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Original Article

Effect of Moringa Oleifera Extract on Behavior Using Male Albino Mice

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ABSTRACT

Background and objectives: Moringa oleifera belonging to the family of Moringaceae, where it is a monotypic taxon with 13 known species. It is widely growing in tropical and subtropical countries, especially in northwest India, Africa, Arabia, South East Asia, the Pacific, and Caribbean Islands, and South America. The present study deals with the preliminary phytochemical screening and investigates the effect of M. oleifera on behavior by applying the Irwin test and maze; also, to find out if the extract works through benzodiazepine receptor by using Flumazenil as the antagonist. Materials and Methods: The leaves of M. oleifera were collected in (September), 2019 at "Tajoura, Libya. The leaves were removed from other parts then dried in a dark place for 3 days and ground. The crude extract subjected for analysis of the presence of active constituents such as tannins, alkaloids, phytosterols, triterpenoids, flavonoids, cardiac glycosides, saponins, carbohydrates, proteins. The Behavior study was carried out using male albino mice. Mice were randomly assigned to seven groups according to the drug administration, intraperitoneal sub-acute administration was applied (24, 5, 1 hour before scoring). In both experiments, Diazepam (5mg/kg) was used as standard and Flumazenil (0.1mg/kg) was used as a benzodiazepine antagonist. Results: Phytochemical screening indicate the presence of alkaloid, triterpenoid, flavonoid, and carbohydrate in the extract. M. oleifera reduced the SMA, Reactivity, and antagonize each of Restlessness, Irritability, and fearfulness in the Irwin test. The combination of M. oleifera with Flumazenil produced a synergistic decrease of SMA in the Plus maze. Conclusion: Moringa oleifera have a CNS depressant like effect; this effect could be through benzodiazepine receptor because this effect is antagonized by flumazenil. The combination of M. oleifera Extract with Flumazenil produces a synergistic effect toward sedation action. This result needs more study to investigate their mechanism of action. M. oleifera extract does not affect anxiety measure but decreases the SMA. This means that the active constituents in moringa extract may act on BZR1 and not on BZR2.

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INTRODUCTION

Moringa oleifera is belonging to the family of *Moringaceae*, where it is a monotypic taxon with 13 known species. *Moringa* is one of the most useful trees in the world, and it is known as a miracle tree [1]. It is a small or medium-sized tree, a fast-growing evergreen or deciduous tree. It usually grows up to 10 to 12m in height; it has an open crown of drooping fragile branches, feathery foliage of tripinnate leaves, and thick corky, whitish bark [2]. It is widely growing in tropical and subtropical countries, especially in northwest India, Africa, Arabia, South East Asia, the Pacific, and Caribbean Islands, and South America [3]. *Moringa* is easily cultivated and can use all parts of the plant (leaves, pods, and seeds) where it contains many essential phytochemicals that are needed for malnutrition [4].

It is considered a source of vitamin A and vitamin C; it contains a high amount of vitamin B and minerals [2]. It is rich in proteins, β-carotene, calcium, potassium; it acts as a good source of natural antioxidants, due to the presence of various types of antioxidant compounds such as ascorbic acid, flavonoids, phenolics, and carotenoids, which enhance the shelf life of fat-containing foods [5]. *Moringa* can be used in the treatment of different types of diseases because it has a wide variety of pharmacological properties in different parts of the tree including leaves, roots, seed, bark, fruit, flowers, and immature pods [5]. The different preparations of *M. oleifera* used for their anti-inflammatory, antihypertensive, diuretic, antimicrobial, antioxidant, antidiabetic, antihyperlipidemic, antineoplastic, antipyretic, antiulcer, cardioprotectant, and hepatoprotectant activities [6]. *M. oleifera* extract was also reported to have a diuretic effect [7]. The aim of this study is to investigate the effect of *M. oleifera* on behavior by applying the Irwin test plus maze, also to find out if the extract works through benzodiazepine receptor by using Flumazenil as an antagonist.

MATERIALS AND METHODS

Chemicals and Drugs

Ethanol was purchased from BDH Limited, England. T80 was purchased from MERCK-Schuchard, Hohenbrunn bei Munchen Germany. Diazepam obtained from Ratiopharm Gmbh, Germany. Flumazenil was obtained from Mylan, Saint-Priest, France.

