impedance spectroscopy

The performance of LSCF nanoparticles onto the CPE towards the study of electron and ion transfer between electrolyte and electrode active surface area was investigated using EIS assay [29, 30]. Nyquist plots obtained are processed with electrochemical Zfit analysis by the EC-Lab program and represented in Figure 5. From the Nyquist plots shown in Figure 5 (a), concerned with the maximal concentration of PCM drug studied in the frequency interval of 10^5 to 10^{-2} Hz, we can observe three principal regions:

a) The primary response of electrolyte resistance (R_1) is observed at frequency levels higher than 10^5 Hz.
b) Between 10^5 to 10^{-1} Hz, a semi-circle of charge transfer (R_2) phenomenon occurs between electrode/electrolyte at these frequencies.
c) At frequency values under 10^{-1} Hz, a straight line is observable related to the adsorption and diffusion phenomenon of PCM species towards our working electrode surface area (R_3).

Figure 5 (b), illustrates all the Nyquist plots drawn between 10^5 to 10^{-1} Hz frequency domains, where the main mass and charge transfer phenomena characterizing the modified prepared electrode were subtracted. The resistances (R) and capacities (CPE) obtained are presented in Table 2. It's found that, with increasing the concentration of PCM drug, the charge transfer resistance R_2 increases directly, which means that the charge transfer resistance R_{ct} is directly proportional to PCM concentrations, which can be explained by the ease of ions mobility and high electronic exchange when the adsorbed concentration is high. Furthermore, the electrolyte resistances R_1 also exhibit proportional increment with raising the concentration levels of PCM drug.

Table 2. Different values of resistances and capacities extracted from the Nyquist plots at 10^5 to 10^{-1} Hz frequency domains and their corresponding fits using electrochemical Zfit analysis by EC-Lab program for different concentrations of paracetamol (3 µM to 180 µM) at LSCF material surface area.

Concentration (µM)	R_1 (Ω)	R_2 (Ω)	CPE_1 (F. s^{-1}).10^{-3}	CPE_2 (F. s^{-1}).10^{-3}
3	88.93	802.0	0.182	0.666
40	235.6	923.0	0.241	1.457
80	265.88	1162	0.547	3.174
120	700.2	1194	2.214	4.865
180	4845	1202	2.833	5.243

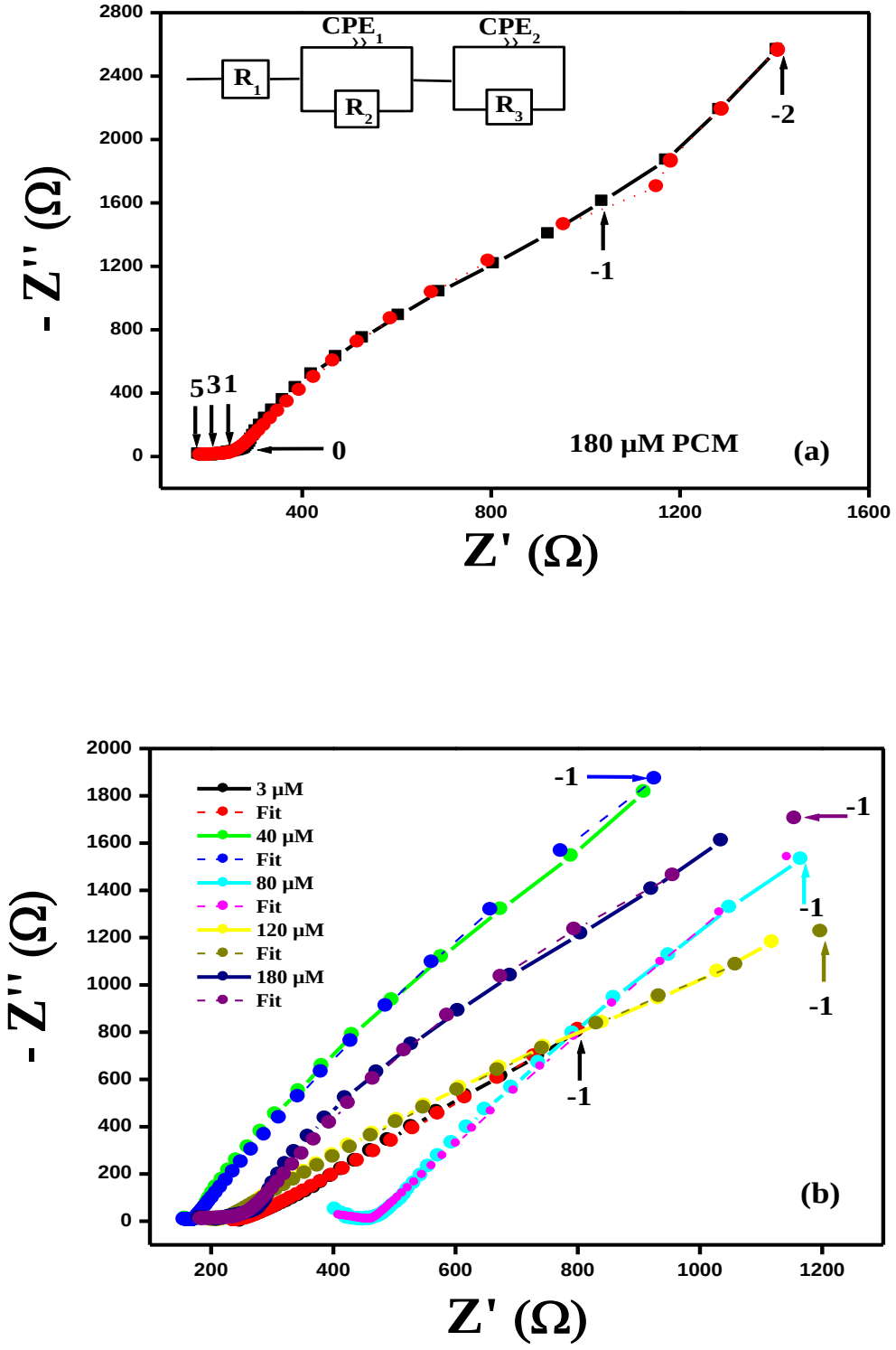


Figure 5. Nyquist plots for PCM drug at its maximal concentrations 180 µM, (a) at 10^{5} to 10^{-2} Hz frequency domains, (b) several plots display the entire concentration range studied in CV technique with their corresponding fits at 10^{5} to 10^{-1} Hz frequency domains.

