Eugenol and Its Liposomal Based Nano-carrier Protects Against Anxiety Disorder in Mice.

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Abstract

Background: Anxiety can also be termed as excessive worry which last for at least more than six months and patients can have difficulty in controlling them. Anxiety is having 31% of life time prevalence and sad part is that the anxiety disorders are not treated and diagnosed well. 3.1% which is 6.8 Million peoples of America are having anxiety disorders. The main part of clove oil is eugenol along with caryophyllene. Eugenol is present at about 70 to 90 percentage and also it contains 15 percentage of total dry weight of bud.

Methodology: Pre-clinical experimental study was conducted for 10-12 Months. Male BALB/c mice were purchased from ICCBS. Sample Size was 42 BALB/c mice and divided into 07 groups.

Results: The animal model was developed and it was found that Eugenol and its liposomal based nano carrier protect anxiety disorders in mice with the help of behavioral tests such as Elevated plus maze and open field tests and was recorded as Mean and SD, frequency and percentages accordingly.

Conclusion: Therefore with the help of behavioral studies i.e. open field test and elevated plus maze test, eugenol and its liposomal based nano particle protect anxiety disorder in the mice by increasing the entry time and stay of mice in open arms for longer duration in elevated maze plus test and in open field the anxiety was reduced after mice spent most of time in the center rather than periphery.

1. Introduction

Anxiety can also be termed as excessive worry which last for at least more than six months and patients can have difficulty in controlling them. Anxiety can also cause physical impairment or distress in important areas of functioning. Sometimes the phrase GAD incorrectly refers that the symptoms can be overall nonspecific. In the 5th volume of the "Diagnostic & Statistical Manual of Medical Disorders" the newest terminology generalized worry disorder was considered but it was not adopted (American Psychiatric Association., 2013). There is rare reporting from patients regarding worry symptom. The most predominant physical symptoms which patients report at primary care settings are gastrointestinal distress or headache (Stein et al., 2005). In children, GAD often displays as frequent abdominal pain etc. Major depression coexists with them, although it is very hard to distinguish it from GAD. For example insomnia and fatigue are the symptoms which occur in both disorders. Patients with GAD mostly suggest a sense of helplessness; in contrast the patient of major depression feels hopeless. Nevertheless the patients suffering from GAD are at the increased risk of suicide and deliberate self-harm (Chartrand et al., 2012). Anxiety is having 31% of life time prevalence and sad part is that the anxiety disorders are not treated and diagnosed well (Katzman et al., 2014). 3.1% which is 6.8 Million peoples of America are having anxiety disorders (Mousa et al., 2016). About 25% of United Stated population is suffering from anxiety disorders. Anxiety (Pathological) is the most frequent mental & psychological disorder which 6 leads to suffering of routine life (Hasanpour-Dehkordi, 2016). About 35 percentages of peoples with GAD medicate with drugs and alcohols to decrease anxiety related signs and this causes increased risk of

drugs and alcohol abuse (Robinson J, et al., 2011). Treatment currently used for anxiety disorders are “serotonin–noradrenaline reuptake inhibitors (SNRIs)”, “serotonin reuptake inhibitors (SSRIs)”, hydroxyzine, phenelzine, buspirone, moclobemide, benzodiazepines, pregabalin and tricyclic antidepressants and opipramol (Bandelow et al., 2015).

The Clove oil is used since ancient times and it is used as an antimicrobial, antiseptic and antispasmodic agent in Chinese traditional medicine. Currently eugenol is used in household products, pharmaceutical products, cosmetics and dental products, fragrance in soaps, flavoring substance for food and skin care products (Nejad et al., 2017). The main part of clove oil is eugenol along with caryophyllene (Malik et al., 2016). Eugenol is present at about 70 to 90 percentage and also it contains 15 percentage of total dry weight of bud (Galal and Abdellatief, 2015). In recent times majority of people are looking to use medicinal plants, native & folk medicine along with their derived products to treat diseases (Nikfarjam et al., 2016). Medicinal plants have one or more organs; they also contain effective compounds along with contain therapeutic effects (Parsaei, 2016; Asadi-Samani, 2015; Bahmani, 2016). The Clove oil is used since ancient times and it is used as an antimicrobial, antiseptic and antispasmodic agent in Chinese traditional medicine. Currently eugenol is used in household products, pharmaceutical products, cosmetics and dental products, fragrance in soaps, flavoring substance for food and skin care products (Nejad et al., 2017). The main part of clove oil is eugenol along with caryophyllene (Malik et al., 2016). Eugenol is present at about 70 to 90 percentage and also it contains 15 percentage of total dry weight of bud (Galal and Abdellatief, 2015). In recent times majority of people are looking to use medicinal plants, native & folk medicine along with their derived products to treat diseases (Nikfarjam et al., 2016). Medicinal plants have one or more organs; they also contain effective compounds along with contain therapeutic effects (Parsaei, 2016; Asadi-Samani, 2015; Bahmani, 2016).

2. Methodology

2.1 STUDY DESIGN: Pre-clinical experimental study

2.2 DURATION OF THE STUDY: 10-12 Months

2.3 ANIMALS: Male BALB/c mice were purchased from ICCBS. Sample Size were 42 BALB/c mice, divided into 07 groups.

2.4 INCLUSION CRITERIA:

- Male BALB/c mice
- Weighing 20-30 gm (Adult).
- No co morbidity

2.5 EXCLUSION CRITERIA

- Male mice weighing less than 20 gm or more than 30 gm.

