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Original Research Article

Integrated OF-HC-EPM approach to assess sedative-anxiolytic potential of honey derived from mustard (*Brassica nigra*) flower

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ABSTRACT

Background: Mustard honey, a monofloral honey derived from mustard flower is considered a great source of nutritional and medicinal values. The honey is traditionally used as ethnomedicine in different parts of the world to cure many health problems. The present study aimed to evaluate its sedative-anxiolytic potential by integrating three conventional methods in a sequential order.
Methods: Open field, hole cross and elevated plus maze experiments were performed in a row with a single oral administration of honey to the Swiss Albino mice. Behavioral parameters like square crossing, rearing, grooming, hole crossing and entry/duration in open arm were observed for each animal in different time intervals.
Results: The findings were compared to that of a standard drug, diazepam (1 mg/kg). Mustard honey at higher doses showed sedative activity (4 g/kg and 6 g/kg) whereas with low doses (2 g/kg) exhibited anxiolytic potential. The physicochemical properties of honey were also screened in this study.
Conclusions: The integrated method proved to be an effective approach for assessment of neuropharmacological potential for crude or standard medicine. However, further analysis was recommended to investigate active compound which may lead to a new drug development.

Keywords: Mustard honey, Open field, Hole cross, Elevated plus maze, Rearing, Grooming

INTRODUCTION

From primitive age, honey is considered a universal healer for all types of ailments. The limitless benefits of honey have been acknowledged by the health practitioners and nutritionists from all parts of the world. Honey derived from mustard (*Brassica nigra*) flower possesses many pharmacological properties. The flower appears as elongating clusters of 1/3 inch yellow flowers with 4 petals and occur during winter.[1] Studies have suggested its antibacterial, antioxidant, detoxifying potential for which it is widely used as a traditional medicine.[2,3] The pharmacological properties of mustard honey are attributed to its nutritional constituents. It is a rich source for carbohydrate, contains polyphenol, flavonoids and vitamin C. Abundance of heavy metals Na, K, Ca, Mg, Fe, Pb has also been reported.[3] So far, many studies have been conducted to investigate the constituents, nutritional value and pharmacology but very few evaluated its neurological potential. Thus, the present study was aimed to assess the sedative-anxiolytic potential of mustard honey.

Depression and anxiety are common neurological disorders that are often treated with sedatives and anxiolytics. However, the need to innovate new agents for such disorders is crucial due to the reports against the side effects, dependency, tolerance and addiction developed with the established synthetic agents.[4,5,6] Therefore, a natural agent can easily serve the demand without any health risks.

METHODS

Collection and preparation of the sample

Mustard honey (approximately 1.5 kg) was obtained from a cultivated hive in the mustard field at Basail upazila (24°13′N 90°3′E) of Tangail District of Bangladesh in the month of February. Collected viscous mustard honey (MH) was sieved through a 0.5 mm mesh to remove non soluble particles (bee particles, wax, pollen, egg) and other coarse material. Then it was kept in a impermeable glass container at 25±2˚C.

Physicochemical properties

Determination of moisture content and total soluble solids

Moisture content and total soluble solids in honey were measured with the method as described by Bogdanov et al.[7] A portable honey refractometer (Biobase BK-PRN3, China) was used having a Brix range of 58–92% which was thermo-regulated and calibrated before use. A drop of honey was applied on the prism of the refractometer and values were obtained after temperature correction.[8]

Determination of pH

pH of the honey was measured using Biobase pH-10S (China) pH meter and calibrated at pH 4.01 and 7.00 prior use. By addition of distilled water, a 10% (w/v) solution was prepared from the honey sample and reading was taken in triplicate.[9]

Determination of optical density (OD)

Like measurement of pH, a 10% (w/v) honey solution was prepared for measuring the Optical density.[10] Absorbance was taken at 530 nm using the Biobase BK-UV1800 UV-VIS spectrophotometer (China) keeping the distilled water as blank. Absorbance values obtain was compared with standard set by United State Department of Agriculture (1985).[11]

Determination of honey density

Honey density was determined by the below formula as described by Kinoo et al where mass of the honey was determined from the difference of both empty and filled weight of a 1 ml syringe filled with honey.[12] An automatic electronic analytical balance Biobase BA2004N (China) was used for the measurement.

$$\text{Density of Honey} = \text{Mass of Honey} / \text{Volume of Honey}$$

Experimental animal

All experiments were performed on female Swiss Albino mice (age: 1.5 month, body weight: 27-32 g) which were kept in ambient temperature, air ventilation, 12 h light/dark cycle and ad libitum food and water at animal house of Institute for Pharmaceutical Skill Development and Research. A total of six groups (n=5) were formed and orally challenged with the following agents.

