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Review

Clinical Perspective on The Effects of *Crocus sativus* in Depression and Attention Deficit Hyperactivity Disorder and Sleep Quality

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Abstract

Crocus sativus L. (saffron) is a valuable plant used as a spice and for therapeutic purposes in many cultures, especially in the Middle East and the Mediterranean, since ancient times. The therapeutic effects of saffron are based on the bioactive compounds found in its stigma—most notably crocin, crocetin, safranal, and picrocrocin. These compounds have been shown to have antioxidant, anti-inflammatory, neuroprotective, and antidepressant properties in various *in vitro, in vivo*, and clinical studies. Recent studies have begun to examine the effects of saffron more systematically, especially on neuropsychiatric disorders such as depression and attention deficit hyperactivity disorder. It has been reported that saffron extracts are effective at a level comparable to some antidepressants in cases of mild to moderate depression; they may also help reduce attention and behavioral problems in attention deficit hyperactivity disorder. This review summarizes the phytochemical structure of saffron, compiles preclinical and clinical data on its antidepressant and attention-modulating effects, and discusses potential areas of application for future research.

Keywords: Attention deficit hyperactivity disorder, *Crocus sativus*, depression, saffron, sleep quality

1. Introduction

The *Crocus* genus is represented by 235 species worldwide, 103 of which grow in Türkiye (1-7). *Crocus sativus* L. (*C. Sativus*), which has a high economic value and is in high demand in the world, is a plant belonging to the Iridaceae family, known as "Saffron" in Turkish, and is grown in Asia (Iran, Azerbaijan, India, Pakistan, and China), Europe (Greece, Italy, France, and Spain), North Africa (Morocco and Egypt), the Middle East (Israel), New Zealand, and Türkiye. The word "zaferan," which is of Arabic origin and means yellow, is used in various languages with minor alterations. For instance, it appears as "safran" in German, Turkish, and French; "saffron" in English; "shafran" in Russian; "zafferano" in Italian; and "zafora" in Greek, all referring to this plant. "Saffron," scientifically known as *C. sativus*, is referred to Kürkum in Middle Eastern agriculture (8).

C. sativus, an agricultural product generally grown in continental and temperate climates, can grow in temperatures between – 10 and 35°C, and it has been reported that it can also withstand high temperatures. The pharmaceutical, dye, cosmetic, and food industries use the plant's stigmas, known

as saffron. The high-profit potential of the saffron trade has led countries other than traditional producer countries to show interest in saffron production. Iran, which accounts for more than 90% of global production, is today the world's largest saffron producer, followed by Spain, Afghanistan, Portugal, and France. The countries that import the most saffron are the USA, the United Arab Emirates, and European countries (9).

C. sativus is the most prominent species of the *Crocus* genus regarding traditional uses and is noted for its widespread use in folk medicine across Egypt, Greece, India, Persia, and Rome. In Islamic traditional medicine and Ayurvedic medicine, saffron is widely used for its aphrodisiac effect, and it has been reported to be used as a nerve tonic, emmenagogue, appetite stimulant, and stimulant, as well as to treat dysmenorrhea, stomach ulcers, and premature ejaculation. In traditional Chinese medicine, the topical application of saffron stigma is recommended in treating asthma, whooping cough, and inflammation. Saffron is used for insomnia, migraines, and metabolism in Iraq; for toothache in Spain, and for indigestion and as a sedative in Italy (10).

Saffron was used in ancient Egypt to strengthen the stomach and liver and to cure digestive system diseases. In ancient Rome, saffron was used for lung and eye inflammation, liver disorders, and to stop coughs. In Turkish Islamic medicine, saffron is known to enhance eyesight, aid digestion, strengthen the heart, alleviate headaches, improve memory, regulate sleep, and soothe the nervous system (11). Additionally, it is a key ingredient in mesir paste, which has been produced in Anatolia since 1539 and is utilized in the treatment of various diseases, particularly infertility (12).

Many studies have been investigated the biological activities and phytochemical profile of *C. sativus*, including its leaves, tepals, petals, stigmas, and bulbs. This review examines the phytochemical content of its stigmas, commonly known as saffron, as well as its effects on anxiety and attention deficit-hyperactivity disorders (ADHD), which have been the focus of more recent research.

2. Botanical Characteristics of *C. sativus*

C. sativus var. *officinalis* L. Species Plantarum 1:36 (1753)

Synonyms

C. officinalis var. *sativus* Huds (1778), *C. sativus* var. *casbmiruanus* Royle (1836), Geanthus autumnalis Raf., Specchio Sci. 1: 116 (1814), *C. sativus* var. *cashmerianus* Royle, Ill. Bot. Himal. Mts. 1: t. 90, f. 1 (1834), *C. orsinii* Parl., Fl. Ital. 3(2): 238 (1860), *C. sativus* var. *orsinii* (Parl.) Maw, Gard. Chron., n.s., 11: 234 (1879), *C. autumnalis* Sm. in Engl. Bot.: t. 343 (1796), nom. Illeg, *C. officinalis* (L.) Honck. in Syn. Pl. Germ. 1: 273 (1792), *C. orsinii* Parl. in Fl. Ital. 3: 238 (1860), *C. pendulus* Stokes in Bot. Comm. 1: 209 (1830), *C. sativus* var. *cashmerianus* Royle in Ill. Bot. Himal. Mts. 1: t. 90, f. 1 (1834), *C. sativus* var. *orsinii* (Parl.) Maw in Gard. Chron., n.s., 11: 234 (1879), *C. sativus* subsp. *orsinii* (Parl.) K.Richt. in Pl. Eur. 1: 248 (1890), *C. setifolius* Stokes in Bot. Mat. Med. 1: 104 (1812), *Geanthus autumnalis* Raf. in Specchio Sci. 1: 116 (1814), *Safran officinarum* Medik. in Hist. & Commentat. Acad. Elect. Sci. Theod.-Palat. 6: 473 (1790) (13, 14).

Cormus is large, measuring up to 32 mm in diameter, and flat-based; the tunic is thin and reticulate fibrous, with no ring present. There are 4 or 5 cataphylls. The leaves number between 5 and 11, reaching up to 2 mm in width, with margins that are ciliated and have a keel. The perigon tube is purple, and the floral segments are obtuse, measuring 50 × 20 mm, bright lilac-purple, dark-veined, and featuring a dark spot near the base; the throat is pubescent, ranging from whitish to purple. The filaments measure 7-11 mm in length, are bare, and purplish; the anthers are 5-20 mm long, yellow, with indistinct connective tissue. The style is located at the base or middle of the anthers and is divided into three long, dark red, soft, hanging branches, each measuring 25-32 mm. The capsule and seed are not present (15).

3. Phytochemical Profile of *C. sativus*

Findings from phytochemical studies conducted to date have shown that saffron contains primary

metabolites such as carbohydrates, minerals, fats, vitamins, amino acids, and proteins, and secondary metabolites such as carotenoids, monoterpenes, and flavonoids. The composition of saffron stigmas consists of 14-16% water, 11-13% nitrogenous compounds (alkaloids), 12-15% carbohydrates (mucilage, starch, and gums), 41-44% nitrogen-free soluble compounds (saponins, anthocyanins, flavonoids), 0.6-0.9% volatile oil, 4-5% cellulose, and 4-6% minerals. Riboflavin (56-138 µg/g) and thiamine (0.7-4 µg/g) are also found in saffron. Additionally, the presence of 150 volatile and aromatic compounds (β-isophorone, linalool, α-isophorone, α, β-dihydro-β-ionone etc.) in saffron stigmas was determined by GC-MS. The compounds responsible for the characteristic color (crocin), smell (safranal), and taste (picrocrocin) of saffron have been identified (Fig 1). Crocin is a hydrophilic carotenoid that, when hydrolyzed, yields gentiobiose and crocetin. Safranal is produced through the enzymatic reaction of picrosin, which is a degradation product of zeaxanthin, releasing glucose (16, 17).

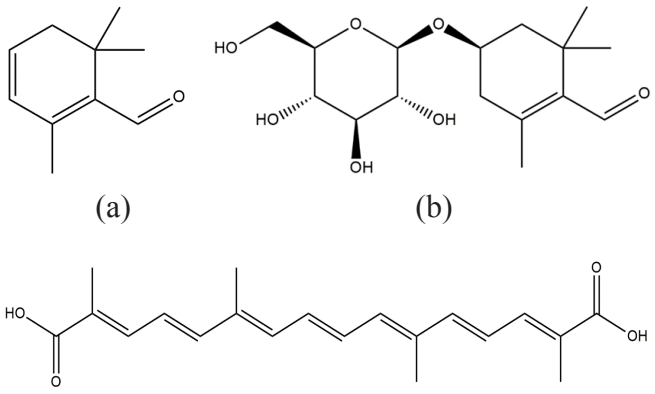

Figure 1. Formulas of (a) safranal, (b) picrocrocin, and (c) crocetin.

The stigmas also contain flavonoids such as kaempferol-3-sophoroside, kaempferol-3-sophoroside-7-glucoside, kaempferol-3,7,4′-triglucoside, kaempferol tetrahexoside, kaempferol-3-dihexoside, isorhamnetin-3-*O*-glucoside, sophoraflavonoloside, quercetin, rutin, quercetin, hesperidin, luteolin, and naringin and coumarins (osthol, isopimpinellin). The stigmas have also been reported to contain some a-hydroxy acids (lactic acid, malic acid, and glycolic acid), fatty esters (methyl arachidate, methyl oleate, methyl palmitate, and methyl stearate), and gallic acid (10,17).

4. Biological Activities of *C. sativus*

To date, numerous studies have been conducted to evaluate the biological activity of extracts obtained from the petals and stigmas of *C. sativus* and the compounds isolated from them (crocin, safranal, and crocetin). These studies are listed below (16,18,19);

- Anticancer/antitumor activity
- Antidepressant and anxiolytic activities
- Antidiabetic activity
- Antigenototoxic and cytotoxic effects
- Antihypertensive activity
- Antinociceptive and anti-inflammatory effects
- Antioxidant activity
- Antitussive activity
- Aphrodisiac effects
- Effects on Alzheimers and Parkinson diseases
- Effects on cardiovascular system
- Effects on gastrointestinal system
- Effects on memory and learning
- Effects on ocular blood flow and retinal function
- Effects on respiratory system
- Effects on urinary system

5. Clinical Studies

5.1. Effects of *C. sativus* in Depression

In the study conducted by Akhondzadeh et al. on 30 patients diagnosed with major depression according to the DSM IV scale, the effects of saffron extract were compared with imipramine. In a 6-week, double-blind, randomized, single-center study, patients were given 30 mg/day saffron extract (Group I) and 100 mg/day imipramine (Group II). Each saffron capsule contains 10 mg of dried 80% ethanol stigma extract. Stigma extract was found to be effective in improving symptoms of mild to

moderate depression, similar to imipramine at a dose of 30 mg/day (F = 2.91, d.f. = 1, p = 0.09). Even anticholinergic side effects (dry mouth and sedation) were more common in the imipramine group (20).

In a six-week double-blind, placebo-controlled, randomized study conducted in Iran in 2005, 40 adult outpatients diagnosed with depression according to DSM IV scale were evaluated with the Hamilton depression rating scale to evaluate the efficacy of saffron stigmas in the treatment of mild to moderate depression. For this purpose, 20 patients were given 30 mg/day saffron capsules every day. The other 20 patients were assigned to the placebo group. At the end of 6 weeks, it was concluded that the improvement in the mood of the saffron group patients was significantly better than the placebo group patients according to the Hamilton depression rating scale. Saffron capsules, which have a low side effect profile, have been reported to be effective in the treatment of mild to moderate depression (21).

In a 6-week double-blind, randomized study, Noorbala et al. investigated the efficacy of standardized saffron stigma aqueous-ethanolic extract on the basis of safranal in the treatment of mild to moderate depression. For this purpose, 19 patients diagnosed with depression according to DSM-IV criteria received capsules containing standardized saffron stigma extract (15 mg) twice a day. To evaluate the effectiveness of the extract, 19 other patients were given 20 mg of fluoxetine per day. In the study evaluating symptoms according to the Hamilton Rating Scale for Depression (HAM-D 17-item), saffron stigma extracts were found to be similarly effective to fluoxetine in the treatment of mild to moderate depression. It was concluded that no significant differences were found in the side effects between the two patient groups. The findings of the study showed that saffron stigma extract may be effective in the treatment of mild to moderate depression, however, these studies should be continued on more subjects (22).

Akhondzadeh Basti et al. studied the efficacy of capsules prepared from *C. sativus* petals in an 8-week pilot, double-blind, randomized clinical trial in 40 patients diagnosed with depression according to DSM-IV criteria. Fluoxetine, a selective serotonin reuptake inhibitor, was utilized for efficacy comparison. Twenty patients were given the petal capsules (15 mg) twice daily. Fluoxetine group patients (n=20) received 10 mg of the drug twice daily, morning and evening. According to the Hamilton Depression Rating Scale, at the end of the treatment, it was found that the petal capsules showed an effect profile similar to fluoxetine in mild to moderate depression. The remission rate for both treatments was determined to be 25%, and there was no notable difference between the two groups regarding side effects (23).

Hausenblas et al. conducted a meta-analysis of randomized controlled trials to evaluate the efficacy of saffron supplements in patients with major depression. The researchers searched the Allied and Complementary Medicine Database, Cumulative Index to Nursing and Related Health Literature, Cochrane Library, EMBASE, MEDLINE, PubMed, and Web of Science databases. Five randomized controlled trials that met the study criteria were included in the meta-analysis, and the quality of the data was assessed using the Jadad score. In studies comparing the effects of imipramine and fluoxetine on depressive symptoms, no difference was found between saffron and these two drugs in terms of their ameliorative effects on depressive symptoms. Researchers emphasized that more controlled, long-term studies with a larger number of subjects should be designed to determine the effectiveness, mechanism of action, and safety of saffron in the treatment of major depression (24).

It is known that 50% of coronary artery patients experience some depressive symptoms, and some are diagnosed with major depression. Shahmansouri et al. evaluated the efficacy of saffron extract (capsule containing 15 mg saffron extract) against fluoxetine in a double-blind, parallel, randomized study on 40 patients diagnosed with mild to moderate depression who underwent percutaneous coronary intervention. In the 6-week study, patients in the positive control group were given 40 mg of fluoxetine per day, while patients in the saffron group were given capsules containing a standardized extract at a dose of 30 mg per day. The Hamilton

Depression Rating Scale (HDRS) was used to assess the efficacy of the treatments administered at 3 and 6 weeks. Despite the relatively small sample size and short observation period, there was no significant difference in the reduction of HDRS scores between the two groups at the beginning and end of treatment. There were also no significant differences between the two groups in terms of adverse reactions (25).

Talaei et al. studied the effect of crocin tablets in a randomized, double-blind, placebo-controlled, pilot clinical trial in 40 patients with major depression (age range 24 to 50 years) for 4 weeks. Patients in the crocin group (n=20) received crocin tablets (30 mg/day) along with a selective serotonin reuptake inhibitor (SSRI) (fluoxetine (20 mg/day), sertraline (50 mg/day), or citalopram (20 mg/day)). On the other hand, patients in the placebo group (n=20) received the same SSRI treatment along with a placebo (two tablets per day).Here, Beck Depression Inventory (BDI), Beck Anxiety Inventory (BAI), General Health Questionnaire (GHQ), Mood Disorder Questionnaire (MDQ), and Side Effect Assessment Questionnaire were used to evaluate the results. Compared with the placebo group, significant improvements were found in BDI, BAI, and GHQ in crocin group patients, and it was concluded that crocin tablets increased the effects of SSRIs in patients with mild to moderate depression ($p< 0.0001$). As a result, it has been reported that crocin can be used as a therapeutic adjuvant in depression because it does not cause significant side effects (26).

Sahraian et al. tested saffron extract against fluoxetine in a randomized, double-blind, placebo-controlled clinical trial in 30 patients diagnosed with major depression according to DSM-IV criteria. In this study, the treatment group received one capsule containing 30 mg of saffron extract along with 20 mg of fluoxetine each day. In contrast, the placebo group received only a placebo and 20 mg of fluoxetine. After four weeks of treatment, improvements in symptoms were evaluated using the Beck Depression Scale, and no significant difference in symptom improvement was observed between the two groups. However, the placebo group reported a higher incidence of side effects, such as headaches, abdominal discomfort, and nausea, compared to the treatment group. Researchers pointed out that different doses need to be tested, more patients need to be included, and longer-term clinical studies need to be conducted to determine the effectiveness of saffron better (27).

The effects of capsules containing 50 mg of dried saffron stigma were evaluated in a double-blind, placebo-controlled clinical trial conducted for 12 weeks in sixty patients with anxiety and depression, compared with placebo. BDI and BAI questionnaires were used to examine the efficacy of saffron capsules, and subjects were asked to answer these questionnaires at baseline and 6 and 12 weeks after starting the medication. It was determined that saffron stigma capsules caused improvements in BDI and BAI scores after 12 weeks of treatment, while very few side effects were observed (28).

