European Review for Medical and Pharmacological Sciences 2023; 27: 2077-2090

Ethnopharmacological evaluation of Poppy seed oil in combination with Tramadol on behavioral paradigm and on dopamine, and cytokines levels

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Abstract. – OBJECTIVE: The present study was taken up to evaluate the combination of two drugs in the management of behavioral disorders such as locomotor activity, muscle relaxation, analgesic, and anxiolytic activity.

MATERIALS AND METHODS: In the methodology, Wistar rats weighing (150-180 g) were divided into six groups of 6 each (n=6). All the animals (groups II to VI) were subjected to stress and analyzed for anxiolytic activity using an elevated plus maze. The animals were treated for 28 days with poppy seed oil in lower and higher doses (1,000 and 2,000 mg/kg), tramadol in lower and higher doses (1.5 and 3 mg/kg) as individual groups, and one group with a combination of both drugs in lower doses.

RESULTS: The results depicted showed that the combined treatment had significantly (p <0.001) improvised behavior deficits, extemporized, and diminished anxiety-like attitude in rats, and showed the analgesic property in a significant manner. The pro-inflammatory cytokines TNF-α and IL-1β were evaluated in the serum and were observed to be lessened the values of both in a significant manner with the co-administration of both the test drugs. The dopamine concentrations were also determined in the serum, which disclosed a decline (**p <0.001) significantly.**

CONCLUSIONS: It was concluded from the results that a combined effect of drugs might be beneficial in the management of behavioral disorders and pain management.

Key Words:
Poppy seed oil, Actophotometer, Rota rod, Elevated Plus Maze, Anxiety, Analgesic, Dopamine, Cytokines.

Introduction

Globally, 1/8th of the total population is suffering from anxiety[1] and 80% of them rely on traditional medicine. Anxiety is defined as a mental condition, accompanied by unpleasantness, discomfort, fear, perplexity, and irritation. It is one of the psychiatric conditions that has been recognized as a mood disorder, related to stress-causing disability and premature death[2]. There is an association between anxiety, aggression, and stress. Animal models are undoubtedly helpful in understanding the molecular underpinnings of anxiety disorders and in the development of new, more effective pharmacological and/or behavioral therapies. A study[3] reported that an animal who failed to conquer (or) overcome experimentally might experience a high level of stress and augmented anxiety-like and depressive attitude. Thus, an elaborate investigation is essential to analyze the possible factors responsible for the development of stress and anxiety related conditions at molecular level, might sift out for better promising treatments.

The biochemical data in stress-induced anxiety is also in conjunction with increased levels of cortisol in serum, with outstanding modification in catechol amines like epinephrine, norepinephrine, and dopamine as they regulate aggressive behavior and emotions. Stress and pro-inflammatory cytokines have a strong relationship with each other as per the evidence[4]. Mental illness in alliance with anxiety is inclined to decrease immune

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function and modifies the profile of pro-inflammatory cytokines. This concept however is poorly understood and stood up as a controversial subject, because of probable and sturdy neuropathological mechanisms[5]. The release of dopamine played an important role in the neuropsychiatric behavioral pattern associated with depression and anxiety[6]. Hence the present study pivoted to implicate the determination of dopamine concentrations in mentally affected experimental animals. Inflammation and pain are associated with each other and have become manifestations of many diseases[7]. Pain is a naturally protective mechanism though it causes distress and discomfort. It is an unpleasant feeling identified by a physician when a patient suffers from pain.

Benzodiazepines were the major compounds used for pain management despite their diminished margin of safety between the therapeutic effect and unfavorable side effects. Hence herbal treatments are being opted as an alternative; an exploration for a new entity or a molecule would be favorable for the treatment of neurological disorders[8]. Using different animal models, the investigation has continued to demonstrate the pharmacological effects of different plant species.

Papaver somniferam L. (opium poppy) has been extensively cultivated and used as a medicinal plant throughout European and Asian countries. Historically, opium poppy served as an aid for pain sufferers in pain management and anesthesia[9]. The seeds were found to be a good source of nutrients and other components, also beneficial for health. Fatty acids and aromatic volatile components, vitamins, and steroidal substances were reported to be the main chemical classes of poppy seed oil[10,11]. It has many properties, such as anti-oxidant, anti-cancer and anti-mutagenic, anti-microbial, anti-parasitic, and anti-inflammatory, exerting its action on the nervous system[12]. Opium alkaloids tend to act on opioid receptors μ, δ, and k receptors which are distributed widely in the central nervous system (CNS) and peripheral tissues. By binding to these receptors, it exerts actions such as analgesia, anti-anxiety, euphoria, and sedation[13]. In a study[14], poppy seed oil was selected for evaluation clinically in school children for thyroid deficiency.

On the contrary, as conventional drug therapy in pain management, many drugs are being used[15]. Amongst these drugs, Tramadol is a centrally acting potent analgesic drug used for the treatment of moderate to severe pain in various diseases such as cancer, osteoarthritis, and other musculoskeletal disorders. Tramadol acts by binding to μ receptors and causes an increase in the concentration of norepinephrine and serotonin[16]. Pain elevates the comorbidities like depression and anxiety, and there is corroboration that emphasizes that preclinical investigations on tramadol were carried out to examine the effects on depression and anxiety-related behaviors using rats as a model. Also, tramadol was proven to lessen anxiety-related and depression-like behaviors and exhibited an anti-nociceptive effect in neuropathological condition[8]. Tramadol was reported to be a powerful centrally acting analgesic, possessing a close structural similarity with the anti-depressants, and could have a possibility of acting by binding to D2 and D3 dopaminergic receptors[17]. Nevertheless, there might be some drug-drug interactions if used in combination with other drugs like anti-depressants or any other. In the long-term therapy, due to the generation of multiple metabolites, there were toxic effects on the liver and kidney[18]. Because of the above considerations, a drive to search for a new compound from plant resources with minimum side effects and efficacy is mandated. The new medicine obtained from a plant source needs to be beneficial in improving the central nervous system (CNS) such as anxiety, depression, Parkinson's disease, and neuromuscular weakness[19]. A safe alternative can be provided if the plant medicine can be used as both herbal and conventional medicine. However, there exists a deficiency in the research of such medicinal plants due to a lack of proper dosing and usage.