Group 1: Blank (No gavage), Group 2: Control (Distilled Water), Group 3: Diazepam (1 mg/kg), Group 4: MH-2 (2 g/kg body weight, equivalent to 25% w/v in 0.15 ml distilled water), Group 5: MH-4 (4g/kg body weight, equivalent to 50% w/v in 0.15 ml distilled water), Group 6: MH-6 (6 g/kg body weight, equivalent to 75% w/v in 0.15 ml distilled water).

Acute toxicity test

Acute toxicity trial was performed prior execution of the in-vivo experimental methods by administering high doses (5 g, 7.5 g, 10 g and 15 g per kg of body weight) of honey to rodents. Afterwards, they were kept in cleaned housing and observed for next 72 hours for any number of deaths or any unusual symptoms or behavior.

The (OF-HC-EPM) experimental design

The new OF-HC-EPM approach was an integration of three conventional experimental fields of open field, hole cross and elevated plus maze arranged in a cascade to omit the likely limitations, which was first described by Billah et al.[13] In this test, all three apparatus were organized sequentially in a row so as to allow rodents to explore each field for 3 minutes.[13] For first interval, mice experienced 0-3rd min at open field, 4-6th min at hole cross (however for simplifying, the time denoted as 0 min for hole cross) and 7-9th min at EPM (the time denoted as 0 min for EPM). The series was repeated in 30, 60, 90 and 120 minute intervals accordingly.

Open field test

Open field represents an open cubic box (60x60x60 cm) with a tiled (5x5 cm) floor alternatively colored black and white.[15] Mice was placed at one corner of the box and allowed to move freely. Behavioral parameters such as number of square crossing, grooming and rearing were observed for investigation of sedative-anxiolytic potential.[16]

Hole cross test

Hole cross field is a rectangular box shaped apparatus (30x20x14 cm) with a partition which divided the box into two equal compartments.[17] Mice were freely allowed to cross a 3 cm hole made on a partition at 7 cm floor

height and the number of holes crossed by the mice was observed as parameter of exploratory behavior.[18]

Elevated plus maze test

Elevated plus maze (EPM) is a plus shaped apparatus with two open arms (14×5 cm) intersecting with two closed arms (14×5×14 cm).[19] Entry and duration in open and close arm were observed as parameter indicating anxiolytic potentials.[20]

Statistical analysis

Statistical analysis of data was done by utilizing the method of one-way analysis of variance (ANOVA) followed by Dunnett's t tests using SPSS 24 for windows. The results obtained were compared with the control group. P values <0.05, 0.01 and 0.001 were considered statistically significant.

RESULTS

Physicochemical properties

The physicochemical analysis of mustard honey revealed that the honey is acidic in nature (pH 4.6) and water white to extra white in color according to USDA gradation by measurement of optical density (0.152). The moisture content found was 19.4 g/100g (Table 1).

Table 1: Physicochemical properties of mustard honey.

Parameters	Observations *
Moisture content (g/100g honey)	19.4±0.32
Total soluble solids (% Brix)	77.5±0.80
Density (w/v)	1.666±0.01
Optical density (at 530 nm)	0.152±0.01
pH (1-14)	4.6