3.5 Effect of pH on the electro-oxidation of PCM drug

The pH of the electrolyte is a crucial parameter for studying the best mechanism of the prepared electrode toward the PCM analyte [42]. To select the best electrolyte pH for paracetamol monitoring, the effect of pH solution on the anodic peak current of PCM at LSCF/BC/CPE from pH 2.0 to pH 12.0 was investigated. The voltametric plots of the peak current vs. pH represented in Figure 6 (a), show that the peak current responses increased at pH 2.0–6.0 with a first linear regression of I_1 = 13.12 pH + 46.56, R^2 = 0.9904 with a good slope of 13.12 µA.pH^{-1}, then reaching the highest level at pH 7.4 resulting a well-shaped, predominant and the lower potential peak at (+399 mV/Ag/AgCl), then decreasing from pH 7.4 to 12.0 with the second linear regression of I_2 = - 20.032 pH + 372.85, R^2 = 0.963 with excellent slope of 20.032 µA.pH^{-1}, herein the values of potentials change in a different way between (399 mV/SCE to 483 mV/SCE). The obtained results show that the estimated proton and electron exchange is low in both acidic and basic media, while the neutral environment (pH 7.4) offers the best appropriate proton media for electrocatalytic oxidation PCM detection. For this reason, all the experiments on paracetamol monitoring were carried out in PBS at pH 7.4.

3.6 Electrochemical performance of LSCF sensor in the real pharmaceutical sample

The performance of the modified $La_{0.6}Sr_{0.4}Co_{0.8}Fe_{0.2}O_3$NPs/BC/CPE sensor was checked using a real sample [43, 44], including pharmaceutical tablets containing paracetamol as the active ingredient and other components, the sample was purchased from PHYSIOPHARM laboratories. The pharmaceutical sample was dissolved in 100 mL PBS pH 7.0(10 µM) at a prepared concentration of 100 µM and analyzed using the developed sensor via CV technique. The scan rates were varied between 10 to 200 mV.s^{-1}. Based on the voltammograms shown in Figure 7 (a), it's clear to see that PCM as a real sample exhibited a reversible system as its active ingredient at (+376.55 mV). The diffusion of PCM drug is directly proportional to the increment of scanning rates, as confirmed by the corresponding linear equations: I_P= -27.39 $V^{1/2}$ + 24.73, R^2 = 0.9972. The diffusion of the pharmaceutical product molecules towards the surface area of the working electrode is well controlled, exhibiting excellent correlation ($R^2 \approx 1$). Based on the voltammograms depicted in Figure 7 (b), comparing the behavior of paracetamol as an active ingredient and as a pharmaceutical tablet at the same concentration of 100 µM, no difference is observed for PCM mechanism; with presenting the same response with a

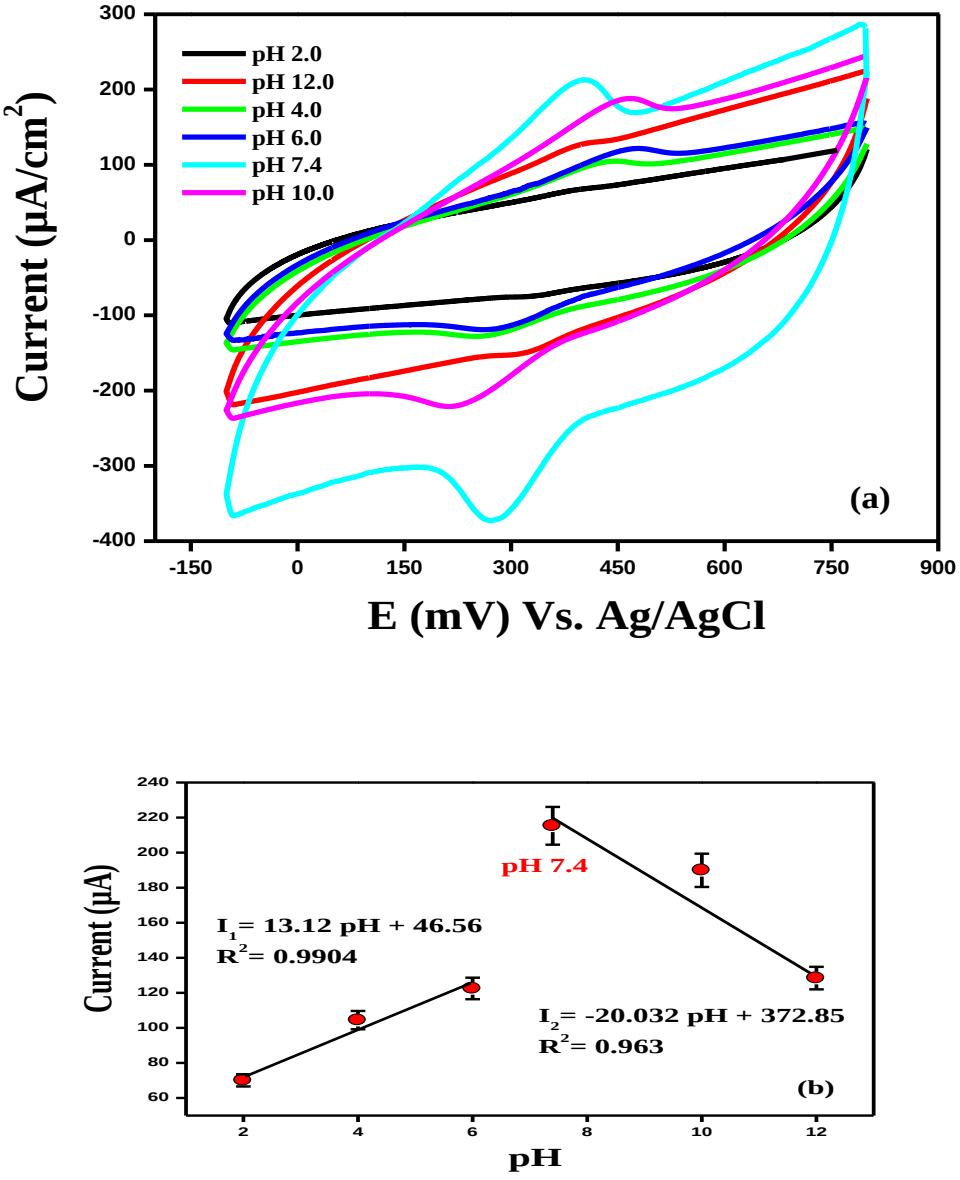


Figure 6. Cyclic voltammetric plots for several solutions of 100 µM PCM with different pH values (**pH 2.0** to **pH 12.0**) in 0.1 mM PBS.

reversible system, with only some difference in peak potentials (ΔE_{AI} = 81.15 mV ΔE_{Tab} = 145.8 mV). Additionally, PCM as an active ingredient exhibits higher current peaks and lower potential compared to PCM tablets, indicating superior electronic and mass transfer efficiency. The results obtained demonstrated high precision, with favorable recovery percentages of 92.24%, and 94.56% for PCM in PBS and as tablets, respectively. These percentages were calculated from equation number (2), with good relative standard deviations of 5.24% and 3.26%. In conclusion, the performance of the fabricated

sensor towards PCM detection in real samples is comparable to its performance with its active ingredient. In addition, these results demonstrate high sensitivity and selectivity, reproducible, reliable precise and the technique is faithful and could be a good alternative for quality controlling of PCM drugs for health and environment preservation.