Therefore, the present study was taken up to compare the efficacy of poppy seed oil and tramadol in low and high doses individually and in combination. A comparative evaluation of the poppy seed oil and tramadol were performed in behavioral patterns such as locomotor activity, motor coordination, anxiety, and analgesia along with the inflammatory biomarkers. To correlate the dopamine levels with behavioral disorders like anxiety and depression, the dopamine concentration was also determined.

Materials and Methods

Preparation of Poppy Seed Oil and Tramadol

Unrefined, cold-pressed poppy (*Papaver somniferum L.)* seed oil was obtained from the Erbology (London, United Kingdom). Tramadol

Hcl (Grunenthal Ltd, Buckinghamshire, UK) was obtained from King Fahd Hospital and dissolved in 0.9% saline before administration.

Characterization of Poppy Seed Oil

The poppy seed oil was characterized by physicochemical parameters such as specific gravity, refractive index, saponification value, iodine value, peroxide value, un-saponifiable matter, and solidification point[10-20].

GC-MS Analysis of Poppy Seed Oil

Thermo Scientific GC-MS (Waltham, MA, USA) fitted with AS 3000 autosampler, trace ultra-GC and ISQ detector was used for the identification of components present in the unrefined poppy seed oil. The injection port was set at 290°C and filtered 2 mL of diluted sample injected in split-less mode into a TR-5MS capillary column (30 m × 0.25 mm ID × 0.25 mm). Helium was used as a carrier gas with a flow rate of 1.2 mL/min. The oven was set with a ramping program having the initial temperature set at 70°C for 5 min and subsequently ramped to 100°C, 150°C, 200°C, 250°C, and 290°C at a rate of 5°C/min with holding time of 10 min at each ramped stage (total run time of 120 minutes). The MS-detector ISQ was set to identify the molecular masses ranging from 40-650 amu at 70 eV in positive ion mode and spectra were recorded with a delay time of 5 min to avoid initial solvent peaks. Ion source temperature and MS transfer line temperature were set at 300°C and 310°C, respectively.

Acute Oral Toxicity Study

Toxicity studies[21] were carried out as per the OECD guidelines No. 423. The poppy seed oil was orally given to various groups of rats at doses of 500, 1,000, and 2,000 mg/kg body weight, respectively. Readings were noted after 48 hours of keen observation to check the normal behavior and examine if there were any issues with nervous problems and any lethality.

Animals and Grouping

Thirty-six adult male Wistar rats (150-180 g) were obtained from the animal breeding unit at the Medical College-Jazan University. Animals were kept in cleansed, clear polypropylene cages in groups of four in each cage maintained at 25 ± 2ºC with 12 hours of light and dark cycle with free access to food pellets and water ad *libitum*. The examination was done between 8.00 am to 3.00 pm. Following the recommendations of Jazan University ethical committee of Scientific Research HAPO-10-Z-001, decision number REC41/1-035, decision date 18/11/2019.

Animals were divided into six groups of six each (n=6).

Group I – Control (0.9% NaCl).
Group II – Poppy seed oil (1,000 mg/kg p.o).
Group III – Poppy seed oil (2,000 mg/kg p.o).
Group IV – Tramadol (1.5 mg/kg p.o).
Group V – Tramadol (3.0 mg/kg p.o).
Group VI – Poppy seed oil (1,000 mg/kg) + Tramadol (1.5 mg/kg p.o).

Treatment and Dosing

The animals of groups II to VI were treated for 28 days with oral administration of test drugs – poppy seed oil and tramadol at lower and higher doses, respectively. Then the animals were subjected to behavioral activities using an actophotometer, rotarod apparatus, and analgesic effect by acetic-acid writhing and tail immersion technique. Also, the animals were induced with stress and tested for anxiety using an elevated plus maze. At the end of the experiment, the stress-induced animals were determined for pro-inflammatory cytokines and dopamine in the serum samples[22].

Induction of Chronic Mild Stress (CMS)

Animals of all the groups except group I were exhibited to a routine of deep-rooted mild restlessness displayed over a portion of time for 14 days, animals stayed in their hutch under usual circumstances according to Chronic mild stress schedule[23-25] as follows:

- Day 1 – Exposed to 4°C for 50 minutes – 11:00 am.
- Day 2 – 60 min cage agitation (60 rpm) – 11:00 am.
- Day 3 – 60 min restrained stress (wire grid) – 11:00 am.
- Day 4 – 12 hrs water deprivation – 11:00 am to 11:00 pm.
- Day 5 – 3 hrs light off daytime – 11:00 am to 02:00 pm.
- Day 6 – 60 min noise stress – 11:00 am.
- Day 7 – 60 min restraint stress (tube) – 11:00 am.

Assessment of Test-Treated Drugs on Behavioral Parameters in Rats

To investigate the test drugs, the following behavioral models were used.

Investigation of Locomotor Activity

Actophotometer

Actophotometer is considered one of the best animal models used to assess spontaneous locomotor activity. It consists of a closed square field arena measuring 30 x 30 x 30 cm in which an animal was placed. It was also furnished with six photocells on the outer wall. The locomotor activity of an animal was recorded with the interruptions of a photocell using a digital counter. On the day of the experiment, to evaluate the locomotor activity of the animal, the instrument was switched on and each rat was placed individually in the activity cage for five minutes. The activity difference before and after drug administration was recorded. The percentage decrease in motor activity was calculated[26].

Rota-Rod Apparatus (Motor Coordination)

The muscle relaxant property was assessed in rats by using the Rota-rod apparatus and could be evaluated by testing their ability to remain on a Rota-rod. Rota-rod apparatus (Dolphin make) is a four-panel techno device with a timer. Animals (4 at a time) were placed on a rod rotating at 20-25 rpm speed. Rats who demonstrated their ability to remain on the revolving rod (20-25 rpm) for 5 min after training sessions during pretest screening, were selected for the study. The fall-off time was recorded in all the groups 30 min after drug administration. A decrease in fall-off time is suggestive of CNS depression. The Rota rod fall-off time difference between control and treatment rats was used as a measure of muscular relaxation[3].