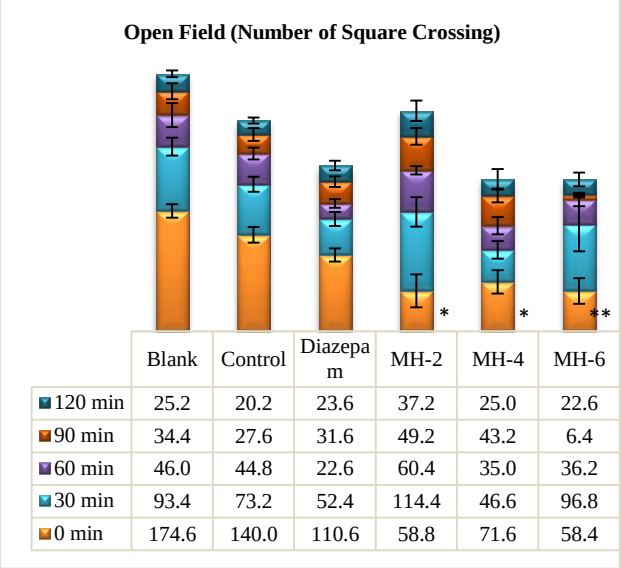
*All methods performed in triplicate.

Acute toxicity test

Mustard honey at high doses caused no unusual symptoms or death within 72 hours of observation. The result suggested designing safe doses for further in-vivo tests.

Open field test

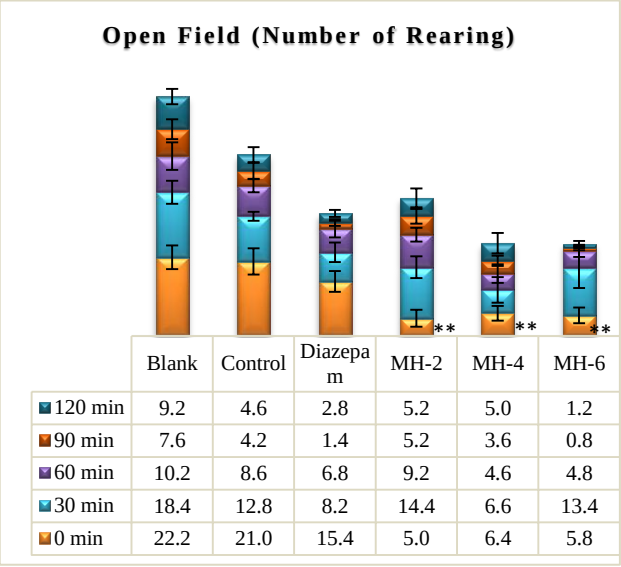
Maximum number of square cross was observed by the MH2 at 30 min (114.4) and cumulatively in all intervals. Diazepam also found with increased ambulation at 0 min. All groups commonly showed a gradual decrease in the number at late intervals. Both MH4 and MH6 showed decreased activity in all intervals compared to controls (Figure 1).



	Blank	Control	Diazepam	MH-2	MH-4	MH-6
120 min	25.2	20.2	23.6	37.2	25.0	22.6
90 min	34.4	27.6	31.6	49.2	43.2	6.4
60 min	46.0	44.8	22.6	60.4	35.0	36.2
30 min	93.4	73.2	52.4	114.4	46.6	96.8
0 min	174.6	140.0	110.6	58.8	71.6	58.4

Figure 1: Number of square crossed by the mice in different time intervals.

Values are mean±S.E.M. (n=5); *p<0.05, **p<0.01; Dunnett t-test (2 sided) treated one group as control (water) and compared all other groups against it.



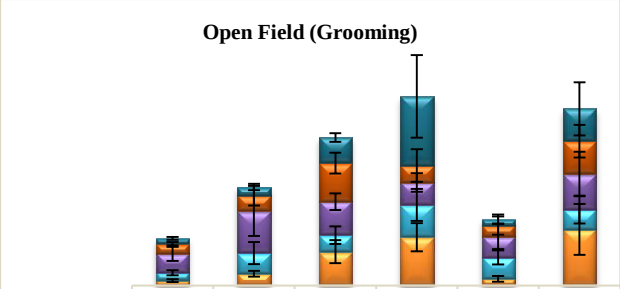
	Blank	Control	Diazepam	MH-2	MH-4	MH-6
120 min	9.2	4.6	2.8	5.2	5.0	1.2
90 min	7.6	4.2	1.4	5.2	3.6	0.8
60 min	10.2	8.6	6.8	9.2	4.6	4.8
30 min	18.4	12.8	8.2	14.4	6.6	13.4
0 min	22.2	21.0	15.4	5.0	6.4	5.8

Figure 2: Number of vertical movement (rearing) performed by the mice in different time intervals.