In a randomized, double-blind, and placebo-controlled study to determine the effect of saffron on the anxiety and sleep quality of diabetic patients, the Spielberger Anxiety Inventory and also the Pittsburgh Sleep Quality Index were used to evaluate the efficacy. For this purpose, fifty diabetic patients were divided into two groups of equal numbers. The intervention group was given a capsule containing 300 mg of saffron, while the control group patients received a placebo. In the one-week clinical study, a significant difference was observed in the anxiety and sleep quality of the intervention group patients when compared to the beginning of the treatment ($p = 0.001$). There were no significant differences in anxiety and sleep quality before and after treatment in the placebo group ($p = 0.001$). The study asserted that saffron can be used as an adjuvant treatment in reducing anxiety and improving sleep quality in diabetic patients (29).

Jafarnia et al. conducted a placebo-controlled, double-blind, randomized controlled trial to evaluate the efficacy of saffron as an adjuvant in 40 patients diagnosed with anxiety according to DSM IV and receiving sertraline treatment (50 mg/day). The treatment group received sertraline and 450 mg/day saffron capsules, while the other group received both sertraline and placebo capsules for six weeks. The results were evaluated with the

Hamilton Anxiety Rating Scale (HAM-A). At the end of the 6th week, the total HAM-A scores of the saffron-treated patients were significantly lower than those of the placebo (2.95 vs. 5.05; p value = 0.005). The findings suggest that the use of saffron as an adjuvant to sertraline treatment may reduce anxiety symptoms, but clinical studies with a larger number of participants and a longer duration are needed to reach a definitive conclusion (30).

Lopresti et al. studied the effects of standardized saffron extract on mild to moderate anxiety and depressive symptoms in a randomized, double-blind, placebo-controlled study of 68 adolescents (ages 12-16) over eight weeks. Youth and parent versions of the Revised Child Anxiety and Depression Scale (RCADS) were used to assess symptom improvements. Each capsule used in the study consisted of a standardized extract (affron®) containing 14 mg of Lepticrosalides® with a concentration exceeding 3.5%. The Lepticrosalides® contained safranal and crocin isomers derived from the stigmas of the plant. These capsules were administered to adolescents twice daily. After eight weeks, adolescents reported that standardized saffron extract improved overall internalizing symptoms (p=0.049), separation anxiety (p=0.003), social phobia (p=0.023), and depression (p=0.016) more than placebo. However, parents did not report improvements in these symptoms as positively as adolescents (31).

Toth et al. conducted a meta-analysis of randomized and controlled clinical trials to assess the efficacy of saffron in treating mild to moderate depression. For this purpose, PubMed, Embase, Cochrane Central Register of Controlled Trials, and Web of Science databases were searched, and the RevMan statistical program was used. Hedges' g was used to calculate effect sizes, while both Cochran's Q test and Higgins' I^2 indicator were used for heterogeneity, and the risk of bias was assessed using the Cochrane Collaboration tool. Nine out of eleven selected randomized trials were reviewed for statistical analysis and concluded that saffron has a significant effect on the severity of depression. Available data support the idea that saffron is significantly more effective than placebo (g = 0.891; 95% CI: 0.369–1.412, p = 0.001), and is competitive with the antidepressant drugs (fluoxetine, citalopram, and imipramine) tested (g = − 0.246; 95% CI:-0.495–0.004, p = 0.053) (32).

In the meta-analysis to evaluate the efficacy of saffron in depression and anxiety, literature was accessed using PRISMA guidelines, and twenty-three clinical studies were found to meet the criteria. Most of the twenty-three studies utilized saffron at a dose of 30 mg per day (n = 19/23). Some studies employed saffron stigma (n = 10), while others used saffron petals (n = 4). Three studies investigated crocin, and the remaining six studies examined saffron powder. In the evaluated studies, findings related to a total of 1237 participants were examined. Treatment durations varied from 4 to 12 weeks, and the studies assessed efficacy by comparing saffron monotherapy to saffron combined with either a placebo or antidepressant medications such as fluoxetine, imipramine, and citalopram. Meta-analysis results indicated that saffron caused a significant improvement compared to placebo for depressive symptoms ($p < 0.001$) and anxiety symptoms ($p < 0.006$) and can be used as an adjuvant to conventional drug therapy in the treatment of depression (33).

Khaksarin et al. conducted a meta-analysis of clinical studies on the efficacy of saffron versus fluoxetine in treating depression. Articles published on this topic up to May 2018 were retrieved from various databases, including the Cochrane Library, Scopus, PubMed/MEDLINE, the Centre for Reviews and Dissemination, EMBASE, and ISI/Web of Science. As a result of the initial literature searches, 157 studies were reached, only 8 of which were found to meet the selection criteria, and data from a total of 368 participants were evaluated. In the studies whose quality was approved according to the Cochrane checklist, it was determined that the participants were given fluoxetine in the dose range of 20-40 mg/day and saffron capsules in the dose range of 30-50 mg/day. Meta-analysis findings demonstrated that saffron was well comparable to fluoxetine and placebo in the treatment of depression (34).

Also, a meta-analysis published in 2023 evaluated the overall effects of saffron on cognition,

depression, anxiety, sleep disorders, attention-deficit/hyperactivity disorder, and obsessive-compulsive disorder. To achieve this, the PubMed/Medline, Web of Science, and Clinical Trials databases were searched for randomized controlled trials published on this topic up to June 2023. RevMan and STATA software programs were utilized for the meta-analysis of forty-six randomized controlled trials involving participants who were either healthy or suffering from various diseases, including neurological and psychiatric disorders. These participants were treated with saffron or its extracts, either alone or in combination with conventional medications, for durations ranging from 4 to 48 weeks. Out of forty-six clinical studies, seven were focused on cognitive disorders, thirty-two on depression, fourteen on anxiety, nine on sleep disorders, four on ADHD, and two on obsessive-compulsive disorders. Cochrane guidelines were used to assess the risk of bias. The findings indicated that the overall effect size of saffron in enhancing cognitive disorders and alleviating depression was 4.26 (95% CI: 5.76, 2.77). In terms of anxiety, the effect size was 3.75 (95% CI: 5.83, 1.67), and for sleep disorders, it was 1.91 (95% CI: 2.88, 0.93). Saffron was also found to be more effective than placebo across all studies. Meta-analysis has highlighted that saffron may be as effective as conventional medications in treating ADHD and obsessive-compulsive disorders, it may also offer preventive effects against neurological and psychiatric disorders, and has a low side effect profile (35).

5.2. Effects of *C. sativus* in ADHD

ADHD is one of the most prevalent neurodevelopmental disorders in childhood, characterized by difficulties with attention, hyperactivity, concentration, and impulse control. The global prevalence of ADHD is approximately 5%, with a higher incidence in boys compared to girls. Pharmacological treatments for ADHD include central nervous system stimulants, antidepressants, antipsychotics, anticonvulsants, anxiolytics, lithium, guanfacine, and clonidine. The side effects associated with these medications can include sleep disturbances, headaches, nausea, eating issues, aggression, malaise, growth retardation, mood swings, tics, and potential cardiac risks. This profile of side effects has prompted investigations into alternative treatment options for ADHD (36).

Fifty-four children, aged 6 to 17, who were diagnosed with ADHD according to the DSM-5, participated in a 6-week randomized, double-blind study designed to compare the effectiveness of saffron with methylphenidate in alleviating ADHD symptoms. Children in the saffron group received saffron capsules at a dosage of 20-30 mg per day, while the other children were administered methylphenidate at the same dosage of 20-30 mg per day. Changes in ADHD symptoms were evaluated using the Teacher and Parent Attention Deficit/Hyperactivity Disorder Rating Scale-IV at baseline and again at weeks 3 and 6. Findings from all evaluation scales indicated that there was no significant difference between the two groups ($F = 0.749$, $df = 1.317$, $p = 0.425$; $F = 0.249$, $df = 1.410$, $p = 0.701$, respectively), and also the frequencies of side effects were comparable (37).

Khaksarian et al. evaluated the efficacy of a combination of methylphenidate and saffron in 70 children (ages 6-16) diagnosed with ADHD. After dividing the patients into two equal groups, both groups received either 20 or 30 mg/day of methylphenidate (20 mg/day for those weighing less than 30 kg and 30 mg/day for those weighing more than 30 kg). Additionally, one of the groups was administered 20 or 30 mg/day of saffron capsules based on body mass index (20 mg/day for those under 30 kg and 30 mg/day for those over 30 kg). According to the Attention Deficit/Hyperactivity Disorder Rating Scale-IV, which was completed by both parents and teachers, both groups of patients exhibited fewer symptoms after eight weeks of treatment. After four weeks, the mean score of the methylphenidate-saffron combination group was lower than that of the methylphenidate group. In light of all the findings, it was concluded that using methylphenidate in combination with saffron would reduce the duration of treatment for ADHD patients while enhancing the treatment's effectiveness (38).

A single-center, prospective, naturalistic, non-randomized, non-blinded, pre-post intervention study was conducted on 63 children diagnosed with

ADHD according to DSM-5 criteria to evaluate the efficacy of saffron extract (Saffr'Activ) versus methylphenidate. Children were divided into two groups, with both groups receiving psychoeducation. Additionally, patients in Group 1 were administered slow-release methylphenidate at a dose of 1 mg/kg/day, while patients in Group 2 received saffron extract at a dosage of 30 mg/day. As a result of evaluations conducted using lens and pen-and-paper tests, it was observed that the effectiveness of saffron extract was comparable to that of methylphenidate. However, it was concluded that saffron was more effective for hyperactivity, while methylphenidate demonstrated greater effectiveness for concentration (39).

Pazoki et al. assessed the effectiveness of saffron extract as an adjunctive treatment in a placebo-controlled, double-blind, randomized clinical trial involving fifty-six patients diagnosed with ADHD over a period of six weeks. Patients were divided equally into two groups: one was given Ritalin plus placebo (30 mg/day), and the other Ritalin plus saffron (15 mg twice daily). The effectiveness of the treatments administered to the patients was assessed using the Conners Adult ADHD Rating Scale (CAARS) and the Adult ADHD Self-Report Scale (ASRS). It was found that there was no significant difference in the CAARS and ASRS scores, nor in the frequency of side effects, between the two groups at baseline and after treatment (40).

As a result of the search studies conducted using the keywords "attention deficit disorder with hyperactivity," "attention deficit," "ADHD," "hyperkinetic," or "minimal brain dysfunction" from the PubMed, EMBASE, Scopus, and Web of Knowledge databases, it was planned to evaluate the effectiveness of saffron in ADHD through a meta-analysis involving a total of four clinical studies. This analysis excluded non-English articles, review articles, comments, letters, observational studies, theses, animal studies, *in vitro* studies, and conference abstracts. The studies compared the effect of saffron with methylphenidate and displayed that the doses of saffron capsules generally ranged from 20 to 30 mg/day. Data from a total of 118 patients indicated that saffron capsules could be used alone or as an adjuvant to methylphenidate in ADHD with an acceptable safety profile (41).

5.3. Effects of *C. sativus* in Premenstrual Syndrome (PMS)

Premenstrual dysphoric disorder (PMDD) is a condition characterized by severe mood and behavioral changes. At present, the correlation between this disorder and alterations in serotonin conductance within the central nervous system has been validated by the positive effects observed with SSRI medications. In addition to the use of antidepressants for PMS, the utilization of herbal medicines with traditional medicinal origins is becoming increasingly common. For this purpose, it is essential to investigate the medicinal plants such as saffron, employed in traditional folk medicine for depressive disorders in clinical trials. Agha-Hosseini et al. conducted a double-blind, placebo-controlled study to investigate the efficacy of capsules containing dried *C. sativus* stigma extract on PMS symptoms. In the study, 78 women aged 20 to 45 who had regular menstrual cycles and experienced PMS symptoms for at least six months were assigned to receive either 15 mg saffron capsules twice daily (group A) or placebo capsules twice daily for two menstrual cycles (cycles 3 and 4). In the study evaluating efficacy using the Daily Symptom Report and the Hamilton Depression Rating Scale, findings indicated that saffron stigma extract was effective in alleviating PMS symptoms during the 3rd and 4th cycles (42).

Fukui et al.(43) designed a clinical study to explain the effects of saffron odor on PMS, dysmenorrhea, and irregular menstruation in 35 women with a normal sense of smell, measured salivary cortisol, testosterone, and 17-β estradiol levels, and used the State-Trait Anxiety Inventory. In the study, women were exposed to saffron odor for 20 minutes. At the end of this period, it was found that cortisol levels decreased significantly in both the follicular and luteal phases, while 17-β estradiol levels increased. Conversely, the State-Trait Anxiety Inventory scores decreased in both phases. The findings support the notion that the scent of saffron may have both physiological and psychological effects on PMS, dysmenorrhea, and irregular menstruation in women.

A randomized, triple-blind controlled clinical trial conducted in Iran on 78 students aged 18-35 investigated the efficacy of capsules containing 30 mg of dried saffron stigma extract in PMS. The study lasted for two menstrual cycles for both the intervention group and the group receiving the placebo capsules. The saffron capsules efficacy of based on the results from the DASS21 scale and the premenstrual symptom assessment forms. At the beginning of the study, there was no significant difference in the mean severity of PMS between the two groups. However, at the end of the study, the change in the mean severity of PMS was found to differ in the intervention group ($p<0.001$) compared to the placebo group ($p = 0.04$) from the beginning. The researchers concluded that saffron effectively reduces the severity of PMS symptoms; however, they noted that further clinical studies are necessary to reach a more definitive conclusion (44).

5.4. Effects of *C. sativus* for sleep quality

Sleep problems occur when a person does not get enough quality sleep. The problem can manifest in symptoms such as difficulty falling asleep, waking up frequently, waking up early, or not feeling refreshed after waking. Various factors, both physical and psychological, can lead to temporary or chronic sleep issues. Since saffron has traditionally been used to combat insomnia, this section of the review discusses several studies and their findings regarding its impact of saffron on sleep quality. It is know that people with diabetes experience deterioration in their sleep quality due to impaired glucose levels. In a placebo-controlled clinical study conducted in Iran in 2016 on 50 diabetic patients, the effects of oral saffron capsules on sleep quality were evaluated. The treatment group received 300 mg saffron capsules, while the control group received placebo capsules for a week. After treatment, the patient's sleep quality was evaluated with the Pittsburgh Sleep Quality Index, and a statistically significant difference was observed between the two groups. The study findings showed that saffron capsules may be effective in improving the sleep quality of diabetic patients (45).

In a meta-analysis conducted on improving sleep quality with saffron, articles published up to 2022 were accessed from PubMed, Central, Google Scholar, and Scopus databases. The Cochrane risk tool was used to prevent bias, and the methodological features of the articles were assessed using the Stevinson and Ernst criteria. As a result, five randomized clinical trials with 379 participants from three countries were found to meet the meta-analysis criteria. The duration of the studies ranged from four to eight weeks; four studies used saffron extract (14-28 mg/day), and one study used crocin (0.6 mg/day). The findings displayed that saffron positively affects the duration and the quality of sleep (46).

6. Toxicity and safety of *C. sativus*

LD_{50} values: 1.6 g/kg intraperitoneally for stigma in mice, >5000 mg/kg orally.

Human safe dose: <1.5 g/day is non-toxic. >5 g is toxic, >20 g is potentially fatal.

Clinical Doses: Generally in the range of 30–50 mg/day; found to be safe.

Common side effects: Dry mouth, dizziness, nausea, diarrhea. Generally mild and transient.

Serious effects (High doses): Numbness, jaundice, spontaneous bleeding, abnormal uterine bleeding (estrogenic effect).

Effects on organ systems: Liver/Kidney: Increased BUN and creatine at high doses; ineffective at low doses.

Coagulation: Decreased platelet count, altered clotting time (adenosine-induced).

Hormones/Lipids: No significant changes were detected.

Drug interactions are not yet known (47).

7. Conclusion

*C. sativus (*saffron), due to its rich profile of bioactive components, has transcended traditional applications and become a promising candidate for modern phytotherapeutic strategies. Recent studies have elucidated the mechanisms of action of its key constituents-crocin, crocetin, safranal, and picrocrocin-in various neuropsychiatric conditions.

Preclinical and clinical evidence supports saffron's effectiveness in managing neurological and psychiatric disorders. The proposed mechanisms of action in these disorders are as follows:

Mechanism of memory-enhancing and neuroprotective effects (in cognitive disorders): Saffron prevents β-amyloid plaque and tau protein accumulation, inhibits acetylcholinesterase activity, reduces neuronal apoptosis, and ameliorates neuroinflammation.

Mechanism of antidepressant effects: Saffron may inhibit the reuptake of dopamine and noradrenaline, enhance levels of brain-derived neurotrophic factor (BDNF), VGF neuropeptide, and cyclic AMP response element-binding protein (CREB), downregulate the hypothalamic-pituitary-adrenal (HPA) axis, and reduce neuroinflammation.

Mechanism of anxiolytic effects: It promotes neuronal growth and survival and may reduce neuronal excitability, contributing to decreased anxiety symptoms.

Mechanism of action in improving sleep disturbances: Saffron exerts sedative and calming effects, regulates the circadian rhythm by enhancing nocturnal melatonin secretion, and supports healthy sleep architecture.

Mechanism of action in ADHD: It modulates monoaminergic and glutamatergic neurotransmission, thereby reducing hyperactivity and impulsivity.