By comparing our prepared modified LSCF/BC/CPE after calculating its detection and quantification limits with previously developed electrodes (Table 4), it was found that our newly developed electrode characterized by: the best-widened concentration range for PCM sensing that can reach even to 36 nM with DPV technique. The highest sensitivity and the lowest detection and quantification limits compared with the literature and our CPE can considered the best performer of previous works with CPE sensors.

Table 3. Determination of the recoveries and relative standard deviations of the prepared modified electrode LSCF NPs/BC/CPE in PBS and real pharmaceutical sample.

Sample	**Original [C] (µM)**	**Found [C] (µM)**	**Recovery[1] (%)**	**R.S.D[2] (%)**
PBS	100	102.24	92.24	5.24
Tablets	100	104.56	94.56	3.26

[1] **Recovery** $= \frac{\text{Found [C]} - \text{Diluted biofluids [C]}}{\text{Original [C]}} \times 100$ (2)

[2] **R.S.D** = Relative Standard Deviation

Diluted biofluids [C] =10 µM PBS

Table 4. Comparison of the detection and quantification limits of LSCF/BC/CPE modified electrode with other previously developed modified carbon paste electrodes.

Electrode	**LOD (µM)**	**LOQ (µM)**	**Linear range (µM)**	**Technique**	**Ref.**
$MnFe_2O_4$ and $CoFe_2O_4$ NPs/GPE	0.25/$MnFe_2O_4$ 0.30/$CoFe_2O_4$	0.825 0.99	3-200 3-160	DPV	[21]
Stevensite-Modified Carbon Paste Electrode (Stv-MCPE)	0.2	0.6	0.6-100	CV, DPV	[22]
ZrO_2 NPs / Modified CPE	0.68	2.244	10-60	CV, DPV	[23]
Ionic Liquid Crystal (ILC)/CPE	2.8	9.24	0-120	DPV	[24]
La_2NiO_4 and Pr_2NiO_4 NPs/CPE	1.99/La_2NiO_4 2.04/Pr_2NiO_4	6.57 6.732	3-200	CV, SWV	[26]
Poly (L-leucine)/ Modified CPE	0.44	1.452	90-200	CV, DPV	[28]
LSCF/BC/CPE	**0.036**	**0.11**	**0.1-180**	**CV, DPV**	**This work**

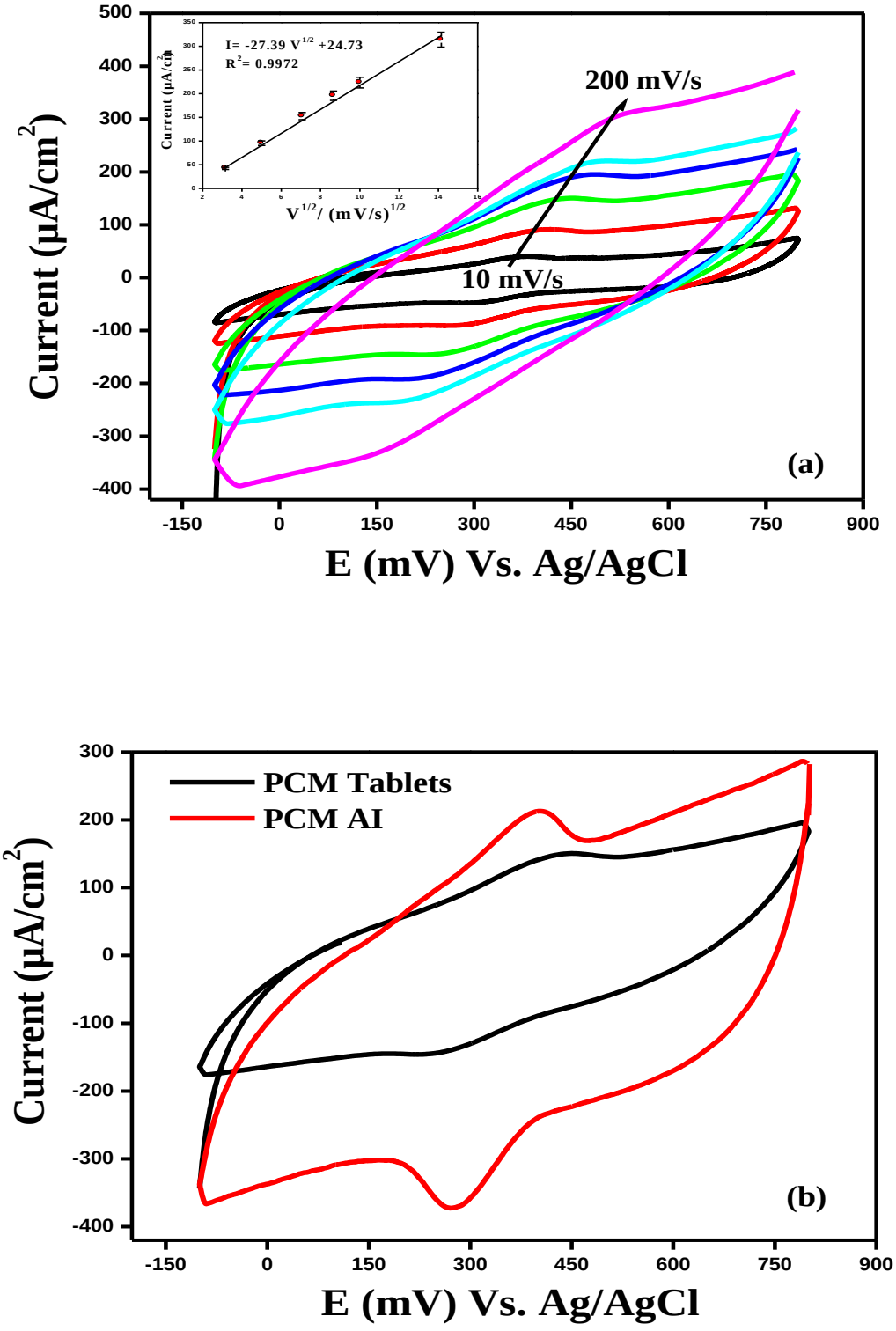


Figure 7. CV plots, (a) using LSCF/BC/CPE at varied scan rates from 10, 25, 50, 75, 100 to 200 mV.s^{-1} in the real sample of PCM drug (100 µM), (b) CV plots of PCM as active ingredient (AI) and as pharmaceutical tablets with 100 µM concentration, at 100 mV.s^{-1} scan rate in 10 µM PBS pH 7.