Values are mean±S.E.M. (n=5); * P<0.05, ** P<0.01; Dunnett t-test (2 sided) treated one group as control (water) and compared all other groups against it.

Lowest rearing response was demonstrated by MH4 and MH6 alongside diazepam (Figure 2). Like square crossing, MH2 also found to increase the activity compared to the standard drug.

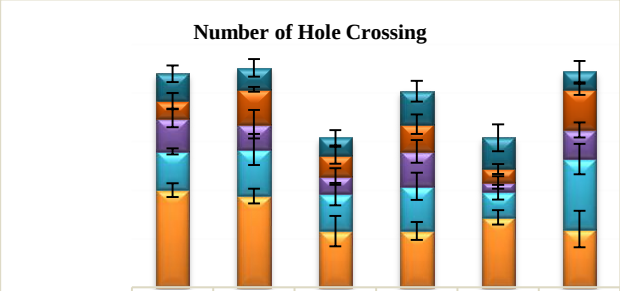
Increased grooming response was observed by diazepam and MH6 followed by maximum activity of MH2 whereas MH4 found with lowest grooming (Figure 3).



	Blank	Control	Diazepam	MH-2	MH-4	MH-6
120 min	4.6	7.6	22.4	60.2	5.8	28.2
90 min	8.8	13.2	33.6	14.8	9.6	28.6
60 min	16.6	36.8	29.2	19.6	18.2	31.0
30 min	6.8	18.0	14.4	28.2	18.6	17.4
0 min	4.4	10.4	29.6	42.2	6.0	49.0

Figure 3: Grooming behavior exhibited by the mice in different time intervals.

Values are mean±S.E.M. (n=5); Dunnett t-test (2 sided) treated one group as control (water) and compared all other groups against it.



	Blank	Control	Diazepam	MH-2	MH-4	MH-6
120 min	2.8	2.2	1.8	3.4	3.2	1.8
90 min	1.8	3.6	2.2	2.8	1.4	4.2
60 min	3.4	2.6	1.8	3.6	1	3
30 min	4	4.8	3.8	4.6	2.6	7.2
0 min	10	9.4	5.8	5.8	7.2	6

Figure 4: Number of hole crossed by the mice in different time intervals.

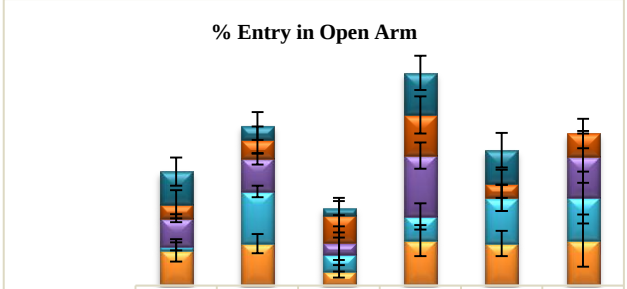
Values are mean±S.E.M. (n=5); Dunnett t-test (2 sided) treated one group as control (water) and compared all other groups against it.

Hole cross test

Observed data indicated that diazepam decreased the number of hole crossing activity and MH4 mimic the pattern comparing to control. On the contrary, MH2 followed by MH4 found with increased response (Figure 4).

Elevated plus maze test

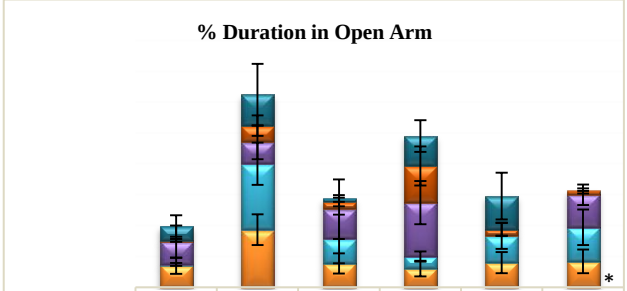
Diazepam reduced the entry in open arm compared to control. However, MH2 increased the entry substantially at all intervals (Figure 5).