Mechanism of action in obsessive-compulsive disorder (OCD): Saffron increases serotonergic activity, elevates brain zinc levels, and may potentiate antipsychotic-like effects.

All of these findings suggest that *C. sativus* may be considered as a complementary option in the treatment of depression and ADHD; however, larger-scale and long-term studies are needed. The biggest shortcoming of clinical studies to date is that few studies have used standardized extract formulations, and there is some methodological heterogeneity. Therefore, large-scale, randomized, placebo-controlled studies are needed to confirm that saffron safety, effectiveness, and its role in pharmacologically predictable treatment for neuropsychiatric disorders such as depression and ADHD. Furthermore, detailed studies on its pharmacokinetics, dose-response relationships, and long-term safety profiles are vital. Future research in these areas will not only strengthen the scientific basis for the use of saffron in modern psychopharmacology but may also accelerate the development of new, plant-based therapeutic interventions.

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Ethics approval: Not applicable

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Original Article

In Silico Investigation of the Effect of Cannabidiolic Acid (CBDA) on Muscarinic Acetylcholine Receptors by Molecular Docking Method

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Abstract

Introduction: *Cannabis sativa* contains phytocannabinoids with therapeutic potential for various diseases. Cannabidiolic acid (CBDA), such as phytocannabinoid, has demonstrated antiemetic effects. Postoperative nausea and vomiting are common complications often mediated by muscarinic acetylcholine receptors (mAChRs) in the vomiting center of the brain. This study aimed to investigate the antiemetic effects of CBDA on mAChRs using in silico methods.

Methods: The molecular structure of CBDA was obtained from the PubChem database. Molecular docking simulations with mAChRs were performed using the AutoDock Vina program. Docking results were visualized and analyzed with the Discovery Studio Visualizer software. N – [methyl – 3 H]scopolamine (NMS), a known muscarinic receptor antagonist, was used as a reference drug for comparison.

Results: CBDA demonstrated strong binding affinity with mAChRs, particularly M1 and M3, through significant hydrogen and hydrophobic interactions. Compared to the reference drug NMS, CBDA exhibited significant binding affinity to the receptors, suggesting possible biological activities.

Conclusions: CBDA demonstrated comparable binding affinities to NMS, indicating its potential as a candidate for further antiemetic research. CBDA demonstrated comparable binding affinities to NMS, suggesting that it may have potential for antiemetic applications. However, further studies are needed to clarify its mechanism of action and clinical relevance. Also, its binding profile suggests potential for antiemetic applications, pending functional confirmation. Further in vitro and in vivo studies are required to validate these findings.

Keywords: Antiemetic, *Cannabis sativa*, in-silico, cannabidiolic acid (CBDA), molecular docking

1. Introduction

Emesis, commonly referred to as vomiting, is a distressing physiological response characterized by the forceful expulsion of gastric contents through the mouth. It is primarily associated with gastrointestinal motor functions and serves as a protective mechanism against harmful substances, illnesses, or pharmacological agents (1). The etiology of vomiting is diverse, encompassing food poisoning, motion sickness, gastroenteritis, intestinal obstructions, head trauma, pregnancy, appendicitis, and alcohol-related hangovers. Furthermore, it can arise as a secondary effect of various medical conditions and treatments, including brain tumors, excessive exposure to ionizing radiation, increased intracranial pressure, chemotherapy and radiotherapy (2,3).

The process of emesis is orchestrated by intricate neural mechanisms, with the brain's vomiting center (VC) playing a central role in initiating nausea and vomiting (4). This center is located within the chemoreceptor trigger zone (CTZ) of the fourth ventricle. In addition to the CTZ, multiple regions such as the gastrointestinal (GI) tract, higher cortical centers, vestibular system, and thalamus contribute to the vomiting reflex. The activation of specific proteins within the VC is crucial for triggering this response (5). During emesis, the gastric muscles relax, hydrochloric acid (HCl) secretion is suppressed, and retrograde contractions of the small intestine generate pressure on the stomach, leading to retching and eventual expulsion of gastric contents (6).

The identification of natural sources for novel antiemetic agents remains an area of active research. Several bioactive compounds, including flavonoids, cannabinoids, chalcones, glycosides, hydroxycinnamic acids, diarylheptanoids, lignans, phenylpropanoids, saponins, polysaccharides, and terpenes, have been investigated for their potential in emesis control (7). Among these, *Cannabis sativa* (*C. sativa)* has gained considerable attention due to its historical and contemporary applications in medicine, textiles, and food industries. Phytocannabinoids such as cannabidiol (CBD) and cannabidiolic acid (CBDA) have demonstrated significant antiemetic effects, particularly in the management of chemotherapy-induced nausea, chronic pain, and inflammation (8,9).

Recent studies highlight the therapeutic potential of *C. sativa* derivatives in overcoming the limitations of conventional antiemetic treatments. CBDA exhibits promising interactions with muscarinic acetylcholine receptors (mAChRs), suggesting the potential to prevent the emesis response by inhibiting the receptors. However, further studies are required to fully elucidate its mechanisms of action, optimize pharmacological applications, and ensure its clinical safety and efficacy (10,11).

Beyond its antiemetic properties, *C. sativa* has been reported to alleviate chronic pain and muscle spasms, enhance appetite in individuals with HIV/AIDS, improve sleep quality, and reduce tics in patients with Tourette syndrome. Among its bioactive constituents, phytocannabinoids are recognized as the most potent and pharmacologically relevant components (12). One of the primary targets of antiemetic drugs is the mAChR system.

Muscarinic acetylcholine receptors (mAChRs) belong to the G-protein-coupled receptor family and regulate critical functions within the central and peripheral nervous systems. Acetylcholine (ACh), the endogenous ligand for these receptors, facilitates neurotransmission via both ligand-gated ion channels (nicotinic receptors) and G-protein-coupled mAChRs (13). These receptors are classified into five subtypes (M1–M5), which exhibit distinct expression patterns across various brain regions and peripheral organs (14). Among these, M1, M3, and M5 couple with Gq/11 family G-proteins, while M2 and M4 preferentially interact with Gi/o proteins. Each subtype mediates unique physiological responses, such as synaptic plasticity (M1), gastric acid secretion and smooth muscle contraction (M3), and neurological modulation via dopaminergic and glutamatergic pathways (M4) (15,16).

M2 and M4 receptors primarily function through Gi/o proteins, leading to reduced cyclic adenosine monophosphate (cAMP) levels and subsequent inhibitory effects on cellular signaling (13). These receptors are crucial in regulating cardiac function (M2) and modulating neurotransmitter release (M4),

influencing both autonomic and central nervous system functions (17). The intricate signaling pathways mediated by mAChRs underscore their significance in physiological processes, particularly in the modulation of gastrointestinal motility and nausea. In conclusion, the inhibition of mAChRs presents a therapeutic potential for the development of antiemetic drugs (11).

The precise roles of mAChR subtypes in nausea and vomiting have not been fully elucidated; consequently, currently available mAChR inhibitors lack subtype selectivity in clinical applications. Current anticholinergic drugs, such as N-methyl scopolamine (NMS), lack selectivity among the M1–M5 subtypes, leading to undesirable side effects such as dry mouth, visual disturbances, and drowsiness (17). Therefore, it is of great significance to investigate each mAChR subtype individually to enhance drug selectivity. This study aimed to evaluate the potential inhibitory effect of each mAChR subtype (M1–M5) on emesis through molecular docking with CBDA using *in silico* methodologies.

2. Methods

The pharmacological properties of the compound were determined using SwissADME, a freely accessible web-based tool for estimating ADME parameters. The SMILES form of the CBDA molecule was taken from PubChem and entered into the SwissADME web tool, and the results were obtained.

2.1. Target protein and ligand preparation

The three-dimensional (3D) structure of the M1, M2, M3, M4 and M5 target proteins were taken from the RCSB PDB. M1-5 crystallographic structures with PDB identity 6WJC(M1) (Resolution: 2.55 Å), 5ZK8(M2) (Resolution: 3.00Å), 4U15(M3) (Resolution: 2.80 Å), 5DSG(M4)(Resolution: 2.60 Å) 6OL9(M5) (Resolution: 2.54 Å) were used. In the BIOVIA Discovery Studio 2021 program, unnecessary residues such as water other than the protein in the receptor obtained from the PDB, were removed from the structure. Energy minimization was carried out to obtain a stable conformation. The structure of the selected ligand, CBDA (PubChem ID: 160570), was obtained from the PubChem chemical compounds database (accessed 17.06.2023) The protein's 3D structure and polarization image were obtained using the PyMOL tool.

2.2. Molecular docking

In the study, the AutoDock Vina tool (version 1.5.7) was used to investigate the molecular interaction between target proteins and the selected ligand. Before docking analysis, the structure of the enzyme was optimized using the BIOVIA Discovery Studio 2021 program. Then all compounds were optimized for energy using the Spartan 14 (Version 1.1.4) program. Polar hydrogens were added to the protein using the AutoDock vina 1.5.7 tool, and Kollman charges were determined as partial charge of compounds calculated using Compute Gasteiger. BIOVIA Discovery was used to determine the active sites of proteins. The x, y, and z coordinates were determined to bind the proteins to the catalytic site. After protein and ligand preparation in Autodock Vina, a grid box was generated.

Target proteins grid box values; for M1 x = 20.4468 y = 13.5360 z = 2.5246 (size:90.00), for M2 :x = 184.1934 y = 27.8428z = 526.0209 (size:62.00) for M3 :x = 25.0037 y = 91.8625 z = 53.4823(size:90.00), for M4: x = 51.6869 y = – 1.0918 z = 79.0402 (size: 90.00), for M5 :x = 35.3866 y = 21.7023 z = – 42.8548 (size 60.00) .

Finally, molecular interactions and binding types between the selected compound and target protein were investigated by using the Discovery Studio visualizer program.

3. Results and Discussion

3.1. 3D Prediction of target proteins

In order to attain a comprehensive understanding of the 3D structure of the target proteins, the 3D conformation and surface electrostatic potential representation of the target protein were derived utilizing the PyMOL software. Determining the surface electrostatic potential map of the receptors is crucial for confirming that the ligand is correctly positioned in the binding pocket after the interaction.

Surface electrostatic potential elucidates the distribution of charges present on the protein surfaces. Regions depicted in blue signify the presence of positive charges, areas depicted in red indicate negative charges, and regions depicted in white denote neutral charges. It is observed that the distribution of negative and positive charges is homogeneously allocated across the surfaces of the M1, M3, and M4 proteins. In contrast, in the M2 and M5 proteins, positive charges are notably concentrated in the central region of the protein, whereas neutral and negative charges demonstrate a more pronounced presence at the termini of the protein. As a result of examining the polarization maps, binding pockets (grid boxes) for proteins were determined (Table 1).

Table 1. 3D structure and polarization image of target proteins.

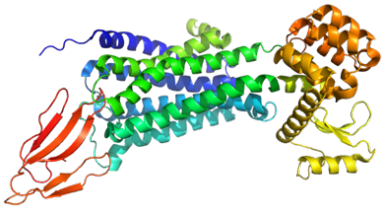
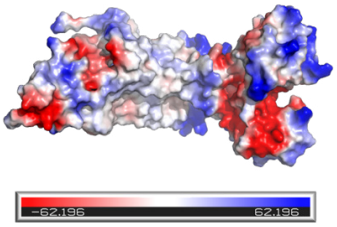
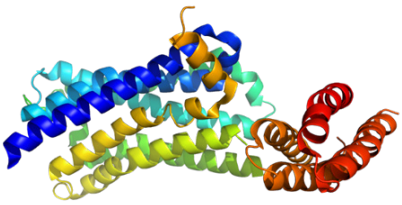
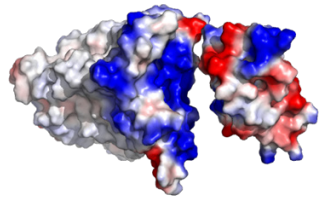
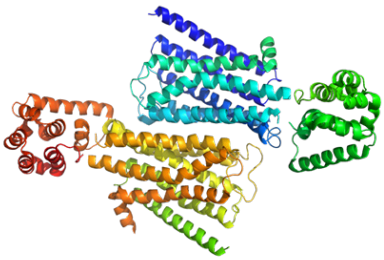
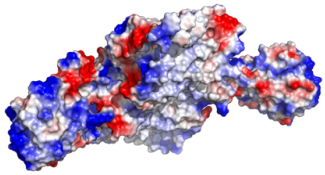
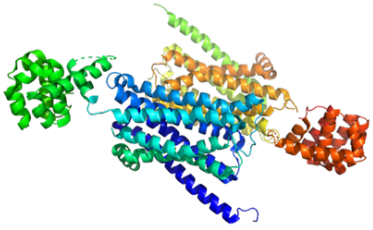
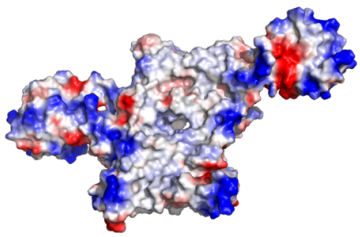
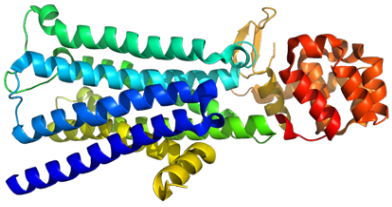
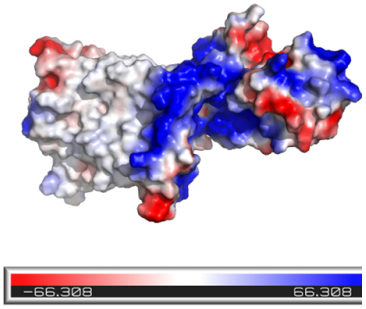
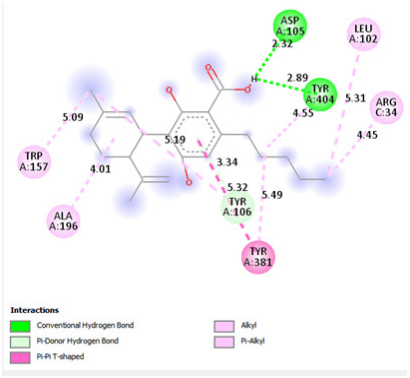
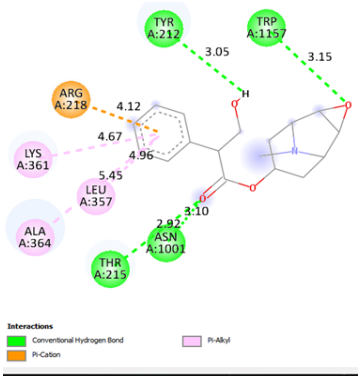
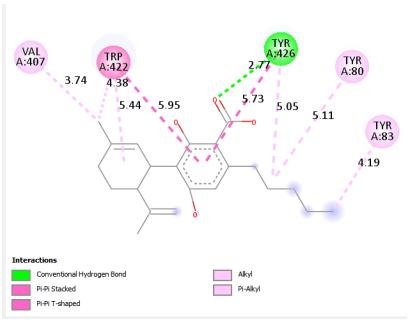
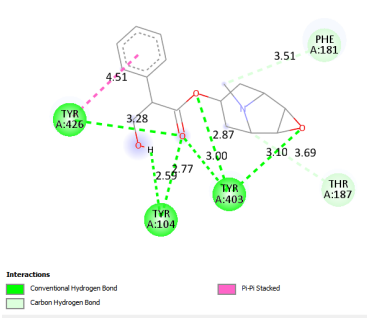
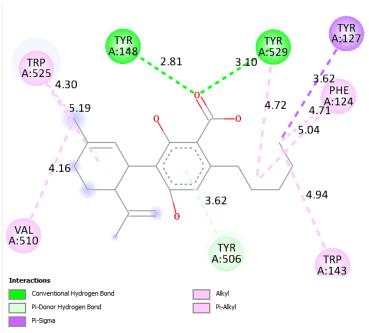
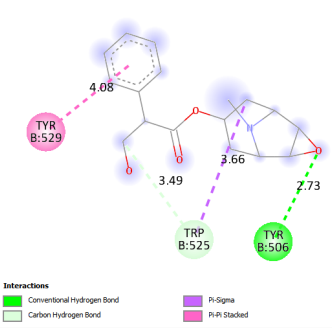
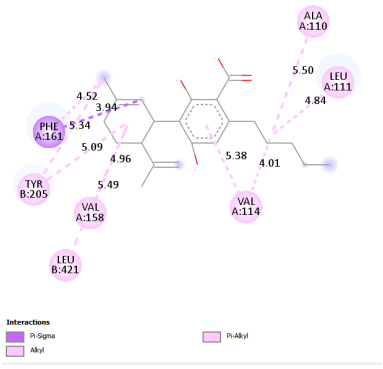
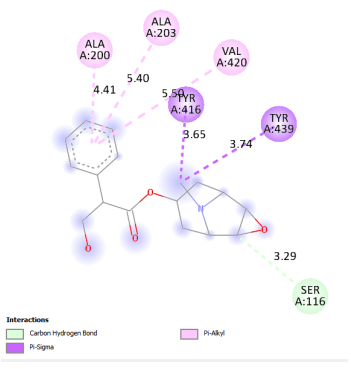
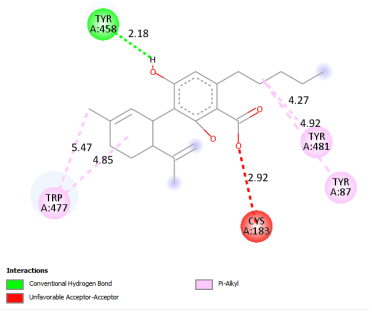
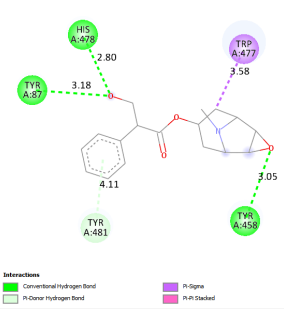
Target protein	3D structure	Polarization image
M1 (6WJC)		
M2 (5ZK8)		
M3 (4U15)		
M4 (5DSG)		
M5 (6OL9)		

Table 2. Interactions of ligands with target proteins.