Investigation of Test Drugs on Analgesia

The animals were evaluated for analgesic activity using two models – the acetic acid-induced writhing test (Peripheral analgesic activity) and Tail Immersion Method (Central analgesic activity).

Acetic Acid-Induced Writhing Test (Peripheral Analgesic Activity)

The assessment of the anti-nociceptive effect of poppy seed oil and tramadol was done using an acetic acid-induced writhing test (abdominal constriction test) in rats. Acetic acid solution (10 ml/kg) was prepared and was injected intra-peritoneal (0.1 ml/10 g, i.p) to induce the Nociception. Animals of Groups II, III, IV, V, and VI were treated orally with low and high doses of poppy seed oil (1,000 and 2,000 mg/kg) and tramadol (1.5 and 3.0 mg/kg) separately and with the combination of two drugs before the experiment for 28 days. On the day of the experiment after 25 min of injection of acetic acid, the animals were subjected to evaluation. The numbers of abdominal contractions along with stretching of hind limbs (writhes) were observed and noted at 10, 20, and 30 minutes, respectively[27,28]. In this method, the same grouping of animals was followed after a washout period of 7 days. Except for the control animals, all the animals were pre-treated with drugs 60 minutes before immersion of the tail.

Tail Immersion Method (Central Analgesic Activity)

A distal portion of 3-5 cm of rat tail was immersed in a beaker with water at a temperature of 55±50°C. The cut-off time of immersion was 10 s with a withdrawal of the tail from the water considered as a reaction time. The rats were administered with subsequent doses of drugs poppy seed oil (1,000 and 2,000 mg/kg) and tramadol at low and high doses (1.5 and 3.0 mg/kg) orally followed by a combination of drugs for 28 days and on the day of the experiment, 60 minutes before the test. Animals were tested at 0, 30, 60, 90, and 120 minutes after the drug administration[28].

Effect of Test Drugs on Anxiety in Stress-Induced Animals

Elevated plus Maze model (EPM)

Elevated plus Maze consists of two open arms (50 cm × 10 cm) and two closed arms (50 cm × 10 cm × 40 cm). A square of 10 cm is present at the center with open and closed arms opposite each other. The maze was elevated at a height of 50 cm above the floor. Each rat was placed individually at the center facing toward the open arm. The time spent in open and closed arms by the animal in 5 minutes was noted. The elevated plus maze apparatus was cleaned with 10% ethanol solution after each reading[29].

Sample Collection

At the end of the experiment, after 24 hours, blood was collected from the retro-orbital plexus for the cytokines assay. Blood samples were allowed to coagulate for 60 minutes at room temperature. Then centrifuged at 1500 g for 15 minutes and crude serum was stored at -20°C until use.

Dopamine Elisa Test

At the end of the experiment, serum samples were collected for measuring dopamine levels using commercialized ELISA kits [cat. No. ab133053; Abcam (Abbott Park, IL, USA)] according to the manufacturer's protocol. All the procedures were performed according to the manufacturer's instructions. The outcome was examined by using an ELISA reader system (Bio-Rad Laboratories, Inc., Hercules, CA, USA)[30].

Cytokines Assay

Pro-inflammatory cytokines, TNF-α, and IL-β1 were estimated in serum samples in terms of picogram per milliliter (pg/ml) using PicoKine ELISA kits. Assays were performed according to the manufacturer's recommendations.

Statistical Analysis

The results of the studies were expressed as Mean ± SEM. Data analysis was done by one-way analysis of variance (ANOVA) followed by the Tukey-Kramer test for multiple comparisons. Probability values $p < 0.05$ was considered significant.

Results

Physicochemical Characterization and Compound Identification by GC-MS Analysis of Poppy Seed Oil

The physicochemical properties of poppy seed oil were performed as per the USP general chapters of fats and fixed oils. The results are depicted as Specific gravity = 0.963, Refractive index = 1.4512, Saponification value = 236, Iodine value = 140.1, Peroxide value = 39, Percent Unsaponiafilabe matter = 1.06, Solidification point = -4°C to -8°C. The components of poppy seed oil were identified by using software-generated match factor (SI) and reverse match factor (RSI) having thresholds of 900 and above. The relative content (%) of each component was calculated by dividing the peak area of each component by the total peak area using Xcalibur software (Arlington, VA, USA) (Figure 1). The peak area was calculated without any internal standard [30]. The details of all identified compounds with their retention time, molecular weight, molecular formula, relative content (%), and chemical class are represented in (Table I).

Acute Oral Toxicity Study

Acute tests revealed that there was no toxicity observed and the poppy seed oil was found to be safe for the study to be carried out because of no signs of death. Hence, rats were administered two doses – lower and higher doses of poppy seed oil (1,000 and 2,000 mg/kg body weight, p.o) in the current study.

Effect of Test Drugs on Locomotor Activity Using Actophotometer

There was a fall in the locomotor activity of the test drugs – poppy seed oil and tramadol at a lower dose and also at higher doses (1,000 and

