Antinociception Induced by *Moringa* *Stenopetela* (Baker f.) Cufod. Leaves Extract and Possible Mechanisms of Action

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*Moringa stenopetala* (Baker f.) Cufod., is an endemic species growing in the south of Ethiopia. *M. stenopetala* is often consumed as food and used in traditional medicine and it has also been traditionally used for relieving of pain in Ethiopia. This study aimed to investigate the antinociceptive effect and mechanisms of action of *M. stenopetala* leaves methanol extract in mice. The per-oral doses of 50, 100, and 200 mg/kg of *M. stenopetala* extract were tested for antinociceptive action by using hot-plate, tail-immersion, and writhing tests. The possible mechanisms of the antinociceptive action were investigated by pre-treatment with 5 mg/kg naloxone (non-selective opioid antagonist), 1 mg/kg ketanserin (5-HT₂A/₂C receptor antagonist), and 1 mg/kg yohimbine (α₂ adrenoceptor antagonist). The methanol extract of *M. stenopetala* showed antinociceptive effect in all tests. The significant involvement of 5-HT₂A/₂C receptors and α₂ adrenoceptors in antinociception induced by *M. stenopetala* extract in the hot-plate and tail-immersion tests, as well as significant contribution of opioid receptors and α₂ adrenoceptors in writhing test, were identified. In conclusion, these findings demonstrate that the methanol extract of *M. stenopetala* has potential in pain management. This study will contribute to new therapeutic approaches and provide guidance for new drug development studies.

**Keywords:** *Moringa stenopetala*. Pain. Adrenoceptor. Serotonin receptor. Opioid receptor.

INTRODUCTION

Pain is an unpleasant sensory and emotional experience associated with actual or potential tissue damage, or described in terms of such damage (Merskey, Boduk, 1994). There are various analgesic drugs in the market, however, patients often complain about side-effects of these drugs. Thus, new analgesic drug discovery studies are in process. Additionally, patients tend to phytotherapy that is thought to have fewer side effects, especially in recent years (Sengupta et al., 2012). The term “phytotherapy” is defined as the treatment of diseases using extracts of fresh or dried plant parts (drug) with therapeutic value. Nowadays, the plants used in phytotherapy are loadstar in the development of modern medicines (Petrovska, 2012). However, the asserted pharmacological effects and mechanisms of action of these medicinal plants should be investigated.

The Moringaceae family comprises 33 different species. *M. stenopetala* (Baker f.) Cufod., African Moringa Tree and locally called Haleko, is an endemic species growing in the south of Ethiopia, north of Kenya, and east of Somalia (Seifu, 2014; Daba, 2016). It grows in rocky areas close to the water as well as in dry areas (Melaku et al., 2017). It has high nutritional value; therefore, the leaves of *M. stenopetala* are consumed as vegetable (Abdull Razis, Ibrahim, Kntayya, 2014). It has also been used as a medicinal plant among folk.
It was reported that *M. stenopetala* have been used by the local people in curing malaria, hypertension and stomach pain (Mekonnen, Gessesse, 1998). Studies have shown at laboratory animals that Moringa species has antihypertensive (Mengistu *et al.*, 2012), antidiabetic (Jaiswal *et al.*, 2009), antispasmodic, antiinflammatory and diuretic activity (Cáceres *et al.*, 1998). Besides, the leaves of *M. stenopetala* have been evaluated for their antihyperglycemic efficacy and reported to decrease the levels of fasting blood sugar, serum total cholesterol and triglyceride in rats (Muhammad *et al.*, 2013; Toma *et al.*, 2015). *M. stenopetala* has also been used for pain management in Ethiopia (Ghebreselassie *et al.*, 2011; Seid, 2013; Seifu, 2014). However, there is no detailed study on its antinociceptive effect. Geremew *et al.* (2015) examined the effect of 80% methanol extract of *M. stenopetala* leaves in the experimental pain models. They have showed that *M. stenopetala* extract generated significant antinociceptive effect in a dose-dependent manner. However, the mechanisms of action of *M. stenopetala* on antinociception have not been elucidated yet. Revealing the mechanism of action of drugs is highly important for rational drug use. Therefore, the present study was performed with a view to investigate the antinociceptive effect and also the mechanisms of action of 80% methanol extract of *M. stenopetala* leaves (MSE) by using hot-plate (integrated supraspinal response), tail-immersion (spinal reflex) tests and writhing (peripheral response) in mice.