	Blank	Control	Diazepam	MH-2	MH-4	MH-6
120 min	23.33	10.00	5.00	30.00	24.00	0.00
90 min	10.00	13.33	20.00	29.05	10.00	16.67
60 min	20.00	23.33	8.00	43.33	0.00	29.05
30 min	2.86	37.17	12.00	17.22	32.50	30.67
0 min	25.08	29.90	10.16	32.00	30.00	32.00

Figure 5: Percentage of open arm entry by the mice in different time intervals.

Values are mean±S.E.M. (n=5); Dunnett t-test (2 sided) treated one group as control (water) and compared all other groups against it.



	Blank	Control	Diazepam	MH-2	MH-4	MH-6
120 min	4.67	10.00	1.11	9.67	10.78	0.00
90 min	0.44	5.33	2.33	11.89	2.11	1.22
60 min	7.33	7.00	9.67	17.44	0.00	10.56
30 min	0.44	21.33	7.89	3.89	8.67	11.00
0 min	7.00	18.67	7.78	6.11	8.00	8.44

Figure 6: Percentage of duration spent in open arm by the mice in different time intervals.

Values are mean±S.E.M. (n=5); *p<0.05, Dunnett t-test (2 sided) treated one group as control (water) and compared all other groups against it.

Data showed that diazepam along with MH4 and MH6 reduced the duration spent by the rodents in open arm compared to control. On the other hand, MH2 raise the duration from 60 min to 120 min (Figure 6).

DISCUSSION

Open field (OF), hole cross (HC) and elevated plus maze (EPM) are among the first experimental choices of neuroscientists for evaluating neurological condition of rodents. Open field is considered for investigating comparative psychology of rodents however it is also used for determination of altered exploration, anxiety and

emotionality.[21] Hole cross apparatus was originated by Takagi et al for measurement of spontaneous movement of rodents.[17] EPM assesses rodents for their anxiety related behavior.[22] The expressions of rodent's behavior are greatly influenced by the test factors like strain, age, sex; prior test experience, presence of stimuli/inducer in test apparatus, forced feeding and handling by experimenter.[23,24] Alongside, the major challenges found to arise are first administration against repeated administration, utilizing same rodent for another experiment but in different time or using different rodent for different experiments. Thus, integrating these three fields allowed to mitigate the limitations by utilizing the same rodents with single oral administration for exposing to different fields which eventually created the scope to nullify the risk of individual and time dependent variance. Incorporation of a blank group reduced the chance for false interpreted results.

Diazepam, among the most popular benzodiazepines, act as positive allosteric modulators of the GABAA (γ-aminobutyric acid type A) receptor complex. It binds to alpha-gamma subunit interface and increases neuronal chloride-ion influx which hyperpolarizes postsynaptic membranes.[25] The anxiolytic effect is attributed to this potentiations of GABAA receptor at α2/α3 subunit isoforms in limbic system, thalamus, hypothalamus and cerebral cortex produce relaxing effects which facilitates the anxiolytic process.[26] Such effects were observed through reduced ambulatory and exploratory behaviors. Mustard honey at different doses mimic the same pattern by two neuropharmacological pathways – sedation and anxiolysis. Sedative potential was evident with low number of square crossing (ambulation), hole crossing (spontaneous movement) and rearing (risk-assessment behavior) when administered in high doses.[16,17,27] However, in low dose, these activities along with grooming (decision-making behavior), open arm entry and duration (mitigation of fear) found to increase as indicating anxiolytic potential of mustard honey.[21,24]

CONCLUSION

The conventional trends in the field of neuro-pharmacological evaluation have been reported to raise substantial additional challenges to sufficiently signify the result of the work. Thus, it was very crucial to address these challenges so as to minimize the variation. The present study drew a new approach utilizing the conventional methods aiming to eliminate the likely limitations. The present study conclude that the mustard honey possesses good sedative-anxiolytic properties however, further investigation is required to find out the responsible compound which may give clue for new drug development.

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Ethical approval: The study was approved by the institutional ethics committee

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