4 CONCLUSIONS

In conclusion, a novel, cost-effective, and highly efficient electrocatalyst ($La_{0.6}Sr_{0.4}Co_{0.8}Fe_{0.2}O_3$), modified with BC and integrated into a CPE, was successfully developed for simultaneous electrochemical sensing of paracetamol. The monitoring electrochemical techniques were CV, DPV, and EIS assay. The developed sensor demonstrated a broad linear detection range (0.1–180 µM) via DPV, ensuring high analytical reliability. This modified electrode was carried out for the precise quantification of PCM in PBS with low detection limits of 36 nM in DPV, and 380 nM in CV, respectively. The sensor's practical applicability was validated using a real pharmaceutical sample, yielding highly reproducible recovery rates of 92.24% (PBS) and 94.56% (tablet form), with relative standard deviations (RSDs) of 5.24% and 3.26%, respectively. The sensor exhibited high sensitivity, selectivity, and stability. Overall, the modified LSCF/BC/CPE sensor exhibited rapid, highly sensitive, and reproducible performance for PCM detection, making it a promising candidate for pharmaceutical quality control applications.

5 ACKNOWLEDGEMENTS

This research was conducted in collaboration with the Laboratory of Materials Interaction and Environment (LIME), Jijel University, Algeria, and the Division of Analytical Chemistry, Faculty of Pharmacy, İnönü University, Malatya, Turkey.

6 AUTHOR CONTRIBUTIONS

Writing – original draft, Validation, Conceptualization, Methodology, Visualization: M.M.; Supervision: M.F.; Investigation, Writing – review-editing: M.F., E.K.S.

7 CONFLICT OF INTEREST

The authors declare no conflict of interest, and all co-authors have approved the final version of this manuscript.

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e-ISSN: 2980-0463
Anat. J. Pharm. Sci., 2025:4(1)
AJPS
Research Article

In Vitro Efficacy of Ceftazidime-Avibactam on Carbapenem-Resistant *Pseudomonas aeruginosa* Isolates

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ABSTRACT: *Pseudomonas aeruginosa* is the primary Pseudomonas species responsible for hospital-acquired infections. Ceftazidime-avibactam (CZA) is a new beta-lactam/beta-lactamase inhibitor combination effective against carbapenem-resistant *P. aeruginosa* isolates. The aim of this study was to evaluate the in vitro activity of CZA against carbapenem-resistant *P. aeruginosa* isolates. In hospitalized patient culture samples, 190 isolates that were evaluated as significant growth and identified as *P. aeruginosa* with the Vitek 2 Compact automated system (BioMérieux, France) and determined as imipenem resistant (≥ 8 mg/L) and meropenem resistant (≥ 16 mg/L)with the same system were included in the study. 88% (167/190) of *P. aeruginosa* strains were isolated from patients in intensive care units and 78% (148/190) from respiratory tract samples. CZA activity was studied using the disk diffusion test (10-4 µg disk) and zone diameters <17 mm were accepted as resistant. 20% (38/190) of the isolates were found to be resistant to CZA. The difference in resistance rates between CZA and all of the studied antimicrobials except amikacin is highly significant (p: 0.006 - <0.001). The low resistance rate found in our study indicates that CZA is a good option for the treatment of carbapenem-resistant *P. aeruginosa* isolates. In addition, amikacin treatment with a low resistance rate may be an appropriate approach for patients requiring combination therapy. Given the growing challenge of carbapenem resistance and multidrug resistance, further studies are warranted to assess the efficacy of new antimicrobials and drug combinations.

Keywords: *Pseudomonas aeruginosa*, ceftazidime-avibactam, carbapenem resistance.

1 INTRODUCTION

Pseudomonas aeruginosa is a Gram-negative, opportunistic pathogen that is responsible for 5–14% of hospital-acquired infections. The risk of infection is particularly high in cases of immunodeficiency, severe burns, prolonged stays in Intensive Care Units (ICUs), cystic fibrosis and bronchiectasis [1]. The development of resistance to antimicrobials—including antipseudomonal cephalosporins, monobactams, beta-lactam/beta-lactamase inhibitors, carbapenems fluoroquinolones, aminoglycosides, and polymyxins—poses significant challenges in the treatment of infections [2,3]. In particular, carbapenem-resistant isolates are defined as critically important pathogens by the World Health Organization (WHO) [4]. *P. aeruginosa* can develop carbapenem resistance through various mechanisms, including gene mutations that regulate the expression of membrane porins, upregulation of efflux pump systems (e.g., MexAB-OprM), and acquisition

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Submitted: 31.01.2025 Accepted: 26.03.2025
doi.org/10.71133/anatphar.1630540

of transferable genes encoding carbapenemases, such as metallo-beta-lactamases (e.g., VIM, IMP, and NDM) [5].

Ceftazidime is an antipseudomonal, semisynthetic third-generation cephalosporin that binds to penicillin-binding proteins and inhibits bacterial cell wall synthesis. However, resistance to third-generation cephalosporins has been increasing due to the emergence of multidrug-resistant Gram-negative bacteria capable of producing extended-spectrum beta-lactamases, chromosomal AmpC cephalosporinases, carbapenemases and metallo-beta-lactamases. Consequently, ceftazidime alone may be insufficient for the treatment of *P. aeruginosa* infections [1,6,7]. Ceftazidime-avibactam (CZA) is a novel beta-lactam/beta-lactamase inhibitor combination effective against carbapenem-resistant *P. aeruginosa* isolates. The combination of ceftazidime with avibactam may be effective against strains producing carbapenemases other than metallo-beta-lactamases [7].

This study aims to evaluate the in vitro activity of CZA against carbapenem-resistant *P. aeruginosa* isolates.

2 MATERIAL AND METHOD

Our study included 190 isolates from Gram-negative, oxidase-positive, R-type colonies that were classified as significant growth in inpatient culture samples sent from various clinics to the Microbiology Laboratory of Malatya Education and Research Hospital in 2022. The isolates were identified as *P. aeruginosa* using the Vitek 2 Compact automated system (BioMérieux, France) and determined to be imipenem-resistant (≥ 8 mg/L) and meropenem-resistant (≥ 16 mg/L) by the same system. The antimicrobial activities of the isolates were evaluated according to ‘‘The European Committee on Antimicrobial Susceptibility Testing’’ (EUCAST) criteria. CZA activity was assessed with disk diffusion test (10–4 µg disk) and zone diameters < 17 mm were accepted as resistant [8]. For statistical analysis, the SPSS 17 software (SPSS Inc., Chicago, IL, USA) was used. Categorical variables were expressed as numbers and percentages, and differences between categorical variables were analyzed using Chi-square tests.