**Phytochemical screening**

**Alkaloid detection**

*Hager's test:* 50 mg of extract was mixed with 3 ml of dilute hydrochloric acid (HCl) and filtered. 1 mL of Hager’s reagent (picric acid solution) added to 2 mL of filtrate. The formation of yellow precipitate indicates the presence of alkaloids.

*Dragendorff test:* A few mL of filtrate was mixed with 1 or 2 mL of the Dragendorff reagent (potassium bismuth iodide solution). The significant yellow precipitate formation is evaluated as the positive test result.

**Carbohydrates detection**

*Molish’s test:* 100 mg of extract was dissolved in 5 mL of distilled water and filtered. 2 mL of filtrate was treated with 2 drops of 15% α-naphthol alcoholic solution and dissolved together. 1 mL of concentrated sulfuric acid added slowly from the edge of the tube. Formation of violate ring indicates the presence of carbohydrate.

*Fehling’s test:* 100 mg of the extract was dissolved in 5 mL of distilled water and filtered. The filtrate was diluted with 6 mL of HCl and neutralized by heating with 2 mL of Fehling A and B solution. Formation of red precipitate indicates the presence of reducing sugar.

**Glycoside detection**

*Bornträger test:* Concentrated HCl was added to 50 mg of extract and left to stand in a water bath for 2 hours. After filtration, 2 mL of filtrate was shaken well with 3 mL of chloroform. When the chloroform layer was separated. 10% ammonia solution was added. A positive reaction is considered when the color of the solution changes to pink.

*Sulfuric acid test:* 100 mg extract was dissolved in 5 mL distilled water and filtered. 1 mL of concentrated sulfuric acid was added to 1 mL of the test solution. Waited for 2 minutes, 100 mg extract was dissolved in 5 mL distilled water and filtered. The reddish color formation is evaluated as the positive test result.

**Saponin detection**

*Frothing test:* In this test, 50 mg of the extract was shaken with 20 mL of distilled water. The formation of

**MATERIAL AND METHODS**

**Plant materials and preparation of the methanol extract of *M. stenopetala* leaves**

The plant was collected from the Arbaminch region of the south of Ethiopia in September 2015. It was identified and deposited with a voucher number AL-001 by Taxonomist Dr Dawit Abebe at Herbarium of Ethiopian Public Health Institute, Addis Ababa, Ethiopia. *M. stenopetala* (Baker f.) Cufod. dried leaves were used to obtain the methanol extract. First, the dry leaves were pulverized. Then, 1000 g of 80% methanol was added to 150 g weighed leaves and the mixture was shaken using a shaker for 72 h at room temperature. The extract was filtered using a filter paper. The methanol in the filtrate was evaporated on the rotavapor, and the remaining aqueous extract was lyophilized, yielding *M. stenopetala* (22.85 g, 15.85% w/v) extract.
approximately 2 cm of foam layer indicates the presence of saponins.

**Phenolic compound detection**

**Lead acetate test:** In this test, 20 mg of the extract was dissolved in distilled water. Then, 3 mL of 10% lead was added to acetic solution. The white precipitate formation indicates the presence of phenolic compounds.

**Detection of total content of phenolic substances**

The Folin–Ciocalteu method was used to determine the total content of phenolic substances. The total phenolic content was expressed as milligram gallic acid (mg gallic acid/graft).

Three different concentrations of the extract (10, 5, and 1 mg/mL) were prepared. After adding 3.9 mL of purified water into 50 μL of the sample, 250 μL of Folin was added to the sample. Then, 750 μL of 20% (w/v) Na₂CO₃ solution was added and incubated at 25 °C for 2 h in the dark. Absorbance was observed at 760 nm using a spectrometer. The procedure was repeated three times for different concentrations. For the gallic acid calibration chart, a stock solution was prepared by dissolving 0.1 g gallic acid in 1 mL of methanol. In the stock solution, dilutions were prepared at concentrations of 0.8, 0.6, 0.4, 0.2, and 0.1 mg/mL. Each procedure was repeated three times for each concentration. Gallic acid concentration was plotted corresponding to the absorbance values (Singleton, 1999).