Plant collection and extraction

Leaves of Moringa oleifera were used for the study. The plant material was identified at the Botany Department, Faculty of Sciences, University of Tripoli. The fresh leaves were collected on September 22, 2019 (Tajoura, Libya), and dried in cool/dark conditions. Then it is ground into coarse powder and stored in a dark area at room temperature. The leaves powder was macerated (500 g) in 2500 ml ethanol for 3days. Then filtered by the filter paper and evaporate filtrate to dryness in a rotary evaporator; a brownish residue will be obtained.

Phytochemical screening

The stock solution is prepared by dissolving 1g of the extract in 100ml of ethanol (1% W/V). The solution is used in tests for analysis of the presence of active constituents such as tannins, alkaloids, phytosterols, triterpenoids, flavonoids, cardiac glycosides, saponins, carbohydrates, proteins following standard methods [8].

Tannins

Ferric chloride test: Add few drops of 5% ferric chloride solution to 2 ml of the test solution. The formation of blue color indicates the presence of hydrolyzable tannins.

Gelatin Test: Add five drops of 1% gelatin containing 10% sodium chloride to 1 ml of the test solution. The formation of white precipitates confirmed the test.

Alkaloids

Approximately 50 mg of extract was dissolved in 5 ml of distilled water. Further, add 2M hydrochloric acid until an acid reaction occurs then filtered. The filtrate was tested for the presence of alkaloids as detailed below.

Dragendorff's Test: To 2 ml of the filtrate, 1 ml of Dragendorff's reagent was added along the side of the test tube. The formation of orange or orange reddish-brown precipitate indicated the test as positive.

Mayer's Test: two drops of Mayer's reagent were added along the sides of the test tube containing 1ml of test solution of the extract. A white or a creamy precipitate confirmed the test as positive.

Hager's Test: To 1 ml of test solution or filtrate, a drop or two of Hager's reagent was added. The formation of yellow precipitate indicated the test as positive.

Wagner Test: Two drops of Wagner's reagent were added to 1ml of the test solution along the side of the test tube. The formation of yellow or brown precipitate confirmed the test as positive for alkaloids.

Phytosterols

Liebermann-Burchard's Test: The extract (2 mg) was dissolved in 2 ml of acetic anhydride, heated to boiling, cooled and then 1 ml of concentrated sulfuric acid was added along the side of the test tube. A brown ring formation at the junction and the turning of the upper layer to dark green color confirmed the test for the presence of phytosterols.

Triterpenoids

Salkowski test: Approximately 2 mg of dry extract was shaken with 1 ml of chloroform, followed by few drops of concentrated sulfuric acid were added along the side of the test tube. A red-brown color formed at the interface indicated the test as positive for triterpenoids.

Flavonoids

Shinoda test: Four pieces of magnesium turnings and 5 drops of concentrated hydrochloric acid were added dropwise to 1 ml of the test solution. A pink, scarlet, crimson red, or occasionally green to blue color appeared after few minutes, confirmed the test.

Alkaline reagent test: The addition of 5 drops of 5% sodium hydroxide to 1 ml of the test solution resulted, an increase in the intensity of the yellow color, which became colorless on the addition of a few drops of 2 M hydrochloric acid, which indicated the presence of flavonoids.

Saponins

Foam Test: 5 ml of the test solution is taken in a test tube was shaken well for five minutes. The formation of stable foam confirmed the presence of saponins.

Olive oil test: Added a few drops of olive oil to 2ml of the test solution and shake well. The formation of soluble emulsion confirmed the presence of saponins.

Cardiac glycosides

Keller -Killiani test: Add 0.4 ml of glacial acetic acid and a few drops of 5% ferric chloride solution to a little dry extract. Further 0.5 ml of concentrated sulfuric acid was added along the side of the test tube carefully. The formation of blue color in the acetic acid layer confirmed the presence of cardiac glycosides.

Carbohydrates

Fehling test: Dissolved 2 mg dry extract in 1 ml of distilled water and added 1ml of Fehling's(A+B) solution, shake and heat on a water bath for 10 minutes. The brick-red precipitate formed confirmed the presence of carbohydrates.

Proteins

Biuret test: To 2 ml of the test solution add 5 drops of 1% copper sulfate solution and 2 ml of 10% NaOH. Mix thoroughly; the formation of purple or violet color confirmed the presence of proteins.