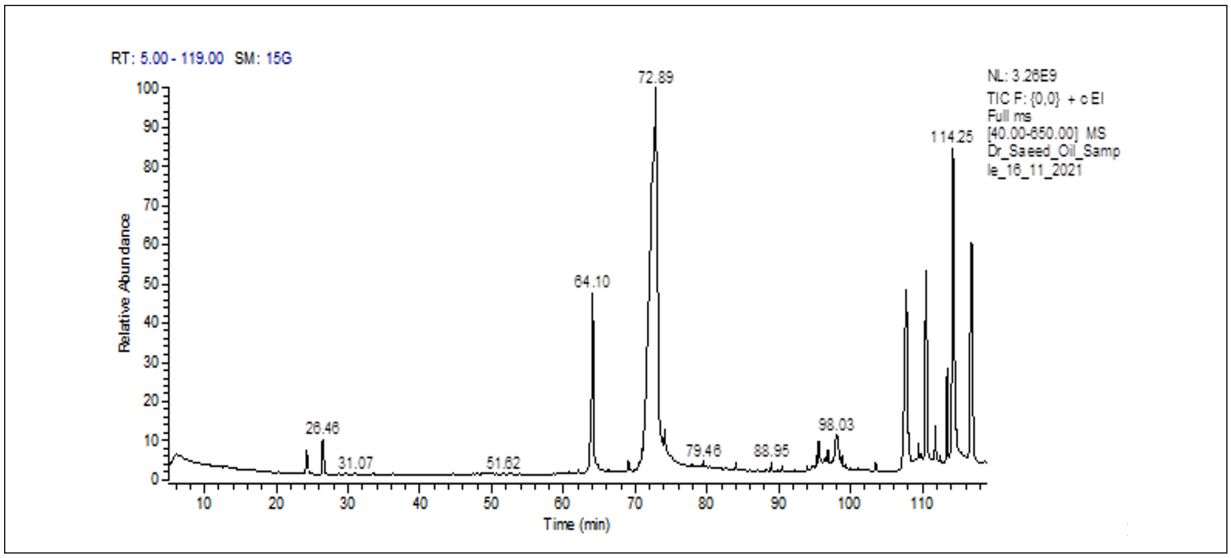

Figure 1. GC-MS Total ion chromatogram of poppy seed oil.

Table I. Chemical compounds of unrefined poppy seed oil identified by gas chromatography-mass spectrometry (GCMS).

S. No.	RT	Name of compound	Molecular formula	Molecular weight	Relative content %	Chemical class
1	14.40	Pentanal	$C_5H_{10}O$	86	1.03	Saturated fatty aldehyde
2	16.88	Ethylbenzene	C_8H_{10}	106	1.01	Aromatic hydrocarbon
3	17.37	2-Methylpyrazine	$C_5H_6N_2$	94	1.62	Pyrazine
4	21.59	2-Pentylfuran	$C_9H_{14}O$	138	1.08	Furan derivative and volatile oil component
5	24.22	2,4-Decadienal	$C_{10}H_{16}O$	152	1.0	Polyunsaturated fatty aldehyde
6	62.10	Methyl palmitate	$C_{17}H_{34}O_2$	270	3.46	Fatty acid methyl ester
7	64.10	Palmitic acid	$C_{16}H_{32}O_2$	256	11.96	Saturated long-chain fatty acid
8	69.05	Methyl linoleate	$C_{19}H_{34}O_2$	294	6.38	Fatty acid methyl ester of linoleic acid.
9	72.89	Linoleic acid	$C_{18}H_{32}O_2$	280	41.8	Saturated long-chain fatty acid
10	88.95	Diisooctyl phthalate	$C_{24}H_{38}O_4$	390	0.30	Phthalate ester and a diester
11	93.96	Glyceryl monolinoleate	$C_{21}H_{38}O_4$	354	4.28	Triglyceride
12	95.26	Glyceryl 2-linoleate	$C_6H_6O_3$	354	2.87	Triglyceride
13	96.43	Diisononylphthalate	$C_{26}H_{42}O_4$	418	1.29	Diisononyl ester of benzene-1,2-dicarboxylic acid (Plasticizer)
14	96.83	Diethylhexyl terephthalate	$C_{24}H_{38}O_4$	390	1.34	Plasticizer
15	109.44	1-Octacosanol	$C_{28}H_{58}O$	410	2.35	Straight-chain aliphatic primary fatty alcohol
16	109.75	Vitamin E	$C_{11}H_{16}O_2$	430	1.17	Fat-soluble vitamin
17	111.81	Campesterol	$C_{28}H^4_8O$	400	3.06	Steroid derivative
18	112.47	Stigmasterol	$C_{29}H_{48}O$	412	1.13	3 beta-sterol

Table II. Effect of test drugs on locomotors activity and muscular Coordination using Actophotometer and Rota rod apparatus.

Groups	Treatment and Dose (mg/kg)	Actophotometer Score			Rota rod apparatus Time of fall off (sec)
		Before the administration of drugs	After the administration of drugs	% of reduction	
I	Control (0.9% NaCl)	150 ± 3.2	155 ± 1.9	-	100 ± 0.8
II	Poppy seed oil (1,000)	430 ± 2.6	**215 ± 2.4	50	**235 ± 1.7
III	Poppy seed oil (2,000)	602 ± 1.5	**212 ± 3.1	64.79	**228 ± 2.4
IV	Tramadol (1.5)	420 ± 1.8	**238 ± 2.5	43.33	**238 ± 1.3
V	Tramadol (3.0)	547 ± 2.6	**230 ± 4.1	57.92	284 ± 1.8
VI	Poppy seed oil (1,000) + Tramadol (1.5)	426 ± 2.9 Df = 5 F= 8261.5	**109 2.9 Df = 5 F = 7152.4	74.41	**175 ± 3.6 Df = 5 F= 6602.17

The data were expressed in Mean ± S.E.M; n = 6 in each group; Values were considered to be statistically significant (**$p < 0.001$) when Group VI was compared to Group II, III, IV, and V. Data was analyzed using a one-way ANOVA test followed by Tukey Test.

2,000 mg/kg of poppy seed oil, 1.5 and 3 mg/kg of tramadol) individually along with the % reduction of 50-64, respectively. Indeed, a combination of both drugs in lower doses (poppy seed oil at 1,000 mg/kg and tramadol at 1.5 kg/kg) had a significant decrease (**p <0.001) in locomotor activity with a huge % of (74) reduction (Table II).

Effect of Test Drugs on Muscular Relaxation Using Rota rod Apparatus

In the Rota rod apparatus, the animals showed a significant decrease (**p <0.001) in muscular co-ordination with the treatment of poppy seed oil at a lower dose (1,000 mg/kg) as compared to the control group and also with a higher dose (2,000 mg/kg) of animals. There was a shrink in the fall of time (238±1.3 sec) with a lower dose of tramadol (1.5 mg/kg) significantly as compared to the control animals. Nevertheless, there was a diminished fall of time significantly (175±3.6 sec) with the integrated administration of both the test drugs – poppy seed oil and tramadol at lower doses (1,000 mg/kg and 1.5 mg/kg) (Table II).