**LCMS-IT-TOF system analysis**

The LCMS-IT-TOF is a new type of mass spectrometer that combines QIT (ion trap) and TOF (time-of-flight) technologies. The LCMS-IT-TOF analysis was performed using a hybrid IT-TOF mass spectrometer with ESI interface (both positive and negative mode; Shimadzu, Kyoto, Japan). The positive ESI conditions were as follows: high-voltage probe, −3.5 kV (for negative ESI, it was 3.5 kV); nebulizing gas flow, 1.5 L/min; CDL temperature, 200 °C; heat block temperature, 200 °C; and drying gas pressure, 200 KPa. The CID parameters were settled 50% for CID energy and 50% for collision gas parameter. Argon gas was used for CID. The detector voltage of TOF was 1.6 kV. A solution of trifluoroacetic acid and sodium hydrate was consumed as the standard sample for postrun calibration. The LC part comprised two LC-20AD dual pump, a DGU-20A3R degasser unit, a CTO-10ASvp column oven, a SIL-20AC autosampler, and an SPD-M20A PDA detector. In the analysis, Inertsil ODS-3 (150 × 1.5 mm², 5 μm) was used as the stationary phase, and a mix of water/acetonitrile (0.1% formic acid) was used gradually as the mobile phase. The data were processed using LCMS solution software (v. 3.80).

**In vivo Experiments**

**Animals**

Male and female Swiss mice (weighing 30–35 g) were equally divided into eleven groups. The mice were housed in a well-ventilated room with controlled temperature (22 °C ± 2 °C) and 12-h light/12-h dark cycle. The animals were supplied from the Osmangazi University Center of Animal Experiments and Research, Eskisehir, Turkey. Animal care and research protocols were based on the principles and guidelines adopted by the Guide for the Care and Use of Laboratory Animals (NIH Publication No. 85-23, revised in 1985) and approved by the local ethics committee of Osmangazi University, Eskisehir, Turkey (Approval No: 470-1).

**Drugs and treatments**

All drugs were purchased from Sigma Aldrich (St. Louis, U.S.A.) and dissolved in saline. The methanol extract of *M. stenopetala* (MSE) was administered orally (p.o.) at the doses of 50, 100, and 200 mg/kg. The control group received only saline. 500 mg/kg dipyrone (p.o.) was used as a reference drug. 5 mg/kg naloxone hydrochloride dihydrate, an opioid receptor antagonist, was injected 15 mins before, serotonin 5HT₂A/2C receptor antagonist 1 mg/kg ketanserin(+)-tartrate and α₂-adrenoceptor antagonist 1 mg/kg yohimbine hydrochloride were injected 30 mins before the saline or 100 mg/kg MSE administrations to investigate the mechanisms underlying the antinociceptive effect of MSE. All antagonists were injected intraperitoneally (i.p.).

**Hot-plate test**

The hot-plate test (No. 7280, Ugo Basile Instruments, Comerio, Italy) developed by Eddy and Leimbach (1953) was applied to evaluate the thermal pain threshold. The mice were placed on a heated plate (55 ± 0.5 °C). The latency of hind paw licking, hind paw
flicking, or jumping were measured as reaction time. Measurements taken before the drug administrations were recorded pre-drug latency. Measurements taken 45 min after the last drug administration were recorded post-drug latency. The cut-off time was limited to 20 s to avoid damage to the hind paw.

**Tail-immersion test**

The spinal reflexes were evaluated using the tail-immersion test described by Schmauss and Yaksh (1984). The painful reactions in mice were induced by thermal stimuli by dipping the tail tips into a hot water bath (Heto, Allerod, Denmark) at 52.5 °C ± 0.2 °C. The withdrawal time of the tail was recorded as the reaction time. Measurements were taken before the first drug administration and 45 min after the last drug administration. The cut-off time was limited to 15 s to avoid tissue damage to the tail.

**Acetic acid–induced writhing test**

The acetic acid–induced abdominal writhing test was used to evaluate the peripheral pain response in mice (Gawade, 2012). This method is based on the contraction of abdominal cavity by injection of acetic acid. 10 mL/kg of 0.6% acetic acid solution was injected i.p. 45 min after the last drug administration. The number of writhing was recorded for 10 min after the 5-min waiting period (Koster, Anderson, Beer 1959).