3 RESULT

88% (167/190) of *P. aeruginosa* strains were isolated from patients in intensive care units and 78% (148/190) from respiratory tract samples. Blood (9%), urine (5%), wound (5%) and catheter (3%) samples were the other sources from which the strains were isolated. 20% (38/190) of the isolates were found to be resistant to CZA. The resistance rates of carbapenem-resistant isolates to other antimicrobials are presented in Table 1.

The difference in resistance rates tween CZA and all other studied antimicrobials, except amikacin, was highly significant ($p = 0.006$ to <0.001). The difference in resistance

Table 1. Antimicrobial resistance rates of 190 carbapenem-resistant *P. aeruginosa* isolates.

Antimicrobial	Rates of resistant isolates (%)
Ceftazidime-avibactam (< 17 mm)	38/190 (20)
Amikacin (>16 mg/L)	54/189 (28.6)
Ceftazidime (>8 mg/L)	80/190 (42.1)
Aztreonam (>16 mg/L)	88/180 (48.9)
Cefepime (>8 mg/L)	141/189 (74.6)
Levofloxacin (>2 mg/L)	150/184 (81.5)
Piperacillin/tazobactam (>16 mg/L)	168/187(89.8)

rates between CZA and amikacin is not statistically significant (p:0.052). 15 (39.5%) of 38 CZA-resistant isolates were found to be susceptible to amikacin.

4 DISCUSSION

The limited treatment options for carbapenem-resistant P. aeruginosa infections pose a significant clinical challenge. Although colistin remains one of the most effective antimicrobials for treating these infections, its nephrotoxicity and unfavorable pharmacokinetic properties limit its widespread use [9]. CZA treatment, one of the combination therapies recommended to solve the treatment problem, has been reported to be effective in many studies [6,7,10,11]. One of these studies was conducted in the United States (US) with 1151 multidrug-resistant isolates, and the CZA resistance rate was reported as 11.8% [11]. Similarly, studies conducted in the US with meropenem-resistant *P. aeruginosa* isolates, the CZA resistance rate was reported as 19% and 26% [12,13].

In studies performed with carbapenem-resistant P. aeruginosa isolates in Turkey, Aydemir et al. reported a CZA resistance rate of 21.8%, Bilgin et al. reported 7.7%, and Mirza et al. reported 16.7% [14–16]. In the present study, we determined a CZA resistance rate of 20% in our hospital, which was found to be consistent with the rates reported in both domestic and international studies.

The low resistance rate observed in our study indicates that CZA may be a valuable treatment option for carbapenem-resistant *P. aeruginosa* isolates. In addition, amikacin treatment with a low resistance rate may be an appropriate approach for patients requiring combination therapy. The increasing problem of carbapenem resistance and multidrug resistance requires more studies to evaluate the effectiveness of new antimicrobials and drug

combinations.

5 AUTHOR CONTRIBUTIONS

Hypotesis: A.M., A.G; Design: A.M., A.G; Literature review: A.M.; Data Collection: A.M., A.G; Analysis and/or interpretation: A.M., A.G; Manuscript writing: A.M.

6 CONFLICT OF INTEREST

Authors declare that there is no conflict of interest.

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e-ISSN: 2980-0463
Anat. J. Pharm. Sci., 2025:4(1)
AJPS
Research Article

Evaluation of the Antiproliferative Effect of Extract from Equisetum arvense L. on Hepatocellular Carcinoma SNU-449 Cells

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ABSTRACT: Equisetum arvense L. (Horsetail) extract (HTE) has been used traditionally in the treatment of various ailments. However, its potential as an anticancer agent, particularly in hepatocellular carcinoma (HCC), is still not well understood. The objective of this study is to investigate the anticancer potential of HTE in the SNU449 HCC cells. To assess the antiproliferative and migratory properties of HTE on HCC, a cell viability was analyzed at 24 and 48nd hours using seven different concentrations of HTE (ranging from 7.81 to 500 ppm). The lowest concentration that effectively affected cell viability was determined, and subsequent experiments were carried out using this dose at the 24-hour mark. The MTT, colony formation, wound healing, and Western blotting assays to measure CASP-3 and Cleaved CASP-3 expressions were also included in the analysis. The MTT assay identified 326 ppm as the minimum effective dose at the 24-hour time point. Colony formation assays showed a notable difference between treated and untreated cells, with a surviving fraction of 46.9% in HTE-treated cells. The wound healing assay indicated that HTE-treated cells exhibited a 43.4% wound closure rate after 24 hours. Western blot analysis revealed the normalized volume ratios for Caspase-3 were 53222328 in the treated cells, and 7948593 in the control, while for Cleaved Caspase-3, the ratios were 707454 in treated cells and 596409 in control cells. The results suggest that HTE has antiproliferative and migratory properties on SNU-449 HCC cells. Further investigations are required to understand the underlying mechanisms of these effects.

Keywords: *Equisetum arvense L., Horsetail, Antiproliferative Effects, SNU-449 Cell Line*.

1 INTRODUCTION

Primary liver carcinoma, particularly hepatocellular carcinoma (HCC), is an epithelial malignancy originating in the liver and accounts for over 80% of all liver cancer cases. In 2020, approximately 9.06 million new cases and 8.3 million deaths were reported globally [1]. HCC, the most common form of primary liver cancer, originates from hepatocytes and typically has a doubling time of 4-5 months [2]. It is a highly invasive tumor that rapidly grows, infiltrates blood vessels, and spreads to distant organs via the bloodstream. HCC often develops in individuals with chronic liver conditions such as hepatitis B and C infections, non-alcoholic fatty liver disease, and cirrhosis [3]. Other common risk factors include exposure to aflatoxins, obesity, diabetes mellitus, and alpha-1 antitrypsin deficiency [4]. Despite advances in treatment, the prognosis for HCC

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Submitted: 17.02.2025 Accepted: 27.03.2025
doi.org/10.71133/anatphar.1641474

remains poor, as current therapeutic strategies are limited by inadequate molecular understanding, lack of early detection biomarkers, and resistance to chemotherapy [5].

The global incidence of HCC continues to rise, with estimates projecting over 1 million cases by 2025. Recent advances in phytomedicine and chemotherapy highlight the anticancer potential of phytochemicals, which possess a wide range of biological activities [6]. Medicinal plants and their extracts offer promising candidates for the development of new drugs and therapies due to their diverse pharmacological properties. Among these, *Equisetum arvense* L. has attracted significant attention due to its favorable chemical composition and therapeutic benefits [7,8].