Behavior study

The experiment was carried out using male albino mice (25-30g). The mice were acclimatized under standard laboratory conditions at temperature 22-25°C and were kept in 12 hr day and night cycle with food and water for 7 days before conducting experiments. All the experimental procedures and protocols used in this study were approved and is carried out within the frame of the department of pharmacology and clinical pharmacy ethics in the faculty of pharmacy. The mice were randomly assigned to seven groups. Intraperitoneal sub-acute administration was applied (24, 5, 1 hour before scoring). Diazepam (5mg/kg) was used as standard and Flumazenil (0.1mg/kg) was used as a benzodiazepine antagonist.

Irwin test: One hour after the last dose of administration, the mouse was observed for one hour [9].

Elevated Plus Maze: it is composed of two open arms (30*5cm) and two close arms (30*5*15cm) that extended from a common central platform (5*5cm). The apparatus was elevated to a height of 45 cm above floor level [10]. Mice were gently handled by the right hand and placed on the center squire of the maze facing into the close arm. The different parameters were scored to evaluate the anxiolytic effect and spontaneous motor activity in the elevated plus-maze, which include: time spent by the mouse in each of the arms, lines crossed in close or open arms, and the number of entries into close or open arms. An arm entry was defined as the entry of all four paws into the arm [11]. The total line crossed and the total number of entries were calculated; the total line crossed and the total arm entries express the spontaneous motor activity [12, 13]. Anxiety measures were calculated by the time spent in close arms by the total time of the test [13]. The duration of the test was 4 minutes.

RESULTS

Phytochemical screening of M. oleifera

Table 1 showed that tannin, alkaloids, triterpenoids, flavonoids, carbohydrates, and proteins are present as active constituents in methanolic extract.

Table 1: Active constituents detected in methanolic extract of Moringa

Tannin	Alkaloids	Phytosterols	Triterpenoids	Flavonoids	Saponins	Carbohydrates	Proteins
Present	Present	Absence	Present	Present	Absence	Present	Present

Irwin test

The preliminary results of the Irwin test showed that there is a significant decrease in spontaneous motor activity (SMA) compared to the Control in each Diazepam treated group (p=0.007), Extract 500mg/kg treated group (p=0.001). Extract 250mg/kg treated group produces a partial decrease in SMA (0.059) compared to the control-treated group. Combined treatment of moringa extract in a dose of 500mg/kg and flumazenil, and moringa extract in a dose of 250mg/kg with flumazenil, also flumazenil alone treated groups did not change the SMA compared to the control-treated group (p> 0.05) (Figure 1).

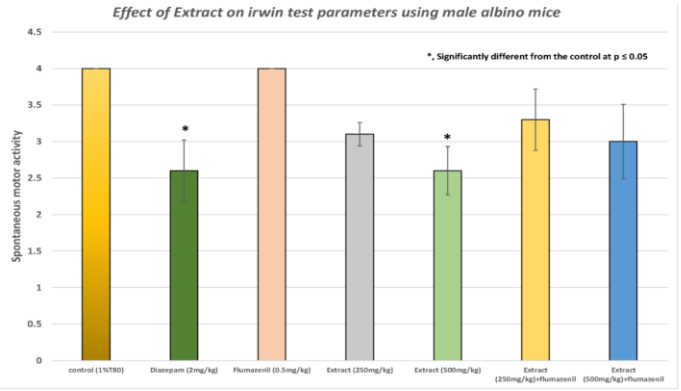

Figure 1, Effect of M. oleifera extract on SMA using male albino mice in Irwin test

The reactivity parameters were decreased significantly in diazepam (p=0.007), M. extract 250mg/kg (p=0.019) and 500mg/kg (p=0.006) treated groups, compared to the control-treated group. Flumazenil treated group alone, flumazenil combined with M. extract with a dose of 250mg/kg and also, combined with M. extract with a dose of 500 mg/kg treated groups, did not change the reactivity compared to the control-treated group (p>0.05) (Figure2).

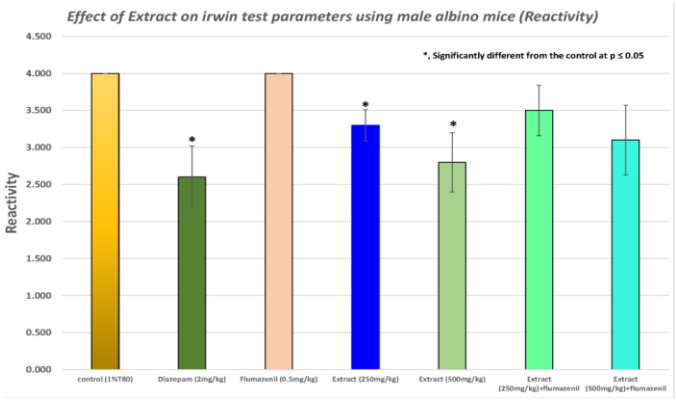

Figure 2, Effect of M. oleifera extract on reactivity parameter using albino mice in Irwin test.