**Statistical analysis**

The results of the hot-plate and tail-immersion tests were expressed as a percentage of the maximal possible effect (MPE %), which was calculated by using the latencies of response to thermal stimuli (Coelho et al., 2005):

\[
\text{MPE}\% = \frac{[(\text{Postdrug latency}) - (\text{Predrug latency})]}{[(\text{Cutoff time}) - (\text{Predrug latency})]} \times 100
\]

All values obtained in this study were determined as the arithmetic means of the individual experimental data (mean ± standard error of mean). Differences between the groups were calculated statistically using the one-way analysis of variance followed by the post-hoc Tukey’s test. The GraphPad Prism version 5.0 software was used for all data analyses. \( P \) value <0.05 was considered as significant.

**RESULTS AND DISCUSSION**

**Phytochemical screening**

**Determination of total phenolic content**

The total phenolic content in methanol extract of *M. stenopetala* was determined using the Folin–Ciocalteu method and calculated as 187.5 ± 12.4 mg GAE/g extract (Table I). Additionally, the results of the qualitative analysis are shown in Table II.

**TABLE I - Total phenolic content in the extract**

| Extract concentrations mg/gallic acid/extract ± STD |
|----------------------------------------------------|
| 10 mg/Ml                                            |
| 5 mg/mL                                             |
| 1 mg/mL                                             |

**TABLE II - Results of qualitative phytochemical analysis**

| Alkaloid       | Carbohydrate | Reducing sugar | Glycoside          | Saponin |
|----------------|--------------|----------------|--------------------|---------|
| Hager’s test   | Molish’s test| Fehling’s test  | Borntrager test    | Frothing test | Gelatin test |
| +              | +            | +              | -                  | -       | -            |
| Dragendorff test|              |                |                    | Sulfuric acid test |
| +              |              |                |                    | -       |              |

(continuing)
TABLE II - Results of qualitative phytochemical analysis

| Phenolic compound | Flavonoid | Mucilage | Phytochemicals | Fixed oil | Terpene |
|-------------------|-----------|----------|----------------|-----------|---------|
| Lead acetate test | Iron chloride test | Whistler and BeMiller test | Libermann-Burchard test | Spot test | Salkowski test |
| +                 | +         | -        |                | +         | +       |
| Iron chloride test |           |          |                |           | +       |

**LCMS-IT-TOF system analysis**

In high-resolution mass spectrometer analysis, the extracts (1 µL) were dissolved in methanol and injected into the system. Both the positive and negative mode results were evaluated based on previous studies and the DrugBank database (https://www.drugbank.ca/). Luteolin, rutin, and quercetin were determined as contents of MSE from the spectrum (Figure 1).

**FIGURE 1** - Selected ion chromatogram of rutin (611.1587), quercetin (303.0487) and luteolin (287.0533). 160x97mm (300 x 300 DPI)
Antinociceptive effect of methanol extract of *M. stenopetala*

The MPE % values calculated from the hot-plate and tail-immersion tests and the number of writhing are shown respectively in Figure 2. 50, 100, and 200 mg/kg MSE significantly (*P* < 0.01, *P* < 0.001 and *P* < 0.001, respectively) extended the response to thermal stimulus as dipyrone was shown to induce (*P* < 0.001) compared to control group in hot-plate test (A). Similarly, it was observed that the MPE % of 50, 100, and 200 mg/kg MSE on thermal threshold were significantly (*P* < 0.001) high in a similar manner as the MPE % of dipyrone in tail-immersion test (B). Administration of 50, 100, and 200 mg/kg MSE significantly (*P* < 0.001) reduced the number of writhing (C) in the acetic acid-induced writhing test. The number of writhing was also significantly (*P* < 0.001) decreased by the injection of 500 mg/kg dipyrone compared to control group.

![Graph showing antinociceptive effect of MSE at different doses](image)

**FIGURE 2** - Antinociceptive effect of MSE. Antinociceptive effect of MSE at the doses of 50, 100, 200mg/kg in hot plate test (A) in tail immersion test (B), in acetic acid induced writhing test (C). Values are expressed as means±SEM. (n=8 each group). **P<0.01 and ***P<0.001: significant difference from control group. MPE%: The percentage of the maximal possible antinociceptive effect.