Equisetum arvense L., a widely distributed medicinal plant [9], is found in regions across America, North Africa, and Asia [10]. Known commonly as horsetail or diarrhea herb, this plant has traditionally been used for various ailments, including as a diuretic, anti-inflammatory, anti-edema, and for treating fractures, wounds, and other conditions [11]. Previous phytochemical studies have declared that It has alkaloids, organic biomolecules, phytosterols, ascorbic acid, silicic acid, phenols, tannins, flavonoids, saponins, triterpenoids, tartaric acid, caffeic acids, apigenin, and kaempferol in its composition [12,13]. The peduncle contained silicic acid and silicates (5-8%), calcium (1.3%), potassium (1.8%), and various minerals [14]. Additionally, compounds such as nicotine, palustrine, and palustrinene have been isolated from the plant [15]. Many experimental and clinical studies report that *E. arvense* L. is a medicinal plant with hopeful therapeutic potential in managing various medical disorders [16,17]. Given its established medicinal uses, *Equisetum arvense* L. was selected for this study to assess its potential antiproliferative and migratory effects against hepatocellular carcinoma cells, specifically the SNU-449 cell line.

2 MATERIAL AND METHOD

2.1 Preparation of SNU449 Cell Culture

The SNU449 human hepatocellular carcinoma cell line (ATCC, CRL 2234) was cultured in RPMI-1640 Medium (Sigma), supplemented with 10% heat-inactivated FBS, (Sigma) and 1% Penicillin-Streptomycin-Neomycin (Sigma), and maintained at 37°C in a 5% CO_2 humidified incubator.

2.2 Cell Viability Assay

The Cell viability was measured using a colorimetric MTT assay as described by van Meerloo et al [18]. SNU449 cells were resuspended in RPMI-1640 medium and plated at a density of 10,000 cells per well in a 96-well plate, after which the plate was incubated

overnight. HTE (Concentrations ranged from 7.81 to 500 ppm) (Solgar, Turkey) was then exposed. After 24 hours MTT solution (5 mg/mL in PBS) was then added, following which the plate was incubated for 2–4 hours. Subsequently, 100 µL of DMSO (Merck) was added, and the absorbances were measured at 570 nm (Biotek, Synergy H1m). The median inhibitory concentration was then determined.

2.3 Colony Formation Assay

Colony formation assay was determined using the method described by Franken et al [19]. SNU449 cells were seeded into 6-well plates at a density of 1000 cells per well and treated with 326 ppm of HTE for 24 hours. Treatment was followed by the replacement of the medium with fresh RPMI-1640, which was refreshed every 2 to 3 days over 14 days. Colonies were then fixed with a solution of methanol: acetic acid (3:1) for 5 minutes. They were subsequently stained with 0.5% crystal violet for 15 minutes, and then counted using a microscope (Leica, DMi8). Plate efficiency (PE) and surviving fraction (SF) were then calculated using the following formulae:

- **PE** = (number of colonies formed / number of cells seeded) * 100
- **SF** = (PE of treated cells / PE of control cells) * 100

2.4 Wound Healing Assay

SNU449 cells were seeded in 60 mm culture dishes and grown to 80% confluence. It was created a scratch with a sterile 100 µL pipette tip in the cell monolayer. Cells were then treated with 326 ppm HTE for 24 hours, and images of the wound area were taken at specific time points using a cell imager (Leica, Paula). The imager's software assessed confluence, gap closure, migration rate, and half-gap time [20].

2.5 Western Blotting Assessment of Caspase 3 and Cleaved Caspase 3

Protein expressions of Caspase-3 (Cell Signaling, no. 14220) and Cleaved Caspase-3 (Cell Signaling, no. 9664) were assessed by Western blotting. Bands were visualized using Clarity Max Western ECL Substrate (Bio-Rad, no. 1705062) and analyzed using the Bio-Rad ChemiDoc system. Band quantification was performed using Bio-Rad Image Lab software (Version 6.1.0 build 7).

2.6 Statistical Analysis

The Kolmogorov-Smirnov test was applied to evaluate the normality. Data are presented as median (IQR). Protein expression levels were normalized and expressed as fold changes relative to the untreated control. Group comparisons were performed using the Kruskal-Wallis test for multiple groups. A p-value of less than 0.05 was considered statistically significant. All statistical analyses were conducted using SPSS 27 (IBM).

3 RESULT

3.1 Cytotoxicity of Horsetail Extract (HTE) in the SNU449 Cell Line

MTT assay was performed to assess the effect of HTE on SNU449 cell viability. The results indicated that the minimum effective dose was 326 ppm at 24 hours. The absorbance values for the HTE-treated and untreated groups were 0.316 (range: 0.234-0.388) and 0.615 (range: 0.481-0.759), respectively (Figure 1).

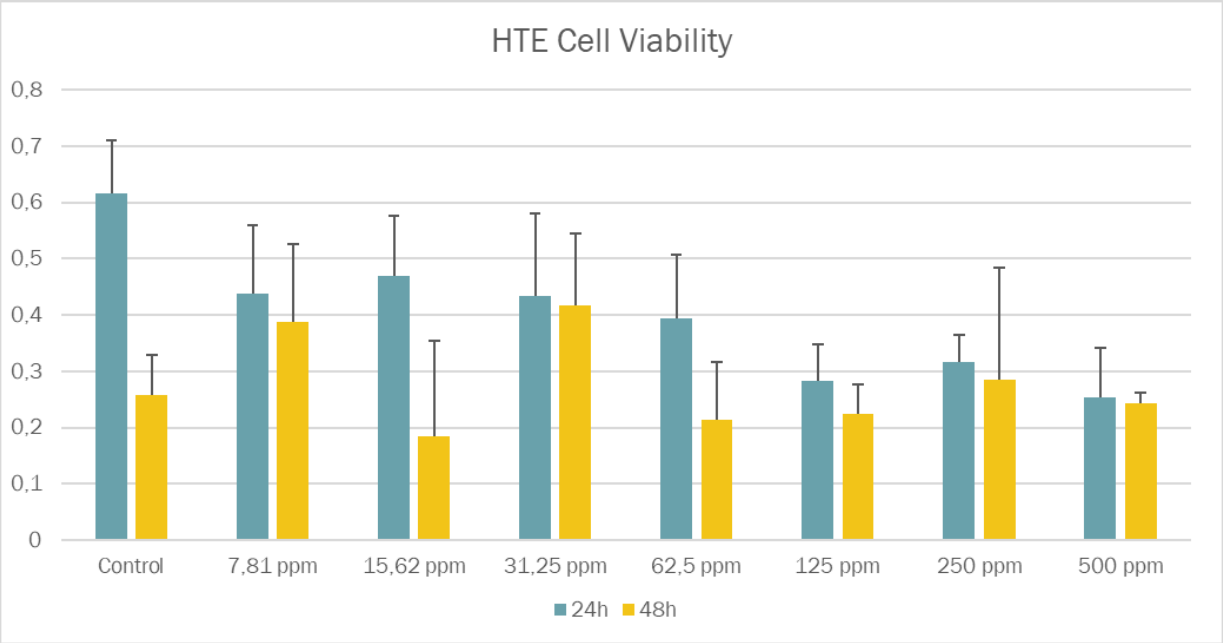

Figure 1. Effects of HTE on Cell viability of SNU449 cells.