The restlessness, irritability, and fearfulness parameters were increased significantly in flumazenil treated group alone (p=0.002), M. extract (250 mg/kg) combined with flumazenil (p=0.021), and M. extract (500 mg/kg) combined with flumazenil (p=0.021) treated groups compared to the control-treated group. Administration of M. extract (250 mg/kg) combined with flumazenil decreased the three parameters significantly compared to the restlessness, irritability, fearfulness induced by flumazenil treated group alone (p=0.022). Diazepam, M. extract with a dose of 250mg/kg, and 500 mg/kg treated groups did not show any change of that parameters and behave as the control-treated group (p>0.05) (Figure 3, 4).

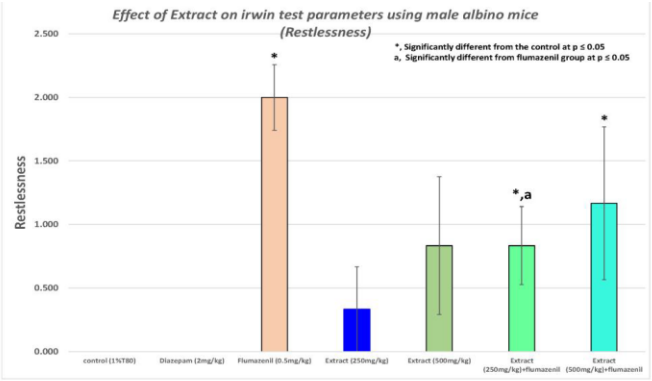

Figure 3, Effect of M. oleifera extract on restlessness using albino mice by applying Irwin test

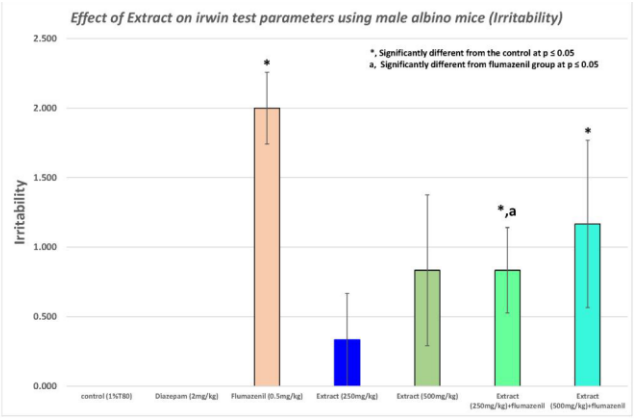

Figure 4, Effect of M.oleifera extract on irritability using albino mice in Irwin test

The other scores in the Irwin table did not show any changes compared to the control. No deaths were recorded over the 30hs observation period, indicating an LD50 above 500mg/kg.

Plus maze

Administration of diazepam produces a significant decrease in anxiety measure (p=0.031) compared to the control-treated group. While M. extract with a dose of 500 mg/kg (p=0.343), M. extract with a dose of 250 mg/kg (p=0.780) and flumazenil (p=0.886) alone treated groups did not show any significant change in anxiety measure compared to the control-treated group.

Combined treatment of flumazenil with M. extract (500 mg/kg) or flumazenil with M. extract (250 mg/kg) did not change anxiety measure compared to flumazenil treated group alone (p=0.546, p=0.922 respectively) (Table 2).

Table 2, Effect of M. oleifera on anxiety measure using the plus-maze

Treatments	Anxiety measure Mean ± SE	P compared to control	P compared to flumazenil	P compared to 500 mg/kg moringa	P compared to 250 mg/kg moringa
Control (1% T80) (5 ml/kg)	0.913 ± 0.0307	-	-	-	-
Diazepam (2mg/kg)	0.709 ± 0.0940	0.031	-	-	-
Flumazenil (0.5mg/kg)	0.900 ± 0.0562	0.886	-	-	-
Moringa (500mg/kg)	0.826 ± 0.0995	0.343	-	-	-
Moringa (250 mg/kg)	0.939 ± 0.0337	0.780	-	0.222	-
Moringa (500mg/kg) + Flumazenil	0.956 ± 0.0125	0.645	0.546	0.163	-
Moringa (250mg/kg) + Flumazenil	0.891 ± 0.0696	0.809	0.922	-	0.602