Target Proteins	CBDA	NMS
M1		
M2		
M3		
M4		
M5		

To assess the reliability and accuracy of the docking protocol employed in this study, a redocking procedure was conducted using NMS, a well-characterized muscarinic receptor antagonist (Table 2). The ligand was redocked into the binding sites of the M2 (PDB ID: 3UON) and M3 (PDB ID: 4DAJ) mAChR crystal structures. The predicted binding poses were compared to the experimentally observed co-crystallized ligand conformations, and the root-mean-square deviation (RMSD) values were calculated. The RMSD values are obtained 0.580 Å for the M2 receptor and 1.339 Å for the M3 receptor, indicating that the docking protocol could reliably reproduce the experimentally determined ligand orientations within an acceptable threshold (RMSD < 2.0 Å).

3.2. Pharmacological properties of CBDA

Results of bioavailability radar, including the chemical structure of the molecule and lipophilicity, size, polarity, solubility, saturation, and flexibility properties, were found on the SwissADME web server (Fig 1). The Log S (ESOL) value of CBDA is – 5.93, classifying it as moderately soluble, whereas NMS has a Log S value of – 2.21, indicating higher solubility. This suggests that NMS exhibits better aqueous solubility than CBDA. CBDA's lower water solubility may limit its absorption and distribution; however, its higher lipophilicity may enhance membrane permeability, which is advantageous for oral bioavailability. The iLOGP (3.45), XLOGP3 (6.60), and MLOGP (3.79) values of CBDA are higher than those of NMS (2.35, 0.98, and 1.19, respectively), indicating that CBDA is more lipophilic and exhibits greater solubility in lipid environments. Increased lipophilicity can enhance membrane permeability but may concurrently reduce aqueous solubility.

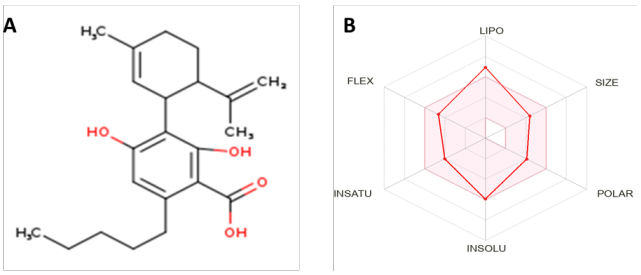


Figure 1. Chemical 2D structure (A) and bioavailability radar of CBDA (B) (19).

Regarding gastrointestinal absorption (GI absorption), both compounds exhibit high absorption rates, suggesting efficient intestinal uptake upon oral administration. In terms of blood-brain barrier (BBB) permeability, SwissADME predictions indicate that neither CBDA nor NMS can cross the BBB. Therefore, the potential antiemetic effects of CBDA might be mediated peripherally. However, these predictions need confirmation through further pharmacokinetic and *in vivo* studies. Although CBDA fulfills drug-likeness criteria such as Lipinski's Rule of Five, its pharmacokinetic profile presents both strengths and limitations. Its high GI absorption and lipophilicity are favorable for oral delivery, but low aqueous solubility and inability to cross the blood-brain barrier (BBB) may restrict systemic or central effects. Therefore, further ADMET profiling, including metabolic stability and bioavailability studies, is needed to support its potential as a therapeutic agent.

Both compounds comply with Lipinski's Rule of Five, with no violations observed. This adherence suggests that they possess favorable oral bioavailability and are suitable candidates for drug development (Table 3) (19).

Table 3. Pharmacokinetic properties of CBDA using the SwissADME web server (18).

Properties	Parameters	CBDA	NMS
Physicochemical Properties	Formula	C22H30O4	C17H21NO4
	MW	358,47 gr/mol	303.35 g/mol
	num. H bond acceptors	7	5
	num. H bond donors	4	1
Lipophilicity	iLOGP	3.45	2.35
	XLOGP3	6.60	0.98
	MLOGP	3.79	1.19
Water Solubility	Log S (ESOL)	-5.93	-2.21
	Class	moderately soluble	Soluble
Pharmacokinetics	GI	high	high
	BBB	No	No
	LogKp	– 3.80 cm/s	-7.45 cm/s
	p – gp substrate	No	No
	CYP1A2 inhibitor	No	No
	CYP2C19 inhibitor	No	No
	CYP2C9 inhibitor	yes	No
	CYP2D6 inhibitor	No	Yes
	CYP3A4 inhibitor	Yes	No
Drug-likeness	Lipinski	Yes; 0 violations	Yes; 0 violation

3.3. Molecular-docking study of the inhibition of M1-5 by CBDA

Nausea and vomiting are triggered by receptors located in the CTZ of the brain. One of these receptors is the mAChR, and the development of inhibitory molecules targeting these receptors is crucial for the treatment of emesis. However, mAChRs are divided into five different subtypes, and M1 and M3 receptor subtypes have been particularly implicated in the emetic response in previous studies. This lack of specificity hinders the development of selective inhibitors, leading to side effects due to non-selective molecular interactions. In this study, the effect of CBDA, which is hypothesized to exhibit inhibitory activity on mAChRs, was investigated for each mAChR subtype using the molecular docking method. The hydrogen, hydrophobic, and other interactions between protein and ligands as a result of the coupling study with the M1-5 receptor with CBDA and reference drug NMS are shown in Table 4.

Table 4. Binding affinity value, hydrogen bonds, hydrophobic and other interactions of mAChRs (M1-5) with CBDA and reference drug.

Target protein	Compound Name	Binding affinity (Kcal/mol)	Hydrogen bonds interactions	Hydrophobic interactions	Others interactions
M1 Receptor interaction	CBDA	– 7.0	ASP105 (2.32Å) TYR404 (2.89Å) TYR106 (3.34Å)	LEU102 (5.31Å) ARG34 (4.45Å) TYR381 (5.49 Å, 5.32Å) TYR106 (5.19Å) ALA196 (4.01Å) TRP157 (5.09 Å)	-
	NMS	-6.1	TYR212 (3.05Å) TRP1157 (3.15Å) ASN1001 (3.10Å) THR215(2.92 Å)	LEU357 (4.96Å) ALA364 (5.45Å) LYS361 (4.67Å)	ARG218 (4.12Å)
M2 Receptor interaction	CBDA	-7.5	TYR426 (2.77Å)	VAL407 (3.74Å) TRP422 (4.38Å, 5.44Å, 5.95Å) TYR426 (5.73Å) TYR80 (5.11Å) TYR83 (4.19Å)	-
	NMS	– 7.4	TYR426 (3.28Å) TYR104 (2.59Å, 2.77Å) TYR403 (3.00Å, 2.87Å, 3.10Å) THR187 (3.69Å) PHE181 (3.51Å)	TYR426 (4.51Å)	-
M3 Receptor interaction	CBDA	-8.0	TYR148 (2.81Å) TYR529 (3.10Å) TYR506 (3.62Å)	TRP525 (4.30Å, 5.19Å) VAL510 (4.16Å) TYR127 (3.62Å) PHE124 (4.71Å, 5.04Å) TRP143 (4.94Å)	-
	NMS	-8.1	TYR506 (2.73Å) TRP525 (3.49Å)	TYR529 (4.08Å) TRP525 (3.66Å)	-
M4 Receptor interaction	CBDA	-8.1	-	PHE161 (3.94Å, 4.52Å) TYR205 (5.09Å, 5.34Å) VAL158 (4.96Å) LEU421 (5.49Å) VAL114 (5.38Å, 4.01Å) LEU111 (4.84Å) ALA110 (5.50Å)	-
	NMS	-8.5	SER116 (3.29Å)	ALA200 (4.41Å) ALA203 (5.40Å) VAL420 (5.50Å) TYR416 (3.65Å) TYR439 (3.74Å)	-
M5 Receptor interaction	CBDA	-7.2	TYR458 (2.18Å)	TYR481(4.27Å) TYR87(4.92Å) TRP477(5.47Å, 4.85Å)	CYS183 (2.92Å)
	NMS	-7.7	TYR458 (3.05Å) TYR87 (3.18Å) HIS478 (2.80Å) TYR481 (4.11Å)	TRP477(3.58Å)	-

In the study, the presence of hydrogen bonds and the binding affinity values expressed with negative values show that a lower value indicates stronger binding compared to the reference drug, thus showing a stronger inhibitory effect.

In the docking study, CBDA was observed to bind well to the M2, M3, M4 and M5 receptors (Fig 2). When compared to the reference drug used in the study, the binding affinity values were found to be remarkably similar. These observed values suggest that CBDA also has the potential to act as a therapeutic agent targeting these receptors. The strong binding of molecules to the receptor indicates effective inhibition of that receptor. Therefore, by suppressing the receptors responsible for initiating nausea and vomiting, the aim is to prevent the onset of these symptoms.

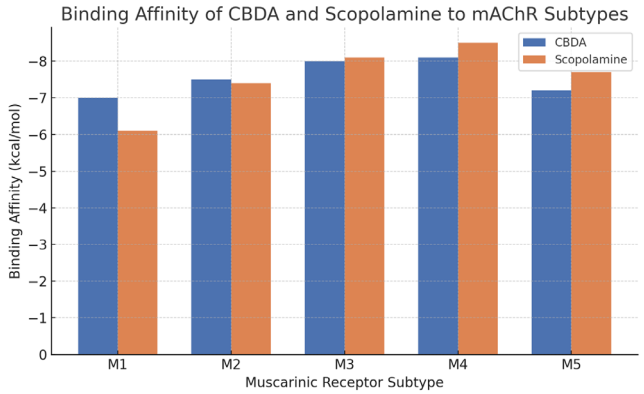


Figure 2. Comparative binding affinities of CBDA and NMS to mAChR subtypes (M1–M5) as predicted by molecular docking. CBDA shows stronger binding to M1 and M4, while NMS exhibits slightly higher affinity for M3 and M5.

At the M1 receptor, CBDA demonstrated a binding affinity of – 7.0 kcal/mol, which is notably higher than that of NMS, measured at – 6.1 kcal/mol. This indicates that CBDA exhibits well receptor binding compared to NMS at this specific receptor. The docking results demonstrated that CBDA exhibited stronger binding affinity to M1, M3, and M4 receptors, particularly M3 and M4. Since M1 and M3 subtypes have been implicated in the emetic reflex, stronger binding to these subtypes may indicate a potential antiemetic effect of CBDA. On the other hand, notable affinity for the M4 receptor, which is involved in dopaminergic and cholinergic modulation, may suggest broader therapeutic implications beyond emesis, possibly in neuropsychiatric or gastrointestinal disorders. Therefore, CBDA's differential binding profile highlights the potential for subtype-selective therapeutic targeting, which could reduce the side effects commonly associated with non-selective muscarinic antagonists like NMS.

In our previous study, the 5HT3A receptor showed a binding affinity of – 7.0 kcal/mol with CBDA (18). Similarly, in the current study, CBDA was observed to form both hydrophobic and hydrogen bonds with mAChRs receptors. When comparing the results, it is difficult to determine which receptor showed better efficacy with CBDA, as the binding affinity values are similar to those of the reference drugs used. Regarding dopamine (D2-D3) receptors, the docking study revealed binding affinities of – 7.8 kcal/mol and – 7.2 kcal/mol, respectively (18). In this study, CBDA was observed to form primarily hydrophobic interactions with both D2 and D3 receptors. Additionally, hydrogen bonds were observed for mAChR receptors, particularly at M1 and M3 receptors. Although these comparisons are not sufficient to definitively determine which receptor CBDA binds to more effectively, they do provide a preliminary basis for further investigation.

In addition, CBDA was observed to form strong hydrogen bonds at M1 and M3 receptors. It was also found to form good hydrophobic interactions between M1–M5 receptors. The effects of CBDA specifically at M1 and M3 receptors may contribute to the development of new treatments for vomiting or other disorders associated with mAChRs.

The binding affinity of CBDA compared to NMS suggests that it may exhibit similar interaction properties. However, functional effects cannot be concluded based on docking results alone. Furthermore, further investigation of factors such as side effects and bioavailability is necessary for clinical application. Additional pharmacokinetic and pharmacodynamic studies should be conducted to advance CBDA into clinical trials.

4. Conclusion

Considering the interactions of CBDA with mAChRs (M1-M5) revealed in this study, CBDA

may exhibit promising binding properties with muscarinic receptors. However, its pharmacological properties await further experimental validation. Molecular dynamics and *in vitro* studies are needed to better evaluate the results obtained in the present study.

Conflict of interest: The authors declare no conflict of interest.

Ethics approval: Not applicable

Funding: None

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Original Article

Identification of Natural Compounds as Potential FGFR2 Inhibitors in Cholangiocarcinoma via Virtual Screening and Network-Based Analysis

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Abstract

Introduction: Cholangiocarcinoma is an aggressive neoplasm of bile duct epithelial cells with poor prognosis due to limited treatment options. Fibroblast growth factor receptor 2 (FGFR2) is critical in cholangiocarcinoma by activating pathways such as MAPK/ERK and PI3K/AKT, marking it as a promising therapeutic target. This study aimed to identify natural FGFR2 inhibitors by using computational methods.

Methods: 46 natural compounds were selected from PubChem based on favorable physicochemical properties and drug-likeness criteria. Molecular docking was performed using SwissDock against FGFR2 (PDB ID: 4J97). The top five compounds were further assessed for pharmacokinetics, pharmacodynamics, and toxicity via SwissADME, pkCSM, and DeepPK tools. Additionally, protein-protein interaction networks and pathway enrichment analyses were conducted using the STRING database and KEGG.

Results: Docking analysis identified Rutecarpine, Palonosetron, Metribolone, 6-Ketoestradiol, and Gestrinone as the top FGFR2 inhibitors, with docking scores between – 6.34 and – 5.95 kcal/mol. ADMET predictions showed favorable drug-like properties, good bioavailability, and acceptable safety profiles. Network and pathway analyses confirmed FGFR2's role in key oncogenic pathways, including MAPK, PI3K/AKT, and Ras.

Conclusions: This study identified promising FGFR2 inhibitors, particularly Rutecarpine, as potential therapeutic candidates for cholangiocarcinoma, warranting further experimental validation.

Keywords: FGFR2, cholangiocarcinoma, molecular docking, natural compounds, ADMET analysis

1. Introduction

Cholangiocarcinoma (CCA), the cancer identified, is distinguished by its remarkably aggressive tendencies, originating from the bile ducts' epithelial surface, and is especially noted for its delayed identification, limited therapeutic possibilities, and unfavorable prognosis (1). In light of recent advancements, the five-year survival percentage is still worryingly low emphasizing the necessity for groundbreaking treatment targets and improved therapeutic strategies (1,2).

The fibroblast growth factor receptor 2 (FGFR2), is a receptor tyrosine kinase and critical for

overseeing cellular development, specialization, and the mechanism of angiogenesis (3). Genetic modifications in FGFR2, comprising gene fusions and rearrangements, have been recorded in nearly 10-16% of intrahepatic CCA cases, profoundly impacting cancer formation and the disease's evolution (4). These genetic modifications render FGFR2 a crucial therapeutic target for the management of CCA (5).

Upon interaction with its ligands, FGFR2 commences a variety of signaling cascades that are fundamental to tumorigenesis and its advancement. The exploration into this subject's pathway enrichment analysis has underscored crucial signaling routes linked to FGFR2 and its partners, notably the PI3K-Akt signaling route, the MAPK signaling route, the Rap1 signaling route, and the Ras signaling route. Imbalances within these pathways are extensively recorded to promote activities such as cellular growth, endurance, new blood vessel development, and tumor spread, confirming their significance in cancer therapy (6-14).

At present, several FGFR inhibitors, including pemigatinib and infigratinib, have received approval for clinical application in the context of FGFR2-altered CCA (15,16). However, the development of resistance and adverse side effects significantly curtail their therapeutic efficacy (17,18). Consequently, the identification of novel FGFR2 inhibitors with enhanced pharmacological properties remains of paramount importance.

Within this present investigation, we executed a unified in silico framework that involved molecular docking (SwissDock, Lausanne, Switzerland) (19-22), evaluations regarding absorption, distribution, metabolism, excretion, and toxicity (ADMET) utilizing SwissADME (Swiss Institute of Bioinformatics, Lausanne, Switzerland) (23), pkCSM (Cambridge Centre for Computational Chemical Engineering, Cambridge, UK) (24), and DeepPK (BioSIG Lab, University of Queensland, Brisbane, Australia) (25), along with protein-protein interaction (PPI) network analysis using the STRING database (Swiss Institute of Bioinformatics, Lausanne, Switzerland) to identify natural compounds with potential inhibitory activity against FGFR2[26]. The enrichment analysis provides valuable insights into the biological processes and signaling pathways involving FGFR2 interactions, enhancing our understanding of their potential therapeutic impacts in CCA (27-29).