3.2 Effects of HTE on the Migratory Characteristics of SNU449 Cells

The migratory characteristics of SNU449 cells were determined via a wound healing assay after 24 hours of HTE treatment. The results are summarized in Figure 2, where the wound closure rate was 43.4% in HTE-treated cells after 24 hours.

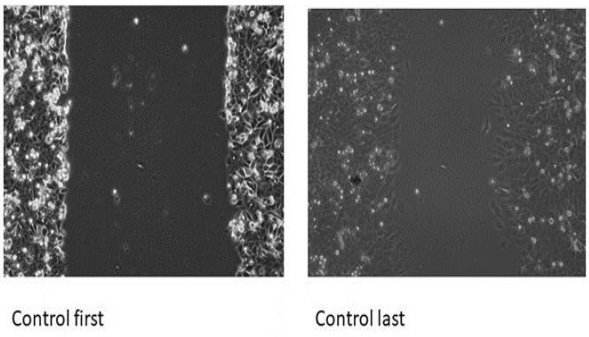

Figure 2a. Control wound healing.

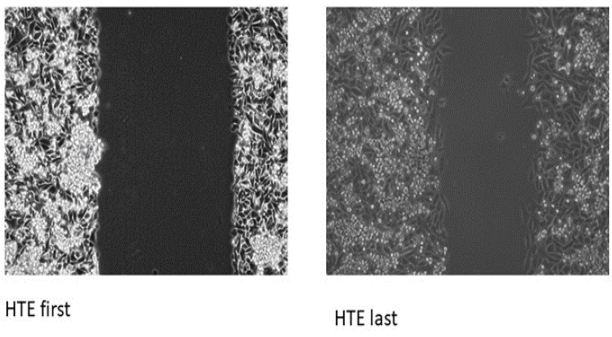

Figure 2b. HTE wound healing.

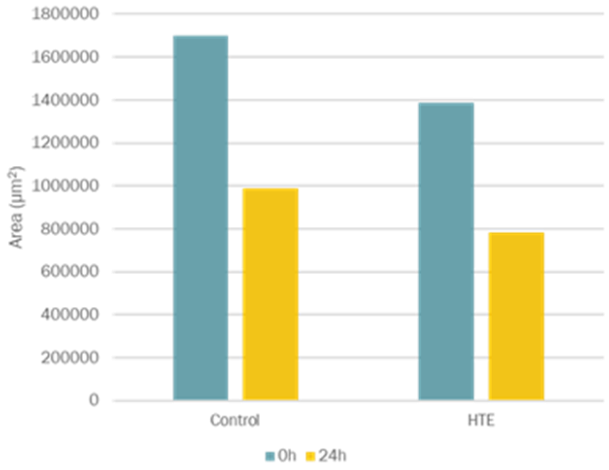

Figure 2c. Wound area.

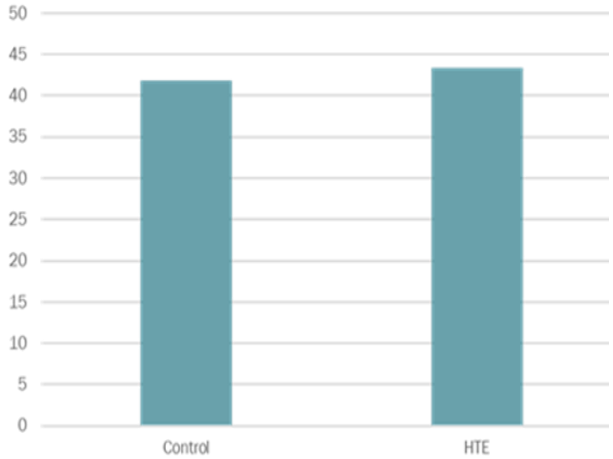

Figure 2d. Wound healing rate.

Figure 2. Migratory characteristics of SNU449 cells following 24-hour treatment with control or HTE.

3.3 Effects of HTE on the Proliferative Potency of SNU449 Cells

Colony formation assays revealed a significant difference in cell proliferation between HTE-treated and untreated cells, with a surviving fraction of 46.9% relative to the control (Figure 3).

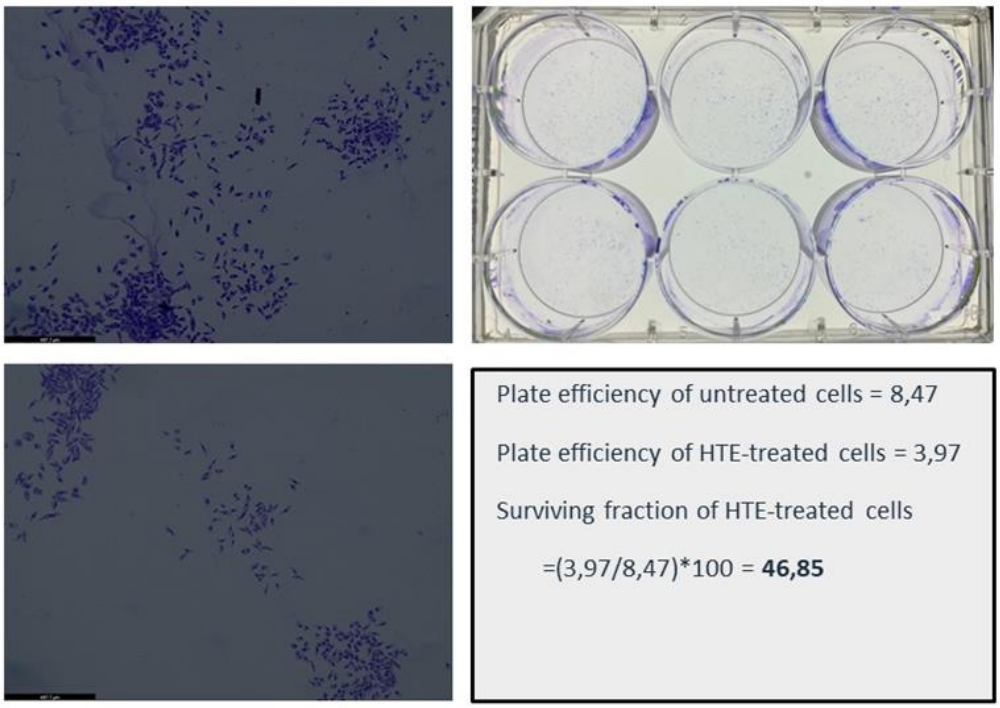

Figure 3. Proliferative potency of SNU449 cells.