Evaluation of Analgesic Activity

The peripheral analgesic effect of the drugs was investigated by inducing acetic acid-induced writhing. The group which was administered with a low dose of Poppy seed oil (1,000 mg/kg orally) exhibited a statistically significant (**p <0.001) reduction in the number of writhing with 2.5±1.01, 2±0.22 and 1.33±0.21 respectively at 10, 20 and 30 min. Also, the group of animals that received a combination of poppy seed oil and tramadol in low doses showed a significant decrease (**p <0.001) in the number of writhing with 2.67±0.33 at 30 min when compared to the group II, III, IV, and V (Table III).

Table III. Effect of test drugs – Poppy seed oil and Tramadol on Acetic acid-induced Nociception.

Groups	Treatment and dose (mg/kg)	10 min	20 min	30 min
I	0.9% NaCl	7.83 ±0.31	7.33 ±0.42	6.22 ± 0.24
II	Poppy seed oil (1,000)	2.5± 1.01**	2 ± 0.22**	1.33 ± 0.49**
III	Poppy seed oil (2,000)	6.8 ± 0.22	4.55 ±0.37	3.33 ± 0.21
IV	Tramadol (1.5)	4.17 ± 0.70	7 ± 0.77	3.5 ± 0.22*
V	Tramadol (3.0)	8.15 ± 0.48	6 ± 0.37	5.17 ± 0.31
VI	Poppy seed oil (1,000) + Tramadol (1.5)	5.5 ± 0.76 Df = 5; F= 4.61	4.0 ± 0.37** Df = 5; F= 5.62	2.67 ± 0.33** Df = 5; F = 3.89

The data were expressed in Mean ± S.E.M; n=6 in each group; Values were considered to be statistically significant at (**$p <$ 0.001) when Group III was compared to Group I, IV, and V. Group VI was found to be significant statistically at (**$p < 0.001$) as compared to control animals (Group I) at 30 min. Data were analyzed using one way ANOVA test followed by Tukey Test.

Tail Immersion Method

To analyze the central analgesic effect of test drugs, the tail immersion method was employed. In this experiment, the group which was administered a low dose of poppy seed oil (1,000 mg/kg orally) exhibited a statistically significant (**p <0.001) increase in the reaction time with 6.33±1.26, 7.17±0.40, and 7.85±0.1, respectively at 60-, 90-, and 120-min. Group IV animals that received tramadol (1.5 mg/kg) low dose showed a significant increase in time for withdrawal of tail with the values 5.33±0.39 at 60 min. Also, the group of animals that received a combination of poppy seed oil and tramadol in low doses showed a significant increase (**p <0.001) in reaction time with 5.43±1.05 at 60 min followed by 5.17±0.7 at 90 min when compared to the groups II, III, IV, and V (Table IV).

Effect of Test Drugs on Anxiety Using Elevated Plus Maze

The animals of all groups were subjected to chronic mild stress and were evaluated for anxiolytic activity. Poppy seed oil at the dose of 1,000 mg/kg body weight, significantly (**p <0.001) reduced the time spent in closed arms as compared to the positive control animals. Tramadol at a lower dose (1.5 mg/kg body weight) decreased the time spent in closed arms significantly (**p <0.001). In conjunction administration of both poppy seed oil and tramadol at lower doses had a significant effect on anxiety which was evident from animals spending more time in the open arm (209 ± 2.6 sec) than in the closed arm (78 ± 1.9 sec), respectively (Figure 2).

Cytokine Assay

At the end of the experiment, the positive control animals exhibited an increase in the pro-inflammatory cytokines TNF-α and IL-1β levels in the serum indicating that stress reduced immunity and provoked inflammation[32]. Besides, stress-induced animals which underwent treatment with the poppy seed oil at the dose of 1,000 mg/kg, showed a significant (**p <0.001) decline in both the parameters with 155.16 ± 0.34 (TNF-α) and 59.13 ± 0.66 pg/ml (IL-1β) values, exhibiting a remarkable blockade effect of pro-inflammatory cytokines. Tramadol at the dose of 1.5 mg/kg, showed a significant (**p <0.001) reduction with 169.17 ± 0.99 (TNF-α) and 72.52 ± 0.54 pg/ml (IL-1β) values, respectively. With the combination of both drugs at lower doses, there was a significant (**p <0.001) decrease in TNF-α and IL-1β with 138.29 ± 0.32 and 48.17 ± 1.51 pg/ml (Figure 3). All the values were comparable to negative and positive control animals.

Effect of Test Drugs on Serum Dopamine Level

At the end of the experiment, all stress-induced animals were estimated with dopamine levels. The stress-induced animals showed a significant rise in dopamine concentrations in the serum. At lower doses, both the drugs – poppy seed oil and tramadol (1,000 mg/kg and 1.5 mg/kg) exhibited a decline with the values 12.56±1.16 and 19.64±1.08 pg/ml in the dopamine concentrations when treated individually[33]. When both the drugs were administered together at a lower dose, there

Table IV. Effect of test drugs on analgesia in Tail immersion test

	Reaction time at different time intervals (sec)				
	0 min	30 min	60 min	90 min	120 min
I Control (0.9% NaCl)	2.3 ± 0.35	1.83 ± 0.89	1.49 ± 0.98	1.22 ± 0.76	2.15 ± 0.76
II Poppy seed oil (1,000)	2.33 ± 0.23	2.9 ± 0.15	6.33 ± 1.26**	7.17 ± 0.40**	7.85 ± 0.1**
III Poppy seed oil (2,000)	2.17 ± 0.65	4.0 ± 0.45	4.33 ± 0.19	4.28 ± 0.21	4.52 ± 0.5
IV Tramadol (1.5)	3.33 ± 0.24	3.8 ± 1.25	5.33 ± 0.39**	4.07 ± 0.41	3.42 ± 0.3
V Tramadol (3.0)	4.18 ± 0.63	3.0 ± 0.44	3.23 ± 0.56	3.12 ± 0.31	2.35 ± 0.1
VI Poppy seed oil (1,000) + Tramadol (1.5)	3.17 ± 0.37	4.17 ± 0.75	5.43 ± 1.05**	5.17 ± 0.7**	4.8 ± 0.36
	Df = 5	Df = 5	Df = 5	Df = 5	Df = 5
	F = 1.75	F = 2.34	F = 3.56	F = 6.87	F = 7.92

The data were expressed in Mean ± S.E.M; n = 6 in each group; Values were considered to be statistically significant (**p < 0.001) when Group VI was compared to Group II, III, IV, and V. Data was analyzed using a one-way ANOVA test followed by Tukey Test.