Therefore, the primary aim of this study was to identify natural compounds with high binding affinity and favorable pharmacokinetic profiles as potential FGFR2 inhibitors for the treatment of CCA. Specifically, the objectives were to conduct molecular docking simulations in order to assess the binding potential of selected natural compounds against FGFR2, to evaluate their ADMET profiles using multiple computational tools, and to explore the biological significance of FGFR2 and its interactors through protein-protein interaction and pathway enrichment analyses. These efforts collectively aim to propose viable lead compounds that enhance further experimental validation for therapeutic development.

2. Methods

2.1. Study Design

This study utilized an integrated *in silico* approach combining molecular docking, pharmacokinetic prediction (ADMET), PPI network, and enrichment analysis to identify potential natural inhibitors targeting FGFR2.

2.2. Compound Selection

Natural compounds were retrieved from the PubChem database (National Center for Biotechnology Information, Bethesda, MD, USA) by applying a multi-parameter filtering strategy to ensure drug-likeness and optimal physicochemical properties suitable for oral bioavailability: molecular weight (280-330 g/mol), H-bond acceptors (≤3), H-bond donors (≤2), rotatable bonds (≤3), polar surface area (≤60 Å²), and XLogP (0-3). Molecular weight between 280-330 g/mol to fall within the range favorable for permeability and metabolic stability. Hydrogen bond acceptors ≤3 and hydrogen bond donors ≤2, to comply with the topological constraints required for membrane permeability and bioavailability, in accordance with Lipinski's Rule of Five Rotatable bonds ≤3, which reduces conformational flexibility and improves binding specificity. Polar surface area (PSA) ≤60 Å², as lower PSA is associated with better cell membrane permeability, especially for passive diffusion. XLogP between 0 and 3, which reflects a

balance between aqueous solubility and lipophilicity, critical for drug absorption and systemic distribution.

These criteria were selected based on widely accepted principles of medicinal chemistry and drug discovery. They are known to enhance the likelihood of identifying compounds with favorable ADME (Absorption, Distribution, Metabolism, and Excretion) profiles and were also supported by previously published studies that successfully applied similar thresholds in virtual screening campaigns (30-33).

2.3. Molecular Docking Analysis

The three-dimensional crystal structure of FGFR2 was retrieved from the Protein Data Bank (PDB ID: 4J97), representing the extracellular domain of FGFR2 in complex with FGF2. Molecular docking simulations were carried out using SwissDock (Swiss Institute of Bioinformatics, Lausanne, Switzerland), utilizing the EADock DSS engine integrated with AutoDock Vina scoring function. Docking calculations were performed across all four chains (A, B, C, and D) of the FGFR2 tetramer to capture all possible binding conformations (19-22).

The top-ranked binding poses were selected based on the fullfitness score and estimated binding free energy (ΔG, kcal/mol). For each docking result, the binding cavity and specific interaction residues were examined to determine pose validity and relevance. To validate the docking results, we utilized PoseView (ProteinsPlus, Bioinformatics Center, Hamburg, Germany) to generate 2D interaction diagrams, highlighting key residues involved in ligand binding. Furthermore, DoGSiteScorer (ProteinsPlus, Bioinformatics Center, Hamburg, Germany) was employed to assess the druggability of the identified binding pockets, analyzing their size, shape, and physicochemical properties. These tools have been validated in previous studies, demonstrating their effectiveness in predicting binding interactions and assessing pocket druggability (34-37). Interaction profiling, including hydrogen bonds, hydrophobic interactions, π-stacking, and electrostatic contacts, was performed using the PoseView tool, which provided detailed 2D schematic diagrams of protein-ligand contacts (38-41).

In particular, Rutecarpine, the top-scoring molecule, was further analyzed for binding pose validation using DoGSiteScorer to confirm its positioning within a druggable cavity of FGFR2 and to evaluate the geometric fit of the ligand. This approach enabled precise identification of key residues (e.g., Glu565B, Leu487B, Leu633B, Ala567B, Asn571B, Arg630B) involved in the stabilization of the ligand-receptor complex, which are critical for rational drug design and understanding molecular mechanisms of inhibition (37,42).

2.4. ADMET Prediction

ADMET properties of the top five candidate molecules were analyzed by using SwissADME (Swiss Institute of Bioinformatics, Lausanne, Switzerland), pkCSM (University of Melbourne, Melbourne, Australia), and Deep-PK software (University of Queensland, Brisbane, Australia) (23–25). Predicted parameters included gastrointestinal (GI) absorption, blood-brain barrier (BBB) permeability, P-glycoprotein (P-gp) substrate and inhibition, cytochrome P450 (CYP) interactions, hepatotoxicity, and general toxicity.

2.5. Protein-Protein Interaction Network Analysis

The PPI network analysis of FGFR2 was performed using the STRING database version 11.5 (ELIXIR, Zürich, Switzerland), which integrates known and predicted protein interactions. Proteins with interaction scores above 0.7 were considered significant and included for further enrichment analysis (26).

2.6. Enrichment Analysis

KEGG (Kyoto Encyclopedia of Genes and Genomes) pathway enrichment analysis was conducted on the significant interactors obtained from STRING by using Enrichr (Icahn School of Medicine at Mount Sinai, New York, NY, USA). Pathways were considered significantly enriched with an adjusted p-value less than 0.05 (27-29).

2.7. Data Analysis

Docking scores and ADMET properties were statistically analyzed to discern significant differences among top-ranking compounds. ANOVA followed by Tukey's post-hoc test demonstrated statistically significant differences in binding affinities ($p<0.05$), notably emphasizing Rutecarpine's superior docking performance compared to other evaluated candidates

Docking affinity scores and ADMET predictions were compared and ranked to identify the most promising FGFR2 inhibitors. Data visualization and statistical analyses were performed by using Microsoft Excel (Microsoft Corporation, Redmond, WA, USA) with in-built statistical functions and charting tools.

3. Results

3.1 Molecular Docking Analysis

The molecular docking analysis identified several natural compounds with significant binding affinities towards the FGFR2 active site. The docking scores of the top five compounds are summarized in Table 1. The compound Rutecarpine demonstrated the strongest binding affinity (-6.348 kcal/mol), followed by Palonosetron (-6.223 kcal/mol), Metribolone (-6.032 kcal/mol), 6-Ketoestradiol (-6.029 kcal/mol), and Gestrinone (-5.959 kcal/mol). Fig 1 specifically illustrates the optimal binding mode and detailed interaction profile of Rutecarpine within the FGFR2 active site.

Table 1. Docking scores and binding affinities of the 46 natural compounds screened as potential FGFR2 inhibitors.

	Molecule Names	Best Score	Top 5 Models (Calculated Affinity (kcal/mol))
1	Rutaecarpine	-6,348	-6.348, – 6.065, – 6.014, – 5.989, – 5.945
2	Palonosetron	-6,223	-6.223, – 6.153, – 5.764, – 5.403, – 5.339
3	Metribolone	-6,032	-6.032, – 5.960, – 5.631, – 5.581, – 5.436
4	6-Ketoestradiol	-6,029	-6.029, – 5.523, – 5.515, – 5.464, – 5.396
5	Gestrinone	-5,959	-5.959, – 5.266, – 5.034, – 5.002, – 4.958
6	Adrenosterone	-5,839	-5.839, – 5.590, – 5.493, – 5.297, – 5.287
7	Trenbolone Acetate	-5,826	-5.826, – 5.448, – 5.433, – 5.336, – 5.294
8	Tibolone	-5,808	-5.808, – 5.358, – 5.333, – 5.167, – 4.972
9	Androstenedione	-5,788	-5.788, – 5.688, – 5.441, – 5.167, – 5.162
10	Ondansetron	-5,764	-5.764, – 5.400, – 5.347, – 5.170, – 5.108
11	Praziquantel	-5,748	-5.748, – 5.681, – 5.678, – 5.667, – 5.632
12	Norethindrone	-5,691	-5.691, – 5.180, – 5.008, – 4.949, – 4.934
13	Gelsemine	-5,674	-5.674, – 5.415, – 5.296, – 5.254, – 5.239
14	16alpha,17-Epoxyprogesterone	-5,661	-5.661, – 5.453, – 5.255, – 5.186, – 5.107
15	Midazolam	-5,645	-5.645, – 5.430, – 5.320, – 5.203, – 5.199
16	Norquetiapine	-5,616	-5.616, – 5.578, – 5.483, – 5.314, – 5.185
17	Eburnamonine	-5,614	-5.614, – 5.430, – 5.412, – 5.362, – 5.352
18	Indoprofen	-5,592	-5.592, – 5.378, – 5.247, – 5.235, – 5.224
19	Boldione	-5,584	-5.584, – 5.581, – 5.340, – 5.253, – 5.175
20	Nile Blue Cation	-5,576	-5.576, – 5.476, – 5.411, – 5.401, – 5.276
21	4-Androsten-3β-Ol-17-One	-5,574	-5.574, – 5.405, – 5.401, – 5.285, – 5.044
22	Formestane	-5,568	-5.568, – 5.395, – 5.393, – 5.263, – 5.243
23	Lerisetron	-5,542	-5.542, – 5.486, – 5.467, – 5.436, – 5.320
24	Altrenogest	-5,535	-5.535, – 5.515, – 5.348, – 5.340, – 5.340
25	Gestodiene	-5,494	-5.494, – 5.341, – 5.018, – 4.968, – 4.961
26	Zolpidem	-5,491	-5.491, – 5.439, – 5.279, – 5.139, – 4.998
27	Cinchonine	-5,486	-5.486, – 5.306, – 5.249, – 5.132, – 5.072
28	19-Hydroxyandrost-4-Ene-3,17-Dione	-5,427	-5.427, – 5.288, – 5.099, – 4.968, – 4.892
29	Cinchonidine	-5,321	-5.321, – 5.130, – 5.116, – 5.049, – 4.961
30	4-Bromo-2,2':6',2''-Terpyridine	-5,321	-5.321, – 5.203, – 5.181, – 5.009, – 4.995
31	Granisetron	-5,307	-5.307, – 5.288, – 5.204, – 4.967, – 4.946
32	Alprazolam	-5,266	-5.266, – 5.257, – 5.147, – 5.098, – 5.071
33	2-(Chloromethyl)-3-(4-Fluorophenyl)Quinazolin-4(3H)-One	-5,24	-5.240, – 5.138, – 5.067, – 4.958, – 4.877
34	1,3-Dicyclohexylbarbituric Acid	-5,225	-5.225, – 5.037, – 4.981, – 4.969, – 4.950
35	Mazindol	-5,196	-5.196, – 5.114, – 4.988, – 4.935, – 4.877
36	1-(6-Chloro-2-Hydroxy-4-Phenylquinolin-3-Yl)Ethanone	-5,185	-5.185, – 4.994, – 4.894, – 4.855, – 4.848
37	1,4-Dibenzoylpiperazine	-5,128	-5.128, – 5.109, – 5.059, – 5.057, – 5.052
38	Methylene Blue Cation	-4,778	-4.778, – 4.705, – 4.685, – 4.616, – 4.603
39	Demoxepam	-4,513	-4.513, – 4.336, – 4.202, – 4.188, – 4.181

40	2-(3-Bromo-2-Oxopropyl)Isoindoline-1,3-Dione	-4,491	-4.491, – 4.462, – 4.348, – 4.290, – 4.247
41	Norfludiazepam	-4,439	-4.439, – 4.313, – 4.074, – 4.001, – 3.952
42	3,5-Dibromo-2-Hydroxybenzoic Acid	-4,118	-4.118, – 4.093, – 3.926, – 3.898, – 3.896
43	3,5-Dibromo-2-Methoxybenzoic Acid	-3,938	-3.938, – 3.931, – 3.852, – 3.813, – 3.766
44	5-Iodo-A-85380	-3,938	-3.938, – 3.926, – 3.848, – 3.692, – 3.636
45	1,3-Dibromo-5,5-Dimethylhydantoin	-3,567	-3.567, – 3.513, – 3.481, – 3.434, – 3.431
46	Tribromoacetic Acid	-2,994	-2.994, – 2.971, – 2.960, – 2.853, – 2.845

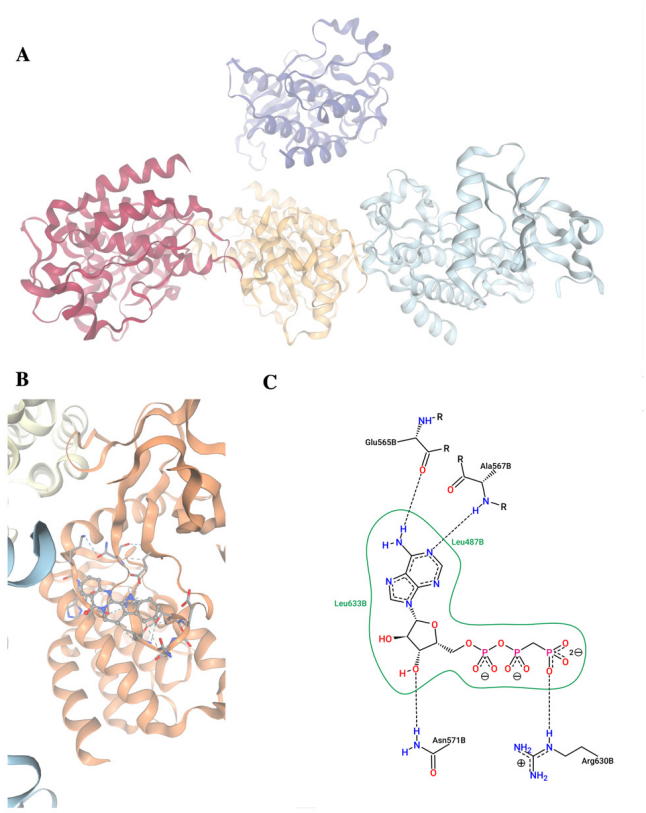

Figure 1. Molecular docking and interaction profile of Rutecarpine with FGFR2 protein (PDB ID: 4J97). A. Three-dimensional structure of the FGFR2 protein tetramer composed of four distinct chains (Chain A: red, Chain B: beige, Chain C: purple, Chain D: blue). B. Three-dimensional representation of Rutecarpine bound within the active site of FGFR2, highlighting hydrogen bonds (blue dashed lines) and hydrophobic interactions (gray dashed lines). C. Detailed two-dimensional interaction map generated by PoseView analysis, illustrating specific amino acid interactions of Rutecarpine with FGFR2. Hydrogen bonds are depicted by black dashed lines, and hydrophobic interactions are represented by green lines. Key interacting amino acid residues (Glu565, Ala567, Leu487, Leu633, Asn571, Arg630) are clearly indicated.

This comprehensive table includes the names of candidate molecules, their best docking scores (expressed in kcal/mol), and the energies of their top five docking poses. Docking simulations were performed by using SwissDock, and the binding energies reflect the stability and strength of interactions between the ligands and the FGFR2 active site, with lower (more negative) values indicating higher affinity. The compounds were ranked from strongest to weakest binding affinity.

Rutecarpine, the top-ranked compound based on docking analysis, exhibited robust interactions within the FGFR2 active site. Detailed visualization of the molecular interactions revealed critical residues involved in binding. Specifically, Rutecarpine formed hydrogen bonds with Glu565, Ala567, Asn571, and Arg630, while establishing hydrophobic contacts with Leu487 and Leu633 (Fig 1C). These interactions underscore Rutecarpine's potential efficacy as an FGFR2 inhibitor, warranting further investigation in preclinical studies.

3.2 ADMET Profiling

Comprehensive ADMET predictions were conducted for the top five compounds using SwissADME, pkCSM, and Deep-PK tools. Results from these analysis were summarized in Table 2. Palonosetron and Rutecarpine showed notably high GI absorption and favorable BBB permeability, indicative of promising pharmacokinetic profiles. Additionally, all top candidates conformed to Lipinski's rule of five, suggesting potential for good oral bioavailability.

Table 2. Updated ADMET Profile Summary of the Top 5 Compounds (Rutecarpine, Palonosetron, Metribolone, 6-Ketoestradiol, Gestrinone).