The mechanisms of action of methanol extract of *M. stenopetala*

The effects of the pre-treatments with serotonin 5-HT<sub>2A/2C</sub> receptor antagonist ketanserin, α<sub>2</sub>-adrenoceptor antagonist yohimbine, and non-selective opioid receptor antagonist naloxone MPE % of 100 mg/kg MSE in the hot-plate (A), tail-immersion (B) and writhing tests (C) are shown in Figure 3. The antagonism did not occur with naloxone pre-treatment in hot-plate and tail-immersion tests while significant (*P*<0.01) antagonism was observed in writhing test. Ketanserin pre-treatment significantly prevented the increase in the MPE % of 100 mg/kg MSE on thermal threshold in hot-plate (*P*<0.05) and tail-immersion (*P*<0.001) tests. However, ketanserin pre-treatment did not antagonized the reducing effect of 100 mg/kg MSE on the number of writhing. In the presence of yohimbine, a significant inhibition in MPE % on thermal thresholds in hot-plate and tail-immersion tests, and also a decrease in number of writhing, were obtained (*P*<0.01; *P*<0.001, *P*<0.001; respectively).

The present study revealed the antinociceptive effect of MSE in acetic acid-induced writhing, tail-immersion, and hot-plate tests and the action mechanisms of antinociception induced by MSE.

The hot-plate and tail-immersion tests are frequently used for evaluating pain response in basic pain research studies (Milind, Monu, 2013). These tests also suggest the level of organization of the effectiveness. It means that the response in tail-immersion test is a simple spinal reflex whereas the hot-plate test is a behavioral model of nociception in which more organized behaviors that are managed by
supraspinal mechanisms, are revealed (Flores et al., 2004). Additionally, the acetic acid-induced writhing test, a visceral pain model, is a common assessment tool that is frequently used to assess peripheral antinociception (Wang et al., 2014). Based on the tests results, it was suggested that MSE has antinociceptive effect managed by central and peripheral organizations since MSE treatment prolonged the latency of pain response in hot-plate and tail-immersion tests and also decreased the pain response in writhing test. It is known that *M. stenopetala* and other *Moringa* species are rich in proteins, amino acids, vitamins, and phenolic substances (Abdull Razis, Ibrahim, Kntayya, 2014). Additionally, *Moringa* species comprise high levels of flavonoids (Mbikay, 2012; Mohammed, Manan, 2015). The contents of MSE were also confirmed in the analysis carried out in the present study. The total phenolic content of methanol extract of *M. stenopetala* is calculated as 187.5 ± 12.4 (mg of gallic acid equivalent-GAE/g extract). As observed from the LCMS-IT-TOF system results, it was determined that MSE contains high amounts of luteolin, quercetin and rutin. These compounds are biologically active substances and it has been reported that they have anticancer, anti-oxidant, anti-inflammatory and antinociceptive effects (Sun et al., 2012; Nazari et al., 2013; Chian et al., 2014). In addition to these pharmacological effects, it has also been shown that luteolin possesses antinociceptive effect in mechanical and cold hyperalgesia tests on neuropathic pain models (Hara et al., 2014; Hashemzaei et al., 2017) and rutin have antinociceptive effect in formalin and glutamate-induced paw-licking tests (Hernandez-Leon, Fernandez-Guasti, Gonzalez-Trujano, 2016, Lapa Fda et al., 2009). On the other hand, it has been demonstrated that quercetin inhibits nociceptive behavior in acetic acid-induced writhing and formalin-induced pain tests (Willain Filho et al., 2008). Therefore, in this study, the antinociception induced by MSE was believed to be mainly a result of presence of luteolin, quercetin, and rutin in MSE.

![FIGURE 3 - Antinociceptive mechanism of MSE.](image-url)