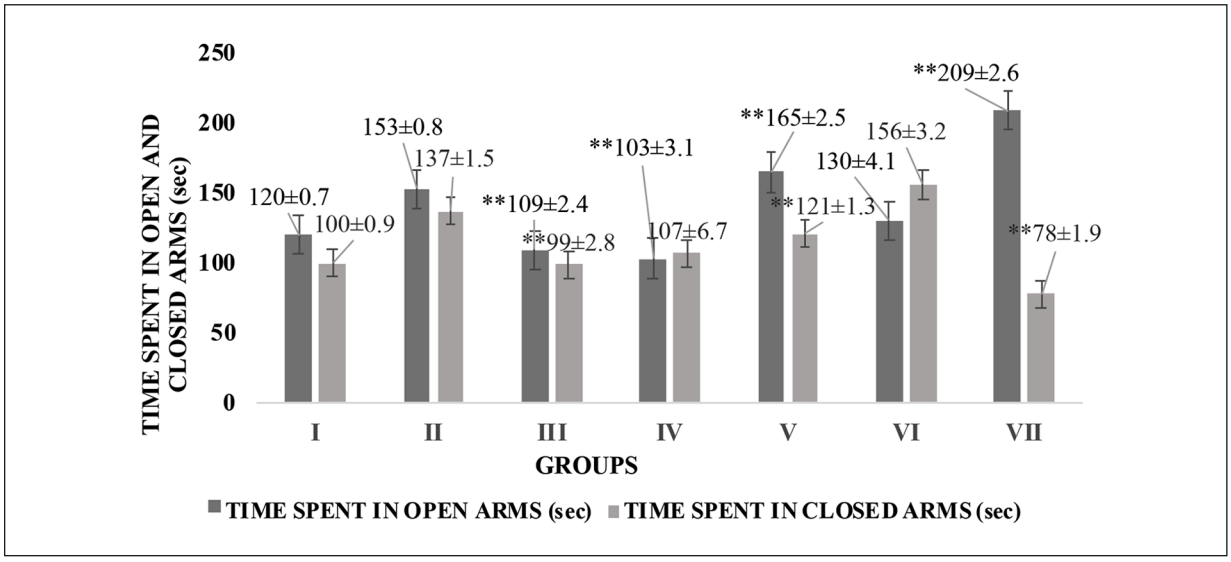

Figure 2. Effect of test drugs on Anxiety using Elevated plus Maze. The data were expressed in Mean ± S.E.M; n=6 in each group; Values were considered to be statistically significant (df =6; F=871.03; df =6; F=862.09; $^{**}p < 0.001$) when Group VII was compared to Group I, II, III, IV, V, and VI. Group VII was found to be significant statistically ($^{**}p < 0.001$) as compared to negative control and positive control animals (Group I & II). Data were analyzed using a one-way ANOVA test followed by Tukey Test.

was a remarkable reduction in the dopamine concentration significantly (6.07±0.16 pg/ml) as compared to the other treated groups and also control animals (Figure 4). All the values were comparable to negative and positive control animals.

Discussion

The poppy seed oil appeared as a pale-yellow liquid having very little aromatic odor. The oil was characterized for various physicochem-

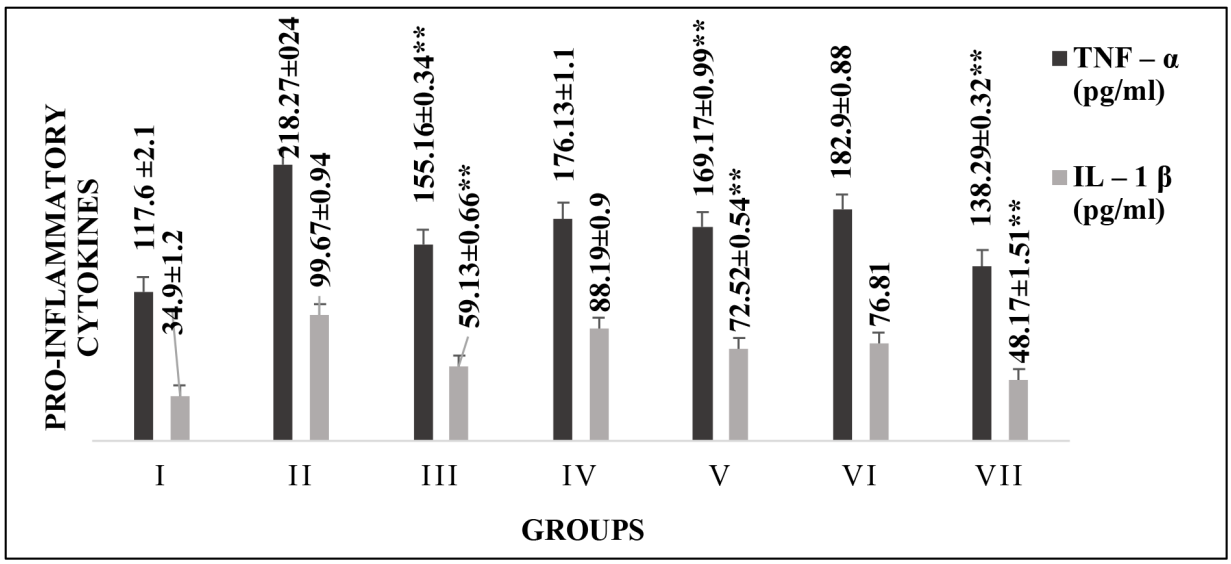

Figure 3. Effect of test drugs on pro-inflammatory cytokines. The data were expressed in Mean ± S.E.M; n=6 in each group; Values were considered to be statistically significant (df =6; F=865.9; df =6; F=1382.86; $^{**}p < 0.001$) when Group VII was compared to Group I, II, III, IV, V and VI. Group VII was found to be significant statistically ($^{**}p < 0.001$) as compared to negative control and positive control animals (Group I & II). Data were analyzed using a one-way ANOVA test followed by Tukey Test.