ADMET Properties	Rutecarpine	Palonosetron	Metribolone	6-Ketoestradiol	Gestrinone
Lipinski Rule Compliance	Yes	Yes	Yes	Yes	Yes
GI Absorption	High	High	High	High	High
BBB Permeability	Yes	Yes	Yes	Yes	Yes
Caco-2 Permeability (log Papp, 10^{-6} cm/s)	1.26	1.12	1.57	1.27	1.64
Water Solubility (log mol/L)	-3.46	-2.64	-4.15	-3.60	-4.46
P-glycoprotein Substrate	Yes	Yes	No	No	No
CYP450 Enzyme Inhibition	CYP1A2, CYP2D6, CYP3A4	CYP2D6	CYP2C19	CYP2D6	CYP2C19, CYP2C9, CYP3A4
AMES Toxicity	Yes	No	No	No	No
Hepatotoxicity	Yes	Yes	No	No	No
Skin Sensitisation	No	No	No	No	No
Max. Tolerated Dose (human, log mg/kg/day)	0.068	-0.453	-0.135	-0.544	-0.395
Synthetic Accessibility	2.78	4.37	4.92	3.60	5.26

The table summarizes the pharmacokinetic (ADMET) profiles of the top five selected compounds: Rutecarpine, Palonosetron, Metribolone, 6-Ketoestradiol, and Gestrinone. ADMET parameters, including Lipinski Rule compliance, GI absorption, BBB permeability, Caco-2 permeability, water solubility, interactions with drug transporters (P-glycoprotein), metabolic enzyme inhibition (CYP450 enzymes), and toxicity profiles (AMES toxicity, hepatotoxicity, skin sensitization, maximum tolerated dose), were evaluated by using integrated computational tools (SwissADME, pkCSM, and Deep-PK). These data provide valuable insights into their potential suitability as drug candidates, focusing on efficacy, pharmacological profile, safety, and synthetic accessibility.

3.3 Protein-Protein Interaction and Enrichment Analysis

Protein-protein interaction network analysis via STRING revealed nine proteins significantly interacting with FGFR2, namely FGF1, FGF2, FGF7, FGF8, FGF9, FGF10, FGFR3, GRB2, and PLCG1 (Fig 2). Pathway enrichment analysis utilizing KEGG pathways further highlighted critical involvement in MAPK signaling, PI3K-Akt signaling, and Ras signaling pathways, which were summarized in Table 3.

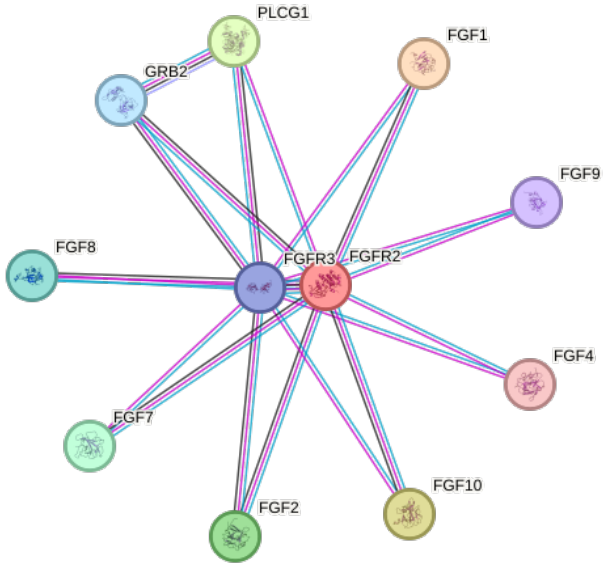

Figure 2. Protein-Protein Interaction Network Analysis of FGFR2. The interaction network demonstrates significant protein interactions involving FGFR2. Notably, strong connections were observed between FGFR2 and various fibroblast growth factors (FGFs), including FGF1, FGF2, FGF7, FGF8, FGF9, and FGF10. Additionally, FGFR2 interactions with FGFR3, another receptor involved in related signaling pathways, and essential adaptor or signaling proteins such as GRB2 and PLCG1, were illustrated. The analysis was conducted using the STRING database, in which nodes represent proteins, edges represented protein-protein interactions, and thicker lines indicate stronger associations. These interactions highlight critical signaling pathways potentially impacted by FGFR2 inhibition.

Table 3. KEGG Pathway Enrichment Analysis of FGFR2-Interacting Proteins.

Pathway	Overlap	P-value	Adjusted P-value	Genes
Ras signaling	9/232	3.25E-18	2.60E-16	FGF7, FGF8, FGF9, GRB2, PLCG1, FGF1, FGF2, FGFR3, FGF10
Rap1 signaling pa	8/210	1.15E-15	4.61E-14	FGF7, FGF8, FGF9, PLCG1, FGF1, FGF2, FGFR3, FGF10
Calcium signaling	8/240	3.41E-15	9.09E-14	FGF7, FGF8, FGF9, PLCG1, FGF1, FGF2, FGFR3, FGF10
Pathways in cancer	9/531	6.13E-15	1.23E-13	FGF7, FGF8, FGF9, GRB2, PLCG1, FGF1, FGF2, FGFR3, FGF10
MAPK signaling	8/294	1.76E-14	2.82E-13	FGF7, FGF8, FGF9, GRB2, FGF1, FGF2, FGFR3, FGF10
Breast cancer	7/147	3.57E-14	4.49E-13	FGF7, FGF8, FGF9, GRB2, FGF1, FGF2, FGF10
Gastric cancer	7/149	3.93E-14	4.49E-13	FGF7, FGF8, FGF9, GRB2, FGF1, FGF2, FGF10
PI3K-Akt signaling	8/354	7.89E-14	7.89E-13	FGF7, FGF8, FGF9, GRB2, FGF1, FGF2, FGFR3, FGF10
Melanoma	6/72	1.46E-13	1.30E-12	FGF7, FGF8, FGF9, FGF1, FGF2, FGF10
Regulation of actin cytoskeleton	7/218	5.86E-13	4.69E-12	FGF7, FGF8, FGF9, FGF1, FGF2, FGFR3, FGF10

The table summarizes the significantly enriched KEGG pathways associated with proteins interacting with FGFR2, including FGF1, FGF2, FGF7, FGF8, FGF9, FGF10, FGFR3, GRB2, and PLCG1. Pathways were ranked according to statistical significance (p-value). The analysis highlights critical signaling pathways such as MAPK, Ras, PI3K-Akt, and Rap1, which are involved in cancer progression and cellular proliferation. The enrichment analysis was performed using Enrichr software.

Docking scores and ADMET properties were statistically analyzed to discern significant differences among top-ranking compounds. ANOVA followed by Tukey's post-hoc test demonstrated statistically significant differences in binding affinities ($p<0.05$), notably emphasizing Rutecarpine's superior docking performance compared to other evaluated candidates (Fig 3).

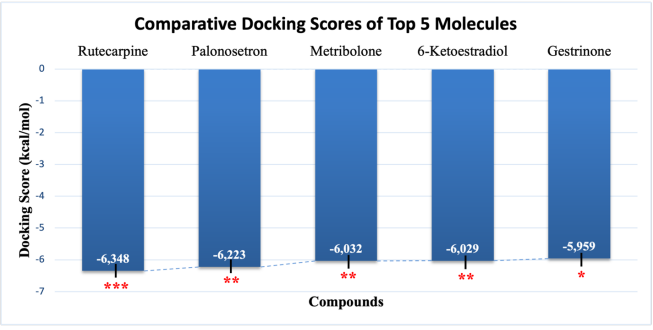

Figure 3. Comparative Docking Scores of Top Five Molecules Against FGFR2. Docking scores for Rutecarpine, Palonosetron, Metribolone, 6-Ketoestradiol, and Gestrinone are displayed with respective standard deviations. Statistical significance (ANOVA, Tukey's post-hoc test) is indicated by red asterisks (* $p<0.05$, ** $p<0.01$, *** $p<0.001$). Rutecarpine exhibited significantly stronger binding affinity compared to other evaluated molecules, highlighting its superior potential as an FGFR2 inhibitor candidate.

4. Discussion

This study provides a comprehensive in silico evaluation of natural compounds as potential FGFR2 inhibitors for CCA, an aggressive cancer with limited therapeutic options and poor prognosis (1,2). FGFR2 has emerged as a critical oncogenic driver, with gene fusions and amplifications contributing to CCA pathogenesis in approximately 10-16% of cases (3-5).

Our molecular docking analysis identified Rutecarpine as the lead compound, exhibiting the highest binding affinity (-6.348 kcal/mol). Detailed analysis of Rutecarpine's binding mode revealed stable interactions within the FGFR2 active site, including hydrogen bonds with key residues such as Glu565, Ala567, Asn571, and Arg630, and hydrophobic contacts involving Leu487 and Leu633. These interactions, distributed across multiple chains of the FGFR2 tetramer, suggest a robust inhibition mechanism that could effectively disrupt FGFR2-mediated oncogenic signaling, potentially enhancing therapeutic outcomes.

The application of validated computational tools such as PoseView and DoGSiteScorer enhances the credibility of our *in silico* findings. PoseView's detailed interaction mappings provide insights into the specific residues involved in ligand binding, while DoGSiteScorer's assessment of pocket druggability offers a quantitative evaluation of the binding site's suitability for therapeutic targeting. The reliability of these tools has been established in prior research, supporting their integration into our study's methodology.

By incorporating these validated methods, we have strengthened the robustness of our computational analysis, providing a solid foundation for the proposed therapeutic potential of the identified compounds.

ADMET predictions further support the drug-likeness of Rutecarpine. Its high gastrointestinal absorption and favorable pharmacokinetic properties make it a strong candidate for oral administration. However, hepatotoxicity and AMES toxicity risks warrant caution and suggest the need for structural modifications or further preclinical studies to ensure safety.

When compared to previous research, our findings align with the well-established principle that natural products are valuable scaffolds for drug discovery, particularly in oncology. Palonosetron, a clinically approved antiemetic, also showed favorable ADMET profiles and significant binding to FGFR2, suggesting a potential repositioning opportunity for targeting CCA.

The protein-protein interaction network analysis revealed FGFR2's association with key oncogenic signaling mediators such as FGFR3, GRB2, and PLCG1, as well as with multiple fibroblast growth factors. KEGG pathway enrichment analysis highlighted critical pathways (MAPK, PI3K-Akt, Ras, and Rap1), all of which are commonly dysregulated in CCA and other solid tumors (6-14). The dual targeting of FGFR2 and its interactors could enhance therapeutic efficacy by disrupting tumor-promoting pathways and reducing the potential for resistance.

While FGFR inhibitors like pemigatinib and infigratinib have advanced clinical outcomes, their use is often hampered by resistance development and adverse events (15-18). Rutecarpine's multi-site binding profile within the FGFR2 tetramer may overcome some of these limitations by stabilizing FGFR2 in an inactive conformation or preventing dimerization-dependent activation.

Strengths of this study include the integration of molecular docking, ADMET profiling, PPI network analysis, and pathway enrichment, which collectively provide a holistic understanding of Rutecarpine's potential. However, limitations must also be acknowledged: (i) in silico predictions require validation through in vitro and in vivo studies, (ii) predicted hepatotoxicity raises concerns about safety, and (iii) dynamic factors such as metabolic biotransformation were not directly modeled.

Future studies should focus on experimental validation of Rutecarpine's inhibitory effect on FGFR2 phosphorylation, downstream signaling suppression, and cytotoxicity in CCA cell lines. *In vivo* pharmacokinetic studies will also be critical to confirm absorption, distribution, metabolism, and excretion properties.

This study identifies Rutecarpine as a promising FGFR2 inhibitor with significant potential for further drug development targeting CCA. The results of this study contribute to the growing body of evidence supporting natural compounds as valuable sources for anticancer therapeutics and underscore the importance of targeting FGFR2-mediated signaling in CCA.

5. Conclusion

In this study, we successfully identified and characterized natural compounds with promising inhibitory potential against FGFR2, a significant molecular target implicated in CCA progression. Rutecarpine emerged as the most potential candidate, exhibiting the highest binding affinity and favorable pharmacokinetic and toxicity profiles. Additional compounds, including Palonosetron, Metribolone, 6-Ketoestradiol, and Gestrinone, also demonstrated considerable inhibitory potential. Protein-protein interaction network analysis and KEGG pathway enrichment indicated that these compounds could broadly modulate oncogenic signaling pathways, such as MAPK, PI3K-Akt, and Ras signaling, reinforcing their therapeutic relevance. These findings provide robust evidence supporting the advancement of Rutecarpine and other identified compounds into further preclinical and clinical studies, potentially contributing to more effective and safer therapeutic strategies for CCA.

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Original Article

Effects of Geographical Origin and Extraction Methods on the Phenolic Content of *Salvia officinalis*

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Abstract

Introduction: *Salvia officinalis L.*, also known as sage, is a widely used medicinal plant known for its rich phenolic content and associated health benefits. It is known that the phenolic profile of sage varies depending on the geographical origin and extraction method. This study aimed to evaluate the phenolic acid composition of S. officinalis samples collected from two geographical locations, Jordan and Isparta-Türkiye, using three extraction techniques.

Methods: Dried plant materials were extracted using infusion, decoction, and organic solvent extraction (methanol/acetonitrile) and the phenolic content was analyzed analyzed using high-performance liquid chromatography with photodiode array detection (HPLC-PDA). Here, 15 phenolic compounds were screened and evaluated.

Results: In this study, rosmarinic acid appeared as the predominant compound in decoction extracts from both locations (19,442 mg/g in Jordan and 17,485 mg/g in Türkiye, respectively), while epicatechin was the major component in infusion extracts. Organic solvent extracts showed moderate levels of phenolics, with rosmarinic acid being the most abundant.

Conclusions: Both geographical origin and extraction technique affected the phenolic content of *S. officinalis*. The decoction method was found to be the most effective extraction method for phenolic compounds, indicating superior efficacy in maximizing the health-promoting properties of the plant.

Keywords: Extraction, Salvia officinalis, HPLC, phenolic compounds

1. Introduction

The genus Salvia L., encompassing over 900 species, is considered the largest genus in the family Lamiaceae. It is distributed worldwide in both subtropical and temperate regions. In Türkiye, Salvia is represented by 86 different species, some of which are perennial and exhibit shrubby or semi-shrub growth forms(1).

Salvia officinalis (*S. officinalis*) holds a significant place in traditional medicine for its health-promoting properties and its therapeutic applications in the treatment of various ailments. Historically, sage has been recommended in the treatment of coughs, as a diuretic, as an emmenagogue, as a wound healer, for managing ulcers, and for maintaining oral health (2). Today, *S. officinalis* continues to be widely used across the world, maintaining its esteemed status in traditional medicine. Recent scientific studies have confirmed that *S. officinalis* possesses antidiabetic (3), antioxidant, gastroprotective (4), anti-inflammatory

(5), antiviral (6), anti-obesity (5), antispasmodic (7), fungicidal and bactericidal (8), and anticarcinogenic (9) activities. Moreover, *S. officinalis* finds wide application in the preparation and preservation of food products (10) as well as serving as a flavoring agent in the perfume and cosmetic industries (8).

It is known that the leaves of *S. officinalis* contain tannic acid, oleic acid, ursonic acid, ursolic acid, carnosol, carnosic acid, fumaric acid, chlorogenic acid, caffeic acid, nicotinamide, flavones, flavonoid glycosides, and estrogenic substances (11). Additionally, the flowers, leaves, and stems of *S. officinalis* contain alkaloids, carbohydrates, fatty acids, glycosidic derivatives, phenolic compounds (such as coumarins, flavonoids, and tannins), polyacetylenes, steroids, terpenes/terpenoids, and waxes (12-14). It is particularly rich in flavonoids, especially rosmarinic acid and luteolin-7-glucoside (15). This phenolic-rich profile underlies sage's potent antioxidant properties and medicinal uses.

The phenolic composition of sage can vary significantly depending on the environmental conditions and the plant's geographical origin. As with other medicinal plants, the phytochemical profile of *S. officinalis* is influenced by factors such as climate, soil characteristics, and altitude (16). In addition to this, the yield and profile of phenolic compounds obtained from *S. officinalis* vary significantly depending on the method of extraction (infusion, decoction, or the use of organic solvents). In general, organic solvent extracts were found to provide higher total phenolic content and antioxidant activity compared to aqueous infusions (17).

This study aimed to determine the phenolic acid content of *S. officinalis* plants from different geographical origins using different extraction methods.

2. Methods

2.1 Plant Materials

The *S. officinalis* L. samples used in this study were freshly collected from different geographical regions. One sample was obtained from an area in Jordan, while the other was gathered from Isparta province, Türkiye. Both samples were harvested during the flowering stage of the plant, in the early morning hours, to minimize exposure to direct sunlight and preserve the integrity of the plant tissue. The fresh collected plant materials (leaves and stems) were subjected to a drying process under controlled conditions to maintain sample integrity and ensure the stability of the phenolic compounds.

2.2 Preparation of Extraction

The dried *S. officinalis* leaves were extracted using three different techniques: infusion, decoction, and organic solvent extraction. For each extraction, 200 mg of plant material was weighed and transferred to separate tubes and then 4 mL of the relevant solvent was added. For organic solvent extraction, methanol/acetonitrile was used in a 1:1 ratio. It was subjected to an overnight shaking process utilizing an orbital shaker. During the decoction method, 4 mL of distilled water was added to each plant material, and then it was heated in a boiling water bath for 15 minutes. In the infusion method, 4 mL of boiling water was added to the plants and the contents were left to infuse at room temperature for 15 min.

2.3 Analysis of phenolic compounds

Quantitative analysis was performed using a Shimadzu Nexera-i LC-2040C 3D Plus HPLC system. A diode array detector (DAD) was employed, with detection carried out at 254 nm. During chromatographic separation, a reversed-phase Phenyl-Hexyl column (4.6 × 150 mm, 3 µm; GL Sciences Intersustain, Japan) was used. The mobile phase consisted of 0.1% formic acid in deionized water (Solvent A) and acetonitrile (Solvent B), both of HPLC grade (Merck), applied according to the gradient program presented in Table 1. The mobile phase flow rate was set at 1.0 mL/min throughout the analysis. Both samples and standards were injected at a volume of 10 µL and the column temperature was maintained at 30 °C.