There were no significant changes in the total lines crossed produced by Diazepam treated group (p=0.847) and flumazenil treated group (p=0.346) compared to the control-treated group. While M. extracts in a dose 500 mg/kg and 250 mg/kg treated group produce a partial insignificant decrease in the total line crossed compared to the control-treated group (p=0.077, p=0.080 respectively). Administration of flumazenil combined with M. extracts in a dose of 500 mg/kg and a dose of 250mg/kg produced a significant decrease in the total line crossed compared to the control-treated group (p=0.034, p=0.037 respectively). While these combined treatments of flumazenil and M. extracts in doses 500 or 250 mg/kg showed no change in the total lines crossed compared to flumazenil treated group (p=0.218 and p=0.231, respectively) (Table 3).

Table 3, *Effect of M. oleifera on the total lines crossed using the plus-maze*

Treatments (n=6)	Total lines crossed Mean ± SE	P compared to control	P compared to flumazenil	P compared to 500 mg/kg moringa	P compared to 250 mg/kg moringa
Control (1% T80) (5 ml/kg)	37.83 ± 6.172	-	-	-	-
Diazepam (2mg/kg)	36.00 ± 7.954	0.847	-	-	-
Flumazenil (0.5mg/kg)	28.83 ± 8.138	0.346	-	-	-

Moringa (500mg/kg)	20.66 ± 7.041	0.077	-	-	-
Moringa (250 mg/kg)	20.83 ± 7.683	0.080	-	0.986	-
Moringa (500mg/kg) + Flumazenil	17.00 ± 3.366	0.034	0.218	0.700	-
Moringa (250mg/kg) + Flumazenil	17.33 ± 4.869	0.037	0.231	-	0.713

The total number of entries was not changed in each of the diazepam treated group (p=0.211), Flumazenil treated group (p=0.504), M. extract in a dose 500mg/kg and dose 250mg/kg (p=0.186, p=0.239 respectively) compared to the control-treated group.

The combined treatment of flumazenil with M. extract in a dose of 500mg/kg (p=0.125) and flumazenil with M. extract in a dose of 250mg/kg (p=0.239) did not show any significant change in the total number of entries compared to the control-treated group and compared to the flumazenil alone treated group (p=0.603, p=0.373 respectively) (Table 4).

Table 4, *Effect of M. oleifera on the total number of entries using the plus-maze*

Treatments (n=6)	Total entries Mean ± SE	P compared to control	P compared to flumazenil	P compared to 500 mg/kg moringa	P compared to 250 mg/kg moringa
Control (1% T80) (5 ml/kg)	6.8 ± 1.44	-	-	-	-
Diazepam (2mg/kg)	9.6 ± 2.37	0.211	-	-	-
Flumazenil (0.5mg/kg)	5.3 ± 1.54	0.504	-	-	-
Moringa (500mg/kg)	3.8 ± 1.53	0.186	-	-	-
Moringa (250 mg/kg)	4.1 ± 1.42	0.239	-	0.882	-
Moringa (500mg/kg) + Flumazenil	3.3 ± 0.66	0.125	0.603	0.823	-
Moringa (250mg/kg) + Flumazenil	4.1 ± 1.53	0.239	0.373	-	1.000

DISSCUSION

M. oleifera can be used in the treatment of different types of diseases because it has a wide variety of pharmacological properties of the different part of the tree, especially in disease related to the CNS disorder [5]. Depressive effect was observed on the CNS, after administration of *M. oleifera* extract. This effect was evaluated by Irwin as the primary observation procedure. Irwin test is generally used to estimate the effects of a new substance on central nervous system activity, minimum lethal dose of the test substance, and the primary effects on behavior and physiological functions. Data from this test are also used to assess the safety pharmacology of drugs [14, 15].

The Central Nervous System depressant effect of *M. oleifera* was estimated by the reduction in each of motor activity and mood in mice; including restlessness, irritability, and fearfulness. This effect may attribute to the presence of phytochemical constituents such as tannins [16], flavonoids, and triterpenoids in *M. oleifera* extract [17].

Tannins have CNS depressant like effect; it produces a dose-dependent decrease in locomotor activity, as tannins have a role in potentiating phenobarbital induced sleeping time [16]. Triterpenoids and flavonoids also have been reported to have CNS depressant effects [18]; where the flavonoids can easily cross the blood-brain barrier and exert various effects on CNS, like memory, cognition, and neurodegeneration. Triterpenoids, saponins, and flavonoids have an agonistic action on the GABAA receptor complex and hence may act like benzodiazepine-like molecules [17].