Pain control is a more complex process that includes numerous peripheral and central mechanisms interacting with each other (Schaible, 2007). In this study, the role of noradrenergic, serotonergic and opioid systems, primary modulatory systems located in inhibitory pain pathways, in antinociceptive effect of MSE was investigated. It was found that noradrenergic and serotonergic systems contribute to central antinociception, whereas noradrenergic and opioid systems contribute to peripheral antinociception induced by MSE. Although various neurochemicals and receptors are located in the ascending and descending inhibitory pain pathways, serotonin and noradrenaline have been accepted as the fundamental
mediators of endogenous antinociceptive mechanisms in the descending pain pathways (Marks et al., 2009). It is known that the activation of presynaptic and postsynaptic $\alpha_2$-adrenoceptors found in noradrenergic inhibitory pain pathways mostly modulate the antinociception induced by noradrenaline (Pertovaara, 2006). Yohimbine, $\alpha_2$-adrenoceptor antagonist, was used to investigate the role of $\alpha_2$-adrenoceptors in the antinociception induced by MSE in this study. It significantly decreased the antinociceptive effect of MSE in the hot-plate and tail-immersion tests. Additionally, serotonin 5-HT$_{2A/C}$ receptor antagonist ketanserin was used to examine the contribution of the serotonergic pathway. Serotonin 5-HT$_{2A/C}$ receptor subtype is abundant in C fibers which transmit the noxious thermal stimuli to the dorsal horn (Sommer, 2010). According to test results, the contribution of serotonin 5-HT$_{2A/C}$ receptors was shown in hot-plate and tail-immersion tests. Thus, it was concluded that both serotonin 5-HT$_{2A/C}$ receptors and $\alpha_2$-adrenoceptors, in serotonergic and noradrenergic descending pathways respectively, are involved in the central antinociceptive effect of MSE. The opioid system also takes a significant part in pain control by own self and modulating descending pathways (Kanjhan, 1995). Non-selective opioid antagonist naloxone was used to investigate the role of opioid system in antinociceptive effect of MSE in this study. Naloxone is a non-specific antagonist for opioid Mu ($\mu$), kappa ($\kappa$), and delta ($\delta$) receptors (Al-Hasani et al., 2011). Although a significant antagonism was not observed in naloxone pre-treated group, relative antagonism was determined and the efficacy of MSE in antinociception was decreased by pre-treatment with naloxone in the hot-plate and tail-immersion tests. When the interaction of serotonin and noradrenaline with opioids in descending inhibitory pathways is considered, it may be claimed that the opioid system also partially contribute to central antinociceptive effect of MSE according to test results.

The relieving of acute nociceptive, neuropathic and inflammatory pain depend on the peripheral activation of primary sensory afferent neurons which express various inhibitory neuronal receptors incorporating $\alpha_2$-adrenergic, cholinergic, opioid, adenosine, and also cannabinoid receptors (Sawynok, 2003). Although noradrenaline was reported to cause peripheral pain through $\alpha_2$-adrenergic receptors, some studies showed that stimulation of peripheral $\alpha_2$-adrenoceptors on inflammatory cells suppressed pain response via the release of endogenous opioid peptides such as $\beta$-endorphin from immune system cells. The release of these peptides results in an analgesic effect by the attenuation of the excitability of sensory nerves via their own receptors ($\mu$, $\kappa$ and $\delta$) or antagonizing the release of pro-inflammatory peptides (Binder et al., 2004; Vadivelu, Mitra, Hines, 2011). The contribution of $\alpha_2$-adrenoceptors and opioid systems to peripheral antinociception induced by MSE was suggested since naloxone and yohimbine pre-treatments prevented the decreasing of writhing count induced by MSE in the acetic acid-induced writhing test. Therefore, it was concluded that the peripheral antinociceptive effect of MSE is mostly exerted via $\alpha_2$-adrenoceptors found in inhibitory noradrenergic pathways and also via opioid system.

**CONCLUSION**

This study demonstrated the central and peripheral antinociceptive effects of *M. stenopetala* induced by centrally originated noradrenergic and serotonergic mechanisms and peripheral originated noradrenergic and opioidergic mechanisms. The antinociceptive effect of MSE may be due to luteolin, rutin, and quercetin contents. In addition to its antinociceptive effect, *M. stenopetala* also has low toxicity profile since it has been demonstrated that the water extract of *M. stenopetala* leaf at different doses did not induce any toxicity/mortality up to 5000 mg/kg dose in the toxicity experiments (Ghebreselassie, et al., 2011). As a whole, these findings reinforce that MSE has potential in pain management. This study also supports the traditional utilization of *M. stenopetala* for relieving pain and, will contribute to new therapeutic approaches and provide guidance for new drug development studies.

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Conflict of interest

The authors declared that there are no conflicts of interest.

Author contributions

Yusuf OZTURK and Rana ARSLAN designed the experiments. Muna Cemal HUSSEIN and Rana ARSLAN performed the experiments. Nurcan BEKTAS and Rana ARSLAN analyzed the data and wrote the manuscript. All of the authors have read and approved the final manuscript.

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