Individual phenolic compound contents of each extract used in the study were screened for 15 standard phenolic compounds. The analysis of these 15 standards, which were detected quantitatively in the extracts were performed separately. These standard compounds were: Vanillic acid, caffeic acid, epicatechin, p-coumaric acid, salicylic acid, cinnamic acid, rosmarinic acid, quercetin, chlorogenic acid, apigenin-7-O-glucoside, rutin, naringenin, 4-hydroxybenzoic acid, gallic acid and ferulic acid.

Table 1. Gradient pump program of mobile phase

Step	Flow Rate (mL/min)	Time (min)	Solvent B %	Solvent A %
1	1.00	0.01	5	95
2	1.00	7.00	9.5	90.5
3	1.00	20.00	17	83
4	1.00	35.00	40	60
5	1.00	40.00	100	0
6	1.00	40.01	Stop	—

3. Results

Using the method described in Table 1, fifteen phenolic compounds were analyzed. The maximum absorbance wavelength for each phenolic compound was determined, and all compounds were scanned at their respective maximum wavelengths (Figure 1).

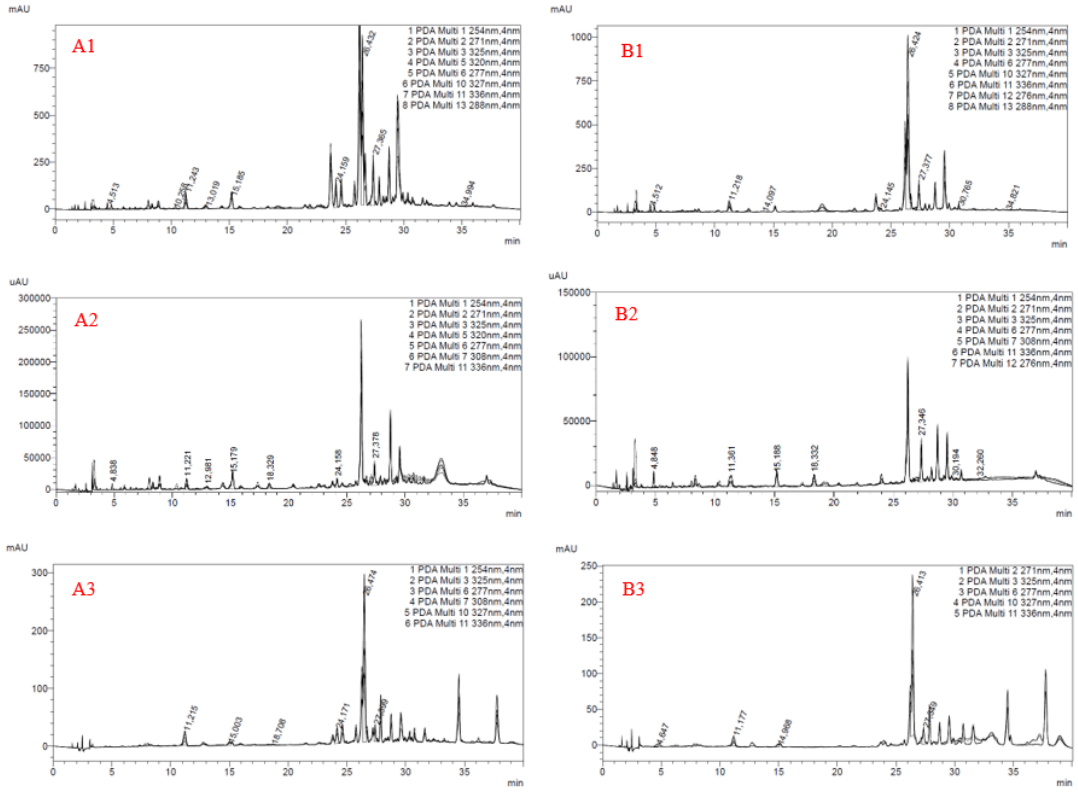

Figure 1. HPLC chromatogram of different extracts of *S. officinalis; **A:** Isparta **B:** Jordan* (1: Decoction, 2: Infusion, 3: Methanol/Acetonitrile).

Table 2. The amounts of phenolic compounds determined in different extracts of *S. officinalis* leaves (mg/g dry plant)

	Compounds	Retention Time (min)	Decoction		Infusion		Methanol/Acetonitrile	
			Isparta	**Jordan**	**Isparta**	**Jordan**	**Isparta**	**Jordan**
1	Gallic acid	4.352	0.115±0,007	0.139±0,002	0.042±0,003	0.051±0,003	-	0.032±0,004
2	4-Hydroxybenzoic acid	10.217	-	-	-	-	-	-
3	Chlorogenic acid	12.073	**0.631**±0,007	**0.587**±0,009	0.119±0,004	0.095±0,006	0.243±0,009	0.129±0,005
4	Vanillic acid	12.437	-	-	-	-	-	-
5	Caffeic acid	12.850	0.129±0,003	-	0.038±0,004	-	-	-
6	Epicatechin	14.150	**2.423**±0,257	0.026±0,002	**0.779**±0,023	**0.293**±0,008	0.214±0,012	0.123±0,006
7	p-Coumaric acid	18.486	-	-	0.031±0,001	0.031±0,005	0.006±0,001	-
8	Ferulic acid	20.971	-	-	-	-	-	-
9	Salicylic acid	21.929	-	-	-	-	-	-
10	Rutin	23.494	1.249±0,009	0.169±0,003	0.134±0,004	-	0.305±0,009	-
11	Rosmarinic acid	26.857	**17.485**±0,793	**19.442**±0,549	-	-	**5.611**±0,074	**4.392**±0,390
12	Apigenin-7-glucoside	27.574	1.142± 0,030	0.709± 0,010	0.153±0,002	0.126±0,006	0.111±0,010	0.094±0,006
13	Cinnamic acid	30.234	-	0.038± 0,005	-	0.001±0,0002	-	-
14	Quercetin	32.008	-	-	-	0.004±0,0003	-	-
15	Naringenin	34.939	0.013± 0,001	0.004±0,0003	-	-	-	-

Qualitative and quantitative analysis of 15 phenolic compounds obtained from different extracts of *S. officinalis* grown in various geographical locations was performed by HPLC-DAD (Table 2). The quantity of each compound was ascertained in milligrams (mg) per gram (g) of dry plant material. According to our HPLC data, rosmarinic acid was found to have the highest concentration in the decoction extracts of the plants grown in both Isparta and Jordan (17.485 mg/g and 19.442 mg/g, respectively). In addition, although rosmarinic acid was the highest phenolic compound in organic solvent extracts (5.611 mg/g and 4.392 mg/g, respectively), it could not be detected in infusion extracts. Epicatechin was found to be the highest phenolic compound in the infusion extracts. In all extracts, p-coumaric acid, cinnamic acid, quercetin, and naringenin were minor components.

4. Discussion

It is known that *S. officinalis* cultivated in different regions or under varying environmental conditions may exhibit distinct phenolic profiles. Genetic differences—such as different cultivars or closely related species—also contribute to this chemotypic diversity. This has been demonstrated in a study comparing various Salvia taxa: although rosmarinic acid was the dominant phenolic compound in all cases, *Salvia africana*, a species native to South Africa, was rich in unique caffeic acid dimers that accounted for approximately 40% of the total phenolics. In contrast, the cultivated variety *S. officinalis "Icterina"* was particularly abundant in flavone glycosides, including glycosides of apigenin, luteolin, and scutellarein (18). It has also been emphasized that the time of harvest significantly affects the levels of phenolic compounds, even in infusion preparations (19).

In this study, we have found notable differences in the phenolic compound content of *S. officinalis* grown under different climatic conditions. Rosmarinic acid was the dominant compound across all samples, with the highest concentrations found in decoctions (Isparta: 17.485 ± 0.793 mg/g DW; Jordan: 19.442 ± 0.549 mg/g DW), consistent with previous findings (20,21). Due to its high polarity and water solubility, it is expected to be efficiently extracted by water-based techniques (22). The investigation revealed that the second major compound was epicatechin. Epicatechin content showed one of the starkest geographic contrasts: Isparta decoction samples yielded 2.423 ± 0.257 mg/g DW, while Jordan samples contained only 0.026 ± 0.002 mg/g DW. This dramatic difference may reflect environmental influences such as altitude, UV exposure, or temperature, which can significantly impact the biosynthesis of flavonoids (23).

It is acknowledged that the yield of phenolic compounds is influenced by the employed extraction methods. For example, to recover chlorogenic acid efficiently traditional extraction methods such as fresh tissue homogenization and leaf decoction have been used (24). Additionally, the use of deep eutectic solvents (DESs) has been explored for extracting phenolic compounds, including chlorogenic acid, from *S. officinalis* (25).

In the current study, chlorogenic acid was efficiently extracted via decoction (0.631 ± 0.007 mg/g DW in Isparta), consistent with its thermal stability and high solubility in hot water. Likewise, apigenin-7-glucoside and rutin were better extracted by decoction and methanol/acetonitrile, likely due to their mid-polarity and affinity for both water and organic solvents. Rutin levels, for example, reached 1.249 ± 0.009 mg/g DW in the Isparta decoction samples compared to only 0.169 ± 0.003 mg/g DW in the Jordan sample. These findings are consistent with the high solubility of these compounds in mid-polar solvents and extended heat exposure, which enhances flavonoid release from cellular matrixes (26). Some phenolics, including 4-hydroxybenzoic acid, ferulic acid, and salicylic acid, were not detected in any of the extracts. These compounds may exist in trace amounts or in conjugated forms not readily released without hydrolysis, as previously suggested in phytochemical surveys of Salvia species (22).

In summary, this study demonstrates that both geographic origin and extraction technique significantly influence the phenolic profile of *S.*

officinalis. The decoction method was found to be the most effective extraction approach in terms of both yield and diversity of phenolic compounds. These findings affirm that solvent polarity, extraction temperature, and duration play critical roles in the efficiency of polyphenol extraction from medicinal plants.

5. Conclusion

It is acknowledged that the chemical composition of plants cultivated in disparate geographical regions is influenced by factors such as climate and soil composition. This study, which utilized sage collected from two different geographical locations, revealed that the presence of phenolic compounds was more prevalent than in other extracts. However, minor variations were observed in the decoction extracts. Sage is a plant that is utilized by the public. In the present study it was demonstrated that sage is more efficacious in terms of phenolic compound content when boiled before consumption.

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Ethics approval: Not applicable

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Original Article

Investigation of the Antiproliferative and Genotoxic Effects of *Serenoa repens* (W. Bartram) Small Extracts Available in the Market

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Abstract

Introduction: *Serenoa repens* (W. Bartram) Small (Saw Palmetto), commonly referred to as the small palm, has been used in both traditional and modern medicine for centuries. Known for its diuretic, endocrinological, anabolic, and anti-inflammatory properties, Saw Palmetto is mostly used to treat benign prostatic hyperplasia (BPH). Known for its diuretic, endocrinological, anabolic, and anti-inflammatory properties, Saw Palmetto is mostly used to treat BPH. There has been a recent surge in interest in these products because herbal products are believed to be safer and have fewer side effects. Pharmacies stock many Saw Palmetto products authorized by the USA Department of Agriculture. People have begun to purchase these herbal products online as technology has advanced, especially during the COVID-19 pandemic, due to faster and easier access and cheaper costs. Many counterfeit products originate from popular e-commerce websites that sell goods that lack the necessary safety inspections. A literature review found that these products have adverse outcomes, including significant outcomes that can lead to death, which poses major public health concerns. The aim of this study was to compare the antiproliferative and genotoxic effects of *Serenoa repens* (W. Bartram) Small extracts obtained from two separate commercial sources, purchased over the internet and from a pharmacy, on human prostate cancer cell line (PC-3).

Methods: The antiproliferative effect of Saw Palmetto extracts at different doses (10-200 µg/ml) was evaluated with the MTT test and the genotoxic effect was determined by the comet assay.

Results: According to MTT results, the extract purchased from the pharmacy did not cause high inhibition in PC-3 cells at he entire dose range, while higher inhibition values were recorded in the extract obtained from the internet. On the other hand, when the genotoxic activities of both extracts were examined, it was determined that the DNA damage caused by the product purchased from the pharmacy in PC-3 cells was lower than the product purchased from the internet.

Conclusions: This research highlights the health risks of herbal products purchased online and the need to source dietary supplements from trusted suppliers. It is crucial to change this view and increase public awareness to ensure safer use of herbal products.

Keywords: *Serenoa repens*, comet assay, antiproliferative effect, genotoxicity, MTT assay

1. Introduction

Serenoa repens, which is native to the Americas and belonging to the Arecaceae family, was first used by Native Americans to treat infertility and erectile dysfunction. Later, colonists began using the berries of this plant as a tonic. The earliest reports in the literature on its use for urinary complaints date back to the early 20th century (1). The fruits of *Serenoa repens* are known to grow in clusters

and ripen from October to December, turning dark purple to black color. The extract of this plant is obtained from the ripe, partially dried fruits. Since different manufacturers use different extraction methods, it is unlikely that one product is equivalent to another (2). Today, Saw Palmetto is considered one of the most widely used herbal remedies for benign prostatic hyperplasia (BPH). Traditionally, it has been used to treat chronic or subacute cystitis, genitourinary rhinitis, testicular atrophy, sex hormone imbalances, and most importantly, prostate enlargement. Other traditional indications include mastitis, eczema, bronchial pathologies, and cough. However, there is no scientific evidence to support these traditional uses of this plant (3, 4).

Saw Palmetto preparations are available in various formulations, including liquids, tablets, capsules, and herbal teas. The plant contains polysaccharides with acidic character and a molecular weight of approximately 10,000 daltons (galactose (38.4%), arabinose (18.7%), uronic acid (14%), invert sugar (28.2%) and mannitol); plant sterols such as campesterol, stigmasterol, β-sitosterol, β-sitosterol-3-O-β-D-diglucoside and several β-sitosterol esters with saturated fatty acids; flavonoids such as isoquercitrin, rutin, kaempferol-3-O-β-D-glucoside, and rhoifolin; triglycerides and fatty acids including capric, caproic, caprylic, lauric, myristic, oleic (oleic and myristoleic acid), and palmitic acid; as well as tannins, volatile oils, resin, carotene, and anthranilic acid (5). Fatty acids are considered the main active components (6). The active ingredients of Saw Palmetto extracts, volatile oils and free fatty acids, are thought to have inhibitory effects on 5-alpha-reductase which blocks the conversion of testosterone to its active metabolite, dihydrotestosterone. The plant is also known to have diuretic, endocrinological, anabolic and anti-inflammatory properties. In addition, rare and generally modest adverse effects of Saw Palmetto include dizziness, headache, nausea, vomiting, constipation and diarrhea. Saw palmetto has been among the top 10 best-selling herbal medicines in the United States since the 1990s, generating approximately $700 million in annual revenue worldwide from *Serenoa repens* preparations (7). With data from more than 35 clinical studies supporting the use of standardized Saw Palmetto extracts, it is the most commonly used herbal treatment for urological symptoms associated with BPH in Europe. It was reported that the use of the extract in the treatment of lower urinary tract symptoms (LUTS) related with BPH has increased peak urine flow and reduced nocturia without elevating serum prostate-specific antigen (PSA) levels (8).

It's known that BPH develops from the proliferation of stromal and epithelial cells in the transition zone of the prostate surrounding the urethra (9). This expansion squeezes the urethra and obstructs the bladder outlet, leading to clinical symptoms such as LUTS, urinary retention, or incomplete bladder emptying, which could cause an infection. The most common clinical presentation of BPH involves LUTS, a group of symptoms that include both obstructive symptoms such as hesitancy, weak stream, incomplete voiding, urinary retention, and overflow incontinence and irritative symptoms such as frequency and urgency (10). If left untreated, the chronic condition may result in high-pressure retention (a potentially life-threatening complication) and irreversible changes in the bladder detrusor muscle (11). Treatment options include pharmacological and surgical interventions. For mild to moderate BPH, pharmacological therapy has become the standard of care, as well-designed clinical studies have shown that 5-alpha-reductase inhibitors (5-ARIs) such as finasteride and alpha-blockers such as tamsulosin, significantly improve LUTS and increase peak urine flow in men with BPH. Subsequently, numerous clinical studies have confirmed the efficacy of two 5-ARIs (finasteride and dutasteride) and five alpha-blockers (including tamsulosin, alfuzosin, and silodosin) approved by the U.S. Food and Drug Administration for the treatment of BPH (12). BPH is a disease whose prevalence increases with age and is emerging as a common health problem in the general population. In response to health concerns, people have increasingly turned to herbal supplements because they believe they have fewer side effects than traditional medications. However, these herbal remedies are sold under numerous brand names by various manufacturers, often lacking adequate

safety assessments. In addition to pharmacies, similar products are also available on reputable internet platforms, but some of these may be illegal, counterfeit, or produced by unauthorized individuals or manufacturers.