Central nervous system depression by the extract was antagonized by flumazenil. Flumazenil is a competitive antagonist toward the effect of benzodiazepine drugs on CNS; it blocks benzodiazepine receptors and inhibits the enhancing effect of gamma-aminobutyric acid (GABA), at postsynaptic membranes [19]. Flumazenil blocks the effect of endogenous benzodiazepine [20] leading to a decrease in GABA release from the nerve ending, where the ethanolic extract of Moringa oleifera leaves possesses CNS depressant possibly mediated through the enhancement of central inhibitory mechanism involving release γ-aminobutyric acid (GABA) [21]. It can be concluded that the antagonizing effect of M. oleifera extract of restlessness, irritability, and fearfulness, that produced by flumazenil, maybe through Benzodiazepine receptor. Flumazenil had also both weak agonist-like and weak inverse agonist-like properties [22, 23].

The result showed that anxiety measure was decreased by diazepam in the plus-maze method; it exerts its effect by allosteric binding at the interface between the alpha and gamma subunits on GABA-A receptor chloride ion channels complex within the limbic system. Where the GABA-A receptor cause decrease excitability of the neuron by an increase in the frequency at which the chloride channel opens, leading to an increased conductance of chloride ions and cause hyperpolarization of the neuronal membrane [24]. However, in the current study, *M. oleifera* did not show a change in anxiety measures by both doses used. Although it was observed that *M. oleifera* has an anxiolytic effect at a dose of 200mg/kg [25]. Bhat and Joy [25] used the soxhlet apparatus for extraction rather than a maceration. In this work, maceration is used. Extraction of plant materials depends on various factors such as solvents, methods, and extraction time to separate different quality and quantity of bioactive components in the crude extracts [26]. Flavonoids showed CNS depressant [18] when the soxhlet apparatus used in the extraction of *M. oleifera*; it was found that the total flavonoids 6.71 IQE/100g, while the total flavonoids obtained by maceration 6.20IQE/100g [26]. This may explain the difference between both experiments, although both results indicated that *M. oleifera* extract has a depressant effect.

Flumazenil alone and in combination with the M. extract by both doses do not affect anxiety measure. The dose of diazepam in this experiment was anxiolytic and not a sedative one, that's why didn't show a change in spontaneous motor activity. *M. oleifera* showed an insignificant decrease in spontaneous motor activity by both

doses; It may produce a sedative effect by using a larger dose above 500mg/kg. Flumazenil alone didn't show any change in spontaneous motor activity compared to the control, but it potentiated the effect of *M. oleifera* in both doses; this could be explained that flumazenil may have the ability to induce agonist or inverse agonist like properties [27].

GABA interneuron [28, 29] may explain the sedative effect produced by the combined treatment of flumazenil and *M. oleifera* extract. Flumazenil may block BZR in the striatum leads to a decrease in the release of GABA from the nerve ending; the second GABA neuron that inputs into the cortex will be free from the inhibitory effect of the first GABA neuron (in the striatum). This GABA interneuron between the striatum and cortex will lead to the excessive release of GABA in the cortex, leading to a decrease in the spontaneous motor activity observed with the combined treatment of flumazenil and *M. oleifera* extract.

It was reported that *M. oleifera* induces sleep via increase the level of 5HT, which is considered as evidence that *M. oleifera* has a role in brain 5-HT in sleeping mechanism [30]. However, the mechanism of *M. oleifera* to produce the sedative effect is not clear with the doses used in this experiment.

The decrease in the spontaneous motor activity by the combination of flumazenil and the extract observed with the total lines crossed was not observed with the total number of entries. It seems that the total number of entries is less sensitive to the changes in spontaneous motor activity.

CONCLUSION

Moringa oleifera has a CNS depressant-like effect; this effect could be through benzodiazepine receptors because this effect is antagonized by flumazenil. The combination of *M. oleifera* extract with flumazenil produces a synergistic effect toward the sedative action. This result needs more study to investigate the mechanism of action of each active constituent. *M. oleifera* extract does not affect anxiety mood but decreases the SMA. It can be concluded the sedative effect of the active constitutes in moringa extract may be through BZR1 and not BZR2.

Disclaimer

The article has not been previously presented or published, and is not part of a thesis project.

Conflict of Interest

There are no financial, personal, or professional conflicts of interest to declare.

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