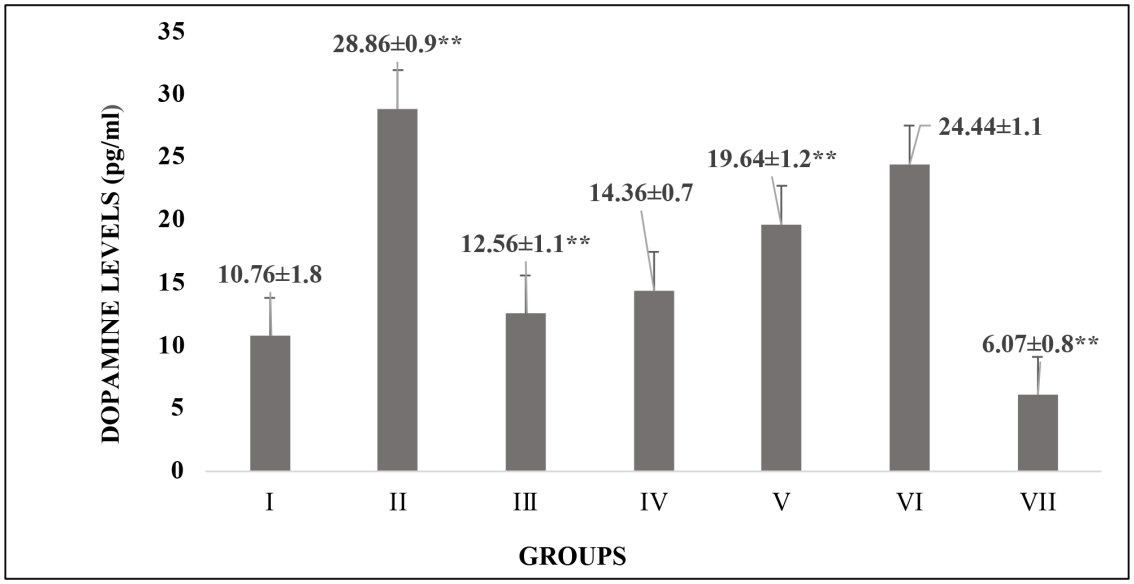

Figure 4. Effect of test drugs on Dopamine levels (pg/ml). The data were expressed in Mean ± S.E.M; n=6 in each group; Values were considered to be statistically significant (df = 6; F= 6.79; ***p* <0.001) when Group VII was compared to Group I, II, III, IV, V, and VI as comparable to negative positive control animals. Data were analyzed using a one-way ANOVA test followed by Tukey Test.

ical parameters as prescribed by USP general chapters. The poppy seed oil monograph is not available in any of the pharmacopeias, so the determined values were compared with those listed in the literature[34], and all values were found to be conforming.

The GC-MS analysis revealed the presence of fatty acids and their esters, and triglycerides as the major chemical class of total components (73.75%) with linoleic acid (41.8%) followed by palmitic acid (11.96%) and triglycerides (7.15%) as the principal components in the poppy seed oil. The volatile components (5.74%), diester (2.30%), plasticizers (2.63%), vitamin E (1.17%), and steroidal substances (4.19%) were also found to be present. Overall, a total of 18 compounds were identified with a mass balance of 92.13%. The details of these compounds are given in (Table III).

In the current study, animals were tested for their behavioral pattern utilizing two models actophotometer for locomotors activity and a Rota rod for muscular coordination. Also, the animals were evaluated for analgesia both peripherally and centrally. In the measurement of locomotor activity, it was found that the poppy seed oil and tramadol test drugs showed reduced locomotor activity at lower doses (1,000 mg/kg and 1.5 mg/kg)[2] when treated in combination. This activity is a measurement of the CNS's excitability. Similarly, the muscle relaxant effect was assessed by the Rota rod apparatus, the test drugs in combination at lower doses showed significant relaxation and muscular coordination by showing a decline in the fall-off time. It also exhibited a depressant effect on the CNS. Subsequently, there was a remarkable effect on locomotor activity and muscle relaxation with the combined treatment of poppy seed oil and tramadol at lower doses.

Inflammation always instigates pain either in acute or chronic form, peripheral or central[35]. Numerous pro-many pro-inflammatory mediators such as interleukin 6 (IL-6), IL-12, interferon (INF-γ), and tumor necrosis factor (TNF) were released in the course of inflammation[36].

Papaver somniferum has gained a lot of medicinal importance of which pain-relieving, anti-anxiety, anti-depressant, anti-tussive and anti-allodynic activities were prominent[37]. Poppy seeds have got a high value and are used for seasoning in Indian cooking, and now the oil obtained from seeds was evaluated in experimental animals. To evaluate the analgesic activity, two different methods were followed, and to experiment with the same animals, a washout period of 7 days was maintained so that the number of animal usage was reduced. The peripheral and central analgesic effects were screened using the acetic-acid writhing method and tail immersion technique. When acetic acid was injected into rats it irritated the serous membrane and provoked a stereotyped

behavior. The abdominal contractions were noted, indicating that the level of pain was due to the release of endogenous substances such as prostaglandins (PGs), serotonin, histamine, bradykinins, and substance P[38]. In the observations, poppy seed oil at a lower dose was found to show a significant analgesic response as compared to tramadol at a low dose. Additionally, the combination of both poppy seed oil and tramadol at low doses was found to be significant in controlling pain impulses. In the tail immersion method, the central analgesic effect was evaluated. This method was found to be effective to differentiate the centrally acting analgesics from peripheral acting. Poppy seed oil at a low dose exhibited a profound increase in reaction time proving a significant analgesic activity effect as compared to the high dose of tramadol. Tramadol as an analgesic showed a significant effect on withdrawal time only at 60 min. On the contrary, the combination of these two drugs showed a significant analgesic effect at 60 and 90 min, respectively. Poppy seed oil at a lower dose showed an anti-nociceptive activity that was due to the presence of stigma sterol which might have a stimulatory action on opioid receptors[39]. Conversely, a study[40] reported that stigma sterol was investigated for its anti-nociceptive action at the receptor level as a cholinergic system is an important modulator of pain. The study also explained that there might be a direct interaction of stigma sterol with the muscarinic receptors or stimulation of acetylcholine release. The analgesic property of poppy seed oil may also be attributed to the presence of vitamin E as this was proved to possess an anti-oxidant effect along with analgesic property[41]. A similar effect was also produced with tramadol but was in combination with poppy seed oil at a lower dose.