In a study examining the inhibition of 5α-reductase type I and II enzymes, the inhibitory capacity of 10 commercially available *Serenoa repens* products was determined. The study revealed that hexanic extracts showed better biological activity compared to other extracts. The findings of this study showed that pharmacological effects differed among drugs of various commercial brands, and this may be important in determining the treatment outcome. The efficacy of the products depends not only on the presence of the plant, but also on the extraction technique used and differences in formulation (13). The effects of *Serenoa repens* extracts of various commercial brands on prostate epithelial and fibroblast cells were subjected to a comparative study. The study showed significant differences in 5α-reductase inhibitory activity among the products, thus emphasizing that these differences may affect their biological activity. It was also noted that low-quality, non-standardized products would not work; this scenario emphasizes the need for quality control in evaluating product efficacy (14).

Recently, online shopping has become more popular due to its affordability and easy accessibility. However, fake products can be found even on highly advertised and reputable websites. Nowadays, numerous reports in both broadcast and print media have highlighted the rise of fake health products (16). To prevent the rise of such incidents and to promote public health protection, stricter regulatory controls should be implemented and the sale of these products through online platforms should be banned.

The aim of this study was to investigate the safety of two *Serenoa repens* extracts, one approved by the Ministry of Agriculture and obtained from a pharmacy, and the other purchased from a well-known e-commerce site in Türkiye, by *in vitro* cell culture techniques using the MTT test for antiproliferative effect and the comet test for genotoxic potential. Although existing studies have addressed the efficacy, biological activity and toxic consequences of *Serenoa repens* and compared various preparations, none of them have evaluated the toxicity of pharmacy and internet-sourced products. By emphasizing the need for regulatory control and the need to restrict uncontrolled internet sales of such products, this study aims to fill this gap and provide information.

2. Methods

2.1. Preparation of plant extract samples

Two separate capsules of *Serenoa repens* extract were used in the study: one purchased from a pharmacy and the other from an online retailer. First, 10 grams of each product were weighed and macerated with ethanol. Then, the liquid phase was filtered with filter paper following maceration; the solvent was evaporated at reduced pressure using a rotary evaporator (Fig 1). The extracts produced were stored in a +4°C refrigerator until further investigation.

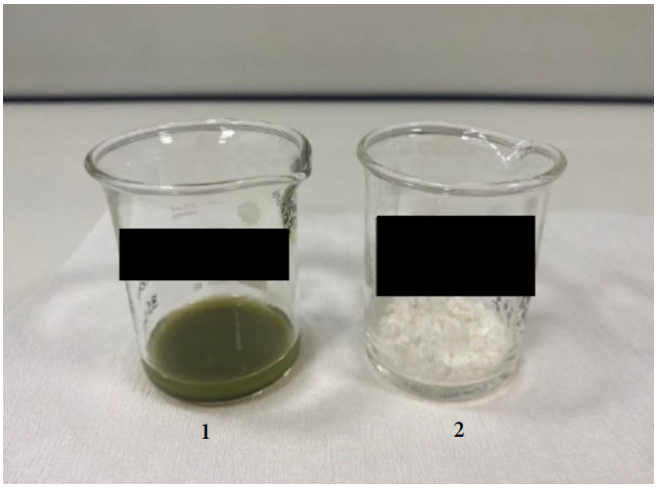

Figure 1. Photograph of the extracts after solvent evaporation using rotary evaporation (number 1 is the herbal supplement purchased from a pharmacy, and number 2 is the one purchased from an online retailer).

2.2. Cell culture studies

In this study, a prostate cancer cell line (PC-3, CRL-1435, ATCC) was used. Under standard incubation conditions at 37°C in a humidified atmosphere containing 5% CO_2, the cells were grown in RPMI 1640 medium (Gibco) supplemented with 10% fetal bovine serum (FBS) and 1%

penicillin-streptomycin. Once the cells reached 80–90% confluency, Trypsin-EDTA was used to extract them from the flask surface and subculture them into 25 cm² flasks.

2.3. Cell viability of *Serenoa repens* extracts on prostate cancer cells

A colorimetric technique based on the measurement of cellular metabolic activity, the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay was used to evaluate antiproliferative effects (17). The impact of *Serenoa repens* extracts, obtained from two different sources (an online and a pharmaceutical product), on cell viability was evaluated using the MTT assay (18). PC-3 cells were seeded into 96-well plates at a density of 1×10^4 cells per well and incubated overnight. The following day, cells were treated with varying concentrations (10, 50, 100, and 200 µg/mL) of each extract for 24 hours. After the incubation period, MTT was added to each well at a final concentration of 0,5 mg/mL, and the cells were incubated for an additional 4 hours. The medium was then aspirated, and 100 µL of SDS buffer was added to each well to solubilize the purple formazan crystals. Absorbance values were measured at 570 nm and 630 nm using a microplate reader (BioTek, Winooski, USA). Cell viability was calculated according to the following equation:

$$\% \text{ Cell Viability} = [(\text{Mean OD of treated cells}) / (\text{Mean OD of control cells})] \times 100$$

2.4. Comet Assay

A modified version of the alkaline single-cell gel electrophoresis technique (Comet Assay), first established by Singh et al., was used to assess the genotoxic effects of the produced *Serenoa repens* extracts on PC-3 cells (19). Control, positive control (hydrogen peroxide), and plant extract-treated groups were used for *in vitro* assay. The positive control was hydrogen peroxide (H_2O_2) at 100 µM, known to cause DNA damage.

PC-3 cells were seeded onto 6-well sterile plates at a density of 2×10^4 cells per well and cultured for 24 hours at 37°C in a humidified environment containing 5% CO_2. The extracts were dissolved in RPMI medium at a stock concentration of 0.5 mg/mL and administered to the cells at 50, 100, and 200 µg/mL after 24 hours. The cells were then cultured for further 24 hours.

At the end of the incubation period, the culture medium was discarded, the cells were trypsinized and then centrifuged at 1800 rpm for ten minutes. The cell pellet was resuspended in PBS and the supernatant was discarded. This washing procedure was repeated two more times. The supernatant was removed after the final wash using cold PBS and the cell concentration was adjusted to 1×10^6 cells/mL.

10 µL aliquot of the cell suspension was combined with 90 µL of 0.6% low-melting-point agarose (LMA) and layered onto slides pre-coated with 1% normal-melting-point agarose. The slides were submerged in cold lysis solution (2.5 M NaCl, 100 mM EDTA, 10 mM Tris, 1% Triton X-100, and 10% DMSO, pH 10) at 4°C for 1 hour after solidification on ice. The slides were rinsed with cold PBS and put in a horizontal electrophoresis tank following lysis.

All slides were covered with cold alkaline electrophoresis buffer (300 mM NaOH, 10 mM Na_2EDTA, pH 13.0) for 20 minutes. Electrophoresis was performed in the same solution at 4°C, 25 V, and 300 mA for a duration of 20 minutes. After electrophoresis, the slides were neutralized by washing three times with neutralization buffer (0.4 M Tris, pH 7.5). After electrophoresis, the slide was drained, neutralized with neutralization buffer (0.4 M Tris-HCl, pH 7.5) for 5 minutes and stained with ethidium bromide (20 µg/mL in distilled water; 50 µL per slide) and covered with a coverslip. Imaging was performed using a fluorescence microscope (Olympus BX51, Japan) at 400× magnification.

The percentage of DNA in the tail (% DNA_T) was quantified using the BAB Bs200Pro image analysis software (BAB LTD., Ankara, Turkey). A total of 100 cells per group (from two slides) were analyzed, and all experiments were performed in triplicate.

2.5. Statistics Analysis

All data were expressed as the mean ± standard error of the mean (SEM), based on at least three independent replicates of each experiment. Statistical analyses were performed using SPSS version 25.0 software. The IC_{50} values obtained

from the MTT assay were evaluated using the Student's t-test. The comet assay findings were evaluated using the Mann-Whitney U test for non-normally distributed data when comparing two groups, and the Kruskal-Wallis analysis of variance for comparisons across many groups. A p-value below 0.05 was deemed statistically significant.

3. Results

3.1. MTT results of *Serenoa repens* extracts

In the present study, the effect of *Serenoa repens* extracts obtained from two different commercial sources (a pharmacy and an online retailer) on the viability of PC-3 cells was evaluated using the MTT assay for 24 hour (Fig 2).

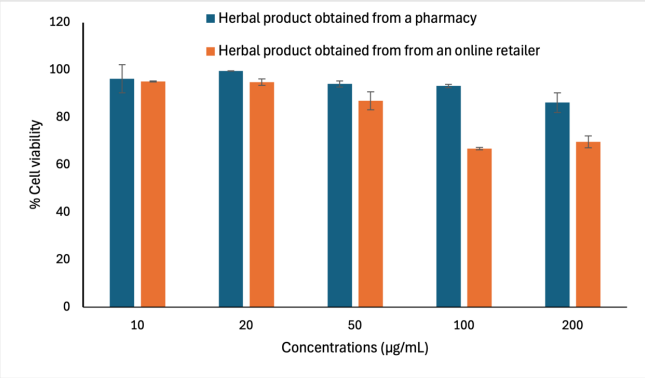

Figure 2. Effect of *Serenoa repens* on the 24-hour viability of PC-3 cells at 24 h.

According to our results, the extract obtained from the pharmacy product did not cause significant inhibition of PC-3 cell viability across the entire concentration range, with 86.25% of the cells remaining viable even at the highest concentration tested (200 µg/mL). In contrast, the extract obtained from the online retailer showed a higher inhibitory effect. Although both extracts produced similar results at lower concentrations (10, 20, and 50 µg/mL), maintaining cell viability around 90%, the online product extract significantly reduced viability at 100 and 200 µg/mL, leading to 32% inhibition.

3.2. Comet assay results

As shown in Table 1, a statistically significant, concentration-dependent increase in DNA damage was observed in cancer cells 24 hours after treatment with *Serenoa repens*, compared to the untreated control group. However, all extracts induced significantly lower levels of DNA damage than the positive control group ($p < 0.001$).

The statistical analysis revealed that both formulations of *Serenoa repens* induced DNA damage in PC-3 cells in a concentration-dependent manner. Upon comparison of the genotoxic effects of the two extracts—one procured from a pharmacy and the other from an internet source—statistically significant differences were observed at all tested doses. The extract from the internet source induced significantly more DNA damage compared to the pharmacy extract at a dose of 50 µg/mL ($p < 0.05$).

The online-sourced extract at doses of 100 and 200 µg/mL induced significantly more DNA damage compared to the pharmacy-derived product ($p < 0.001$ for both concentrations). The results indicate that alterations in the source or formulation of *Serenoa repens* extracts may influence their biological activity, particularly regarding their capacity to induce DNA damage.

Table 1. Genotoxic effects of *Serenoa repens* extracts on PC-3 cells

		$\%DNA_T$
Control		25.22 ± 0.34
Positive control (100 µM H_2O_2)		65.69 ± 0.81
Herbal product obtained from a pharmacy	**50 µg/mL**	$30.03 \pm 0.65^{***,+++,¶}$
	100 µg/mL	$35.01 \pm 0.62^{***,+++,¶¶}$
	200 µg/mL	$37.24 \pm 0.75^{***,+++,¶¶}$
Herbal product obtained from an online retailer	**50 µg/mL**	$31.78 \pm 0.65^{***,+++}$
	100 µg/mL	$38.71 \pm 0.67^{***,+++}$
	200 µg/mL	$43.87 \pm 0.81^{***,+++}$

The results are expressed as the mean ± standard deviation of three independent measurements.
$^{*}p < 0.05$, $^{**}p < 0.01$, and $^{***}p < 0.001$ compared to the control group; $^{+}p < 0.05$, $^{++}p < 0.01$, and $^{+++}p < 0.001$ compared to the positive control group.
¶$p < 0.05$, ¶¶$p < 0.01$, and ¶¶¶$p < 0.001$ compared to the online retailer group
$\%DNA_T$: Percentage of DNA in the tail

4. Discussion

In this study, the antiproliferative and genotoxic effects of *Serenoa repens* (Saw Palmetto) extracts obtained from pharmacy and online retailer were investigated on human prostate cancer cells (PC-3) using MTT and comet assays, respectively. Our

findings revealed significant differences in both antiproliferative and genotoxic profiles between the two products, highlighting potential public health concerns regarding the unregulated online sale of herbal supplements.

The MTT test results revealed that the product extract purchased from a pharmacy did not exhibit antiproliferative activity even at the highest concentrations tested. In contrast, the product extract purchased from an online retailer showed significantly higher cytotoxicity, indicating a stronger inhibitory effect on the cell viability. While moderate cytotoxicity may be interpreted as a desirable antiproliferative activity in the context of prostate cancer, the unregulated nature of products purchased online raises concerns regarding product composition, concentration of active compounds, and the presence of toxic imposters.

The observed differences in antiproliferative activity can be attributed to several factors, including differences in extraction methods, raw material quality, manufacturing standards, and potential contamination or counterfeiting. The fact that both products claim to contain the same active ingredient highlights the lack of standardization in the herbal supplement market.

Booker et al. examined the chemical components of Saw Palmetto products from various brands using gas chromatography and and ^{1}H NMR metabolomic methods (6). Despite coming from the same plant source, significant differences were found in the amounts of important components such as free fatty acids, phytosterols, and volatile chemicals. These variations in composition have a direct impact on the biological activity and bioavailability of the product. The article also emphasizes that marketed products should be evaluated not only by their botanical names but also by their structures.

Another study compared the fatty acid and sterol levels of *Serenoa repens* extracts from various commercial brands. The results revealed significant differences in chemical content between the products, and the researchers suggested that these differences may be important for pharmacological outcomes. Some brands of products showed low levels of active chemicals and reported that these products did not provide the desired effects. In line with our findings, the results of this study suggest that therapeutic efficacy depends on research on product quality and content (15). Previous studies have emphasized that different extraction techniques may lead to different concentrations of bioactive fatty acids and sterols, which may directly affect the biological activity (6, 2).

De Monte et al (2014) also examined how various extraction methods, including supercritical CO_2, hexane, and ethanol, affected *Serenoa repens* extracts. The researchers found that extracts from the supercritical CO_2 method exhibited less antiandrogenic activity compared to the other techniques. Hexanic extracts produced better biological effects, leading to improved therapeutic efficacy. These findings highlight how the extraction process affects the biological efficacy of the product (20).

In the comet assay, which evaluates DNA damage at the single-cell level, the extract purchased from pharmacy caused significantly less genotoxicity compared to the extract purchased from an online retailer. The DNA tail intensity was markedly increased in cells treated with the online-purchased extract, suggesting higher levels of strand breaks and overall genomic instability. This outcome further supports the hypothesis that products sourced from unverified online sellers may contain impurities or unregulated additives that pose a genotoxic risk.

Interestingly, despite the increasing popularity of *Serenoa repens* in the treatment of BPH, most of the existing studies have focused on efficacy rather than safety, especially with regard to genotoxicity. Our study addresses this gap by providing comparative toxicity data and also highlights that perceived naturalness is not the same as safety. It also highlights the critical importance of post-market surveillance and quality control, especially in light of recent reports of counterfeit and adulterated health products sold online.

A study conducted in Italy between 2011 and 2013 assessed the health risks of pharmaceuticals and nutritional supplements sold illegally or online. The results showed that supplements sold online were highly risky, both in terms of the health of

customers and the safety of the ingredients. The researchers reported that inadequate regulatory oversight may negatively impact public health and that stricter regulation was needed (21).

Veatch-Blohm et al (2021) evaluated the levels of contamination and consistency of content in 29 herbal supplements commonly used in the United States. The study revealed significant variability in antioxidant activity, phenolic and flavonoid content, and undesirable impurities among different supplements. The findings emphasize the necessity for government regulation of these products in order to avoid harming public health (22).

Researchers at King Saud University's College of Pharmacy investigated the frequency of online purchases of health products, herbs, and medicines in Saudi Arabia, the motivations behind these purchases, and perceptions of product safety. While the online health product market is rapidly expanding, individuals have expressed concerns about the safety and quality of these products. Most of the participants agreed that purchasing products online is easy; however, many expressed uncertainty about the reliability and accuracy of product information. The study highlights the importance of safety guidelines for online health product sales and consumer awareness of these regulations (23).

The PC-3 cell line used in this study provides a relevant model to evaluate the potential therapeutic and toxicological effects of prostate-targeting agents. Previous findings emphasize the importance of ongoing research comparing online purchases with those from pharmacies, underscoring their public health importance. There are very few studies in the literature comparing internet products from online and pharmacy sources, as in our study. The previous studies support the importance of our study, as it is the first research of its kind in this area. However, it should be noted that *in vitro* assays have limitations and may not fully replicate *in vivo* responses. Therefore, future studies involving animal models or clinical samples may further confirm the findings and provide insight into pharmacokinetic and metabolic parameters.

5. Conclusion

In conclusion, the significant antiproliferative and genotoxic differences observed between *Serenoa repens* extracts purchased from pharmacies and online sources highlight the urgent need for stricter regulation and monitoring of online herbal supplement sales. Public awareness campaigns and strict regulatory regulations are essential to protect consumers from potentially harmful products and to ensure that herbal therapies maintain both efficacy and safety standards. On the other hand, herbal medicines are becoming more important in phytovigilance systems due to their therapeutic potential. More comprehensive studies on a wider range of brands and formulations are needed to support regulatory decision-making and public health recommendations.

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