3.4 Effects of HTE on Protein Expressions of Caspase 3 and Cleaved Caspase 3

Normalized volume ratios for Caspase-3 were 53222328 and 7948593 in HTE-treated and control cells, respectively. For Cleaved Caspase-3, normalized volume ratios were 320607 and 707454 in HTE-treated and control cells, respectively (Figure 4).

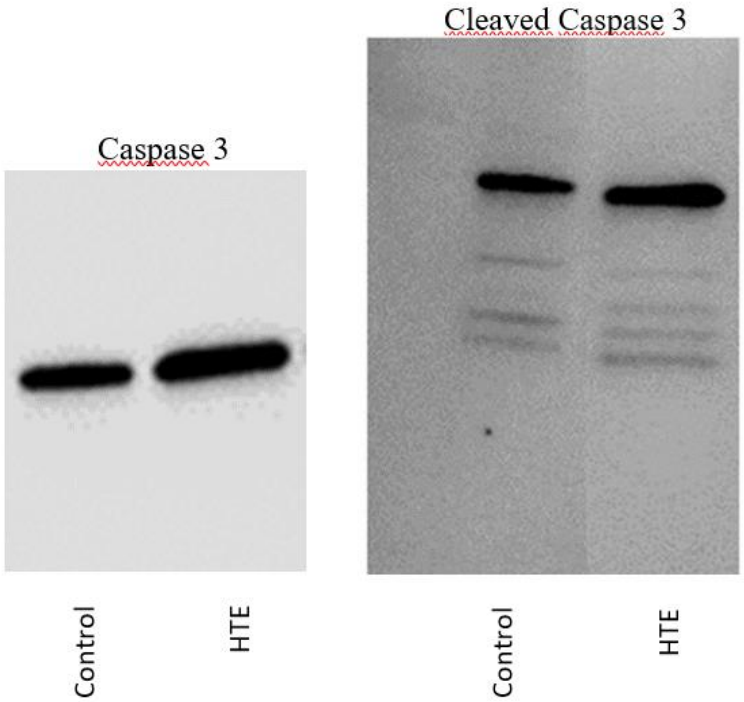

Figure 4. Changes in Caspase 3 and Cleaved Caspase 3 levels of control and HTE treatment groups

4 DISCUSSION

Several studies have reported the various biological activities of hydroalcoholic extracts of *Equisetum arvense* L., including analgesic, sedative, anti-inflammatory, antioxidant, antiseptic, antidepressant, hypoglycemic, and diuretic effects [11]. Additionally, *Equisetum arvense* L. has shown positive effects in treating liver conditions [12]. Notably, the methanolic extract of *Equisetum arvense* L. has exhibited hepatoprotective effects on tacrine-induced cytotoxicity in HepG2 cells [21]. Studies have also demonstrated that *Equisetum arvense* L. possesses anticancer properties [22].

The antiproliferative properties of *Equisetum arvense* L. extracts were evaluated on HeLa, HT-29, and MCF7 human cancer cells using the sulforhodamine B assay. The inhibition of cell growth was found to be contingent upon the type of cell line, extract type, and extract concentration [15]. Yamamoto et al. also observed cytotoxic effects of *Equisetum arvense* L. through apoptosis in the human leukemia U937 cell line. Furthermore, protein extracts from

Equisetum arvense L. have been shown to inhibit proliferation in cancer cell cultures [23].

Additionally, *Equisetum arvense* extract exhibited significant antiproliferative effects against melanoma B16 cells [24]. In general, the extract has demonstrated inhibitory effects on tumor cells, which depend on factors such as concentration, extract type, and cancer cell sensitivity. The inhibition rate increases with higher extract concentrations, likely due to the presence of compounds in the extract that alter the physiological state of the cells, arrest the cell cycle at specific stages, prevent proliferation, or induce apoptosis in cancer cells [25]. The hepatoprotective properties of phenolic compounds and flavonoids isolated from *Equisetum arvense* L. also support the use of this plant in traditional medicine for treating liver diseases [21].

In this study, the antiproliferative and migratory properties of *Equisetum arvense* L. extract were evaluated against the human hepatocellular carcinoma SNU449 cell line. Initially, we investigated whether HTE influenced the proliferative capacity of SNU449 cells. We found that low doses of HTE stimulated cell proliferation, while higher doses exhibited cytotoxic effects. The MTT assay revealed that 326 ppm was the effective minimum dose at 24 hours. The colony formation assay further confirmed a significant difference between the extract of *Equisetum arvense* L. exposed and control cells, suggesting that HTE affects the proliferative capacity of SNU449 cells. However, HTE did not alter the levels of caspase-3 and cleaved caspase-3 proteins. This suggests that HTE may exert its effects through alternative mechanisms, which warrant further investigation in future studies.

5 CONCLUSION

HTE has a moderate antiproliferative effect independent of the apoptotic pathway. In future studies, it may be considered to investigate the effect of the combination of the HTE with other chemotherapeutic agents and its effect on different signaling pathway mechanisms in tumor cells.

6 CONFLICT OF INTEREST

The authors declare that there is no conflict of interest.

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AJPS
e-ISSN: 2980-0463
Anat. J. Pharm. Sci., 2025:4(1)
Erratum

ERRATUM

In the article by Çalışkan et al., entitled 'Antimicrobial Resistance of E. coli Strains Isolated from Urine Cultures' (Çalışkan A, Öner SZ, Demir M, Kaleli İ, Mete E, Ergin C, October 1, 2024, *Antimicrobial Resistance of E. coli Strains Isolated from Urine Cultures*, Anatolian Journal of Pharmaceutical Sciences, 3(2), 212–219), published in the second issue of 2024 of *Anatolian Journal of Pharmaceutical Sciences*, the name of the author, İlknur Kaleli, was erroneously written as İlker Kaleli in the PDF file. The corresponding author of the article informed the journal editorial secretary. The editors reviewed the case and approved the correction of the author list, which has now been updated in the PDF file. The corrected author list is provided below:

Ahmet Çalışkan, Sedef Zeliha Öner, Melek Demir, İlknur Kaleli, Ergun Mete, Çağrı Ergin