Rodents are exposed to chronic mild stress (CMS) which has a series of unpredictable compact stressors like deprivation of food and water, cage agitation, and noise over some time. In this, an array of behavioral changes like fear, confusion, and blooming of anxiety-like behavior are remarkable, hence used as a suitable model for induction of stress in an investigation of anxiety and depression. All groups of animals were subjected to stress chronically and were tested for an anti-anxiety property using an elevated plus maze. In the elevated plus maze model, an animal is explored to an unfamiliar ambiance which enables it to have altered behavior like conflict, avoidance, social withdrawal, and fear. In the current study, the stress-induced rats opted to be in closed arms for more time avoiding open arms. This made clear their fear and aversion towards open spaces. After chronic treatment with the test drugs, the anxiety dropped down, elevating the time spent in open arms[24]. A combined treatment of poppy seed oil and tramadol at lower doses (1,000 mg/kg and 1.5 mg/kg) reduced the fear, anxiety, and aversive feeling which indicated an anti-anxiety-like effect as compared to the stress-induced animals. The effects evinced by poppy seed oil were attributed to the presence of flavonoids, and polyphenolic substances, which might have an effect at the receptor level in the CNS[2]. In the same animals, parameters like pro-inflammatory cytokines and dopamine levels in serum were determined. It was evident that there was an elevation in the serum levels of IL-1β and TNF-α in the stress-induced animals. Few studies[42] had reported that IL-1β as a pro-inflammatory cytokine that might act on CNS, transmitting pain, was also implicated in hyperalgesia, pain, and inflammatory responses. Also, there exists pre-clinical data elaborating the rise in serum TNF-α associated with many psychiatric conditions, reduced societal and analytic behavior, and contextual fear including depression. The inflammation process takes place in two phases. In the first phase, there is the release of histamine and serotonin, and in the second phase, bradykinin, protease, and prostaglandins[5,28]. From the above results, it was observed that there was a potential effect of alleviated levels of both IL-1β and TNF-α with the lower doses of poppy seed oil and tramadol and also with their combination at lower doses. Hence these drugs may be useful in the management of acute and chronic inflammatory musculoskeletal disorders associated with pain, and so might be in the comorbid conditions[43].

Catechol amines play an important role in conflict behavior, with an altered signaling pathway that is related to mental disorders like anxiety. A modified behavior is instigated by stress-induced catecholamine release[30]. The dopaminergic system has a responsible role when a subject is exposed socially, responsible for aversive events, social distress, and anxiety-like attitude, with involvement of the release of dopamine release. Besides, alterations in the receptors of dopamine were noticeable in anxiety disorders and fearful/introverted behaviors[44]. The current study emphasized decreased levels of dopamine significantly with the combined treatment of poppy seed oil and tramadol at lower doses (1,000 and 1.5 mg/kg).

There is a burden of diseases on humankind and needs to be focused on the different treatment strategies. In this regard, many treatments have come into the limelight of which conventional drug therapy, herbal therapy, and complementary therapy were nailed[29]. However, there must be minimum adverse events or side effects with patient compliance. For decades, the innate worth of Phyto medicine has been under investigation and was found successful due to the presence of phytochemicals or secondary metabolites with respective therapeutic value. In the treatment of chronic diseases like type 2 diabetes mellitus, asthma, cancer and certain CNS disorders, long term therapies are needed. In this regard, a combination of medications may be utilized or sometimes required to manage the disease and combat the disease complications. Hence, investigation on combination of drugs might be more advantageous to analyze the efficacy and potentiality of a drug. The present study was taken up to assess the effect of combined therapy besides individual drug treatment using rats as experimental animals.

The present study explored the effect of either poppy seed oil (herbal medicine) or tramadol at lower doses individually or a combination of poppy seed oil and tramadol (herbal and conventional drug) at low doses. Single drug use at higher doses may lead to drug resistance. Hence, the combination of drugs at a lower dose with therapeutic efficacy might be of more value. Moreover, certain diseases like cancer, osteoarthritis, and certain musculoskeletal disorders associated with pain; also mentally related ailments may require chronic treatment for long-term therapy. In such conditions, using any conventional drug therapy for the long term has an impact on various organs of the body, like the kidney and liver, which were the most affected, producing additional side effects[45]. Hence, to minimize the side effects, a combination of drugs may be preferred. The underlying mechanism might be because of synergistic or additive effect[46].

Conclusions

The current study exemplified that treatment with the conjunction of drugs could be advantageous in terms of side effects, efficacy, and safety. Certainly, despite opting for a higher dose of a single drug, a combination of drugs at a low dose could be preferable to clear off the untoward and unexpected outcome along with the drug resistance. Thus, combined therapy may be recommended for the management of chronic diseases and disorders related to behavior, anxiety, pain, and inflammation. Further, it is a need to focus elaborately on the possible interactions (herbal drug and synthetic drug) between these drugs by evaluating the pharmacokinetic parameters. Hence more pre-clinical data is essential to establish its long-term use and safety.

Conflict of Interest

The Authors declare that they have no conflict of interests.

Ethics Approval

This research study was given approval by the standing committee for Scientific Research Ethics of the university HAPO-10-Z-001 number (REC41/1-035).

Authors' Contribution

YN designs the work-ES handles animals-YN, GK, WH, AM, RK, and HR perform behavior tests-MF, and TA perform Elisa experiments-ZR performs the chromatography technique-YN, and KK perform the dopamine test-YN collection of data-YN, SR, KK and SS analysis and interpretation of data-YN and SR writing the original draft, SS review and edit the original draft.

Data Availability Statement

The combined datasets were available from the corresponding author (Yousra Nomier) on reasonable request.

Funding

Authors are thankful to the Future scientist 7 program-Deanship of Scientific Research-Pharmacy College-Jazan University, KSA for support, continuous encouragement and funding project No. FS10-045.

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