- All female mice were excluded due to hormonal level which might cause data variability from estrous cycles.

2.6 GROUPING OF ANIMALS: Animals were placed into subsequent groups with six animals per group:

- Group I: (Vehicle control) Animals were given Normal Saline, per oral
- Group II: (Anxiety Group) Animals were given Chronic restraint stress and Normal Saline, per oral
- Group III: (Treatment Group) Animals were given Chronic restraint stress and Eugenol, 50 mg/kg, per oral
- Group IV: (Treatment Group) Animals were given Chronic restraint stress and Eugenol, 100 mg/kg, per oral
- Group V: (Treatment Group) Animals were given Chronic restraint stress and EUGVes or Eug-lypo 15 mg/kg, per oral
- Group VI: (Treatment group) Animals were given Chronic restraint stress and EUGVes or Eug-lypo 30 mg/kg, per oral Group
- Group VII: (Treatment group) Animals were given Chronic restraint stress and Diazepam, 01 mg/kg, per oral

2.7 HOUSING OF ANIMALS: BALB/c mice were maintained and kept in the animal house facility of Faculty of Pharmacy, Ziauddin University Karachi. Animals were given free access to water and rodent chow in their conventional cages kept at 22–23 °C with 12-hr light–dark cycle. Prior to the start of experiment, animals were acclimatized with the experimenter and the environment for few days.

2.8 CONVENTIONAL RESTRAIN MODEL: it was done in accordance with the protocol mentioned in (Siyal *et al.*, 2020).

2.9 SYNTHESIS: The synthesis was done in accordance with the protocol mentioned in (Siyal *et al.*, 2020).

3. Behavioral Tests

- **ELEVATED PLUS MAZE:** It is built with grey colored woody planks. Compromising of two open arms, 10 (cm in width) x 50 (cm in length) along with 02 arms enclosed, 40 (cm in height) x 10 (cm in width) x 50 cm (length) enclosed by a portable lid, in order that the both arms (closed and open) were opposite The maze was inflated to an altitude of 50 cm against the floor. In the 5-min test time, the subsequent measurements were taken: the no of entry in the both arms and the time consumed in the both arms. An entry was defined as all 4 feet into one arm. The increase in open arm entry along with increase in time spent in open arms revealed the anxiolytic activity because mice normally like the closed arms (Komada, Takao and Miyakawa, 2008).
- **OPEN FIELD TEST:** Spontaneous exploratory and locomotor activity was checked in open field (the apparatus was composed of PVC chamber 60x60x60) and the platform consists 25 squares (the

centre containing nine and periphery containing 16) surrounded by a wall. The animals were located in the intermediate or centre of the arena and the animal were recorded with a webcam for 5 min. The videos were later visualized and the periphery versus centre distance travelled would determine to assess anxiolytic behavior (Ali et al., 2019).

4. Statistical Analysis

Data was analyzed by MS Excel 2010, Numerical variables were represented as means and standard deviation (SD) and categorical variables were presented as frequency and percentages. Appropriate test of significance was applied (ANOVA Single factor and by t-test). p value <0.05 was considered statistically significant.

5. Results

- **ELEVATED PLUS MAZE:** Figure 1 showed the mean value for stay in Open arm regarding Normal group as 190.83 ± 44.10 seconds (s) (Mean $\pm$ SD). The mean value for Eug 50+ANX was 195.5 ± 23.40 s ($p < 0.01$), for EUG 100 + ANX the mean value was 199.66 ± 23.20 s ($p < 0.01$), regarding Eug-Lypo15+ ANX it was 196 ± 12.79 s ($p < 0.01$) along with the mean value for Eug-lypo 30 + ANX was 202 ± 20.77 s ($p < 0.01$) when compared with anxiety group it was 50.16 ± 52.90 s ($P < 0.001$) and diazepam was used as reference drug having mean value of 191.66 ± 44.88 s ($p < 0.01$) (Table 1). The Mean value for stay in Close arm regarding for Normal group was about 101.66 ± 40.93 s. The mean value for Eug 50+ANX was 84.16 ± 27.89 s ($p < 0.01$) for EUG 100+ ANX the mean value was 82 ± 23.91 s ($p < 0.01$). The mean value for Eug-Lypo15 + ANX was 87.66 ± 8.80 s ($p < 0.01$) along with the mean value for Eug-lypo 30+ ANX was 81 ± 15.21 s ($p < 0.01$) when compared with anxiety group it was 217 ± 60.53 s ($P < 0.01$) and diazepam was used as reference drug having mean value of 93.16 ± 44.64 s ($p < 0.01$). (Table 2). As per figure 2, the Mean for Open arm entries of Normal group was 9.33 ± 1.50 seconds (s) (Mean $\pm$ SD). The mean value for Eug 50+ANX was 10.5 ± 1.76 s ($p < 0.001$), for EUG 100+ ANX it was 11.33 ± 2.25 s ($p < 0.001$), for Eug-Lypo 15+ANX it was 10.33 ± 1.75 s ($p < 0.001$) and for Eug-Lypo 30+ANX it was 12 ± 2.36 s ($p < 0.001$) when compared with anxiety group it was 4.33 ± 0.51 s ($p < 0.001$), and diazepam was used as reference drug having mean value of 10.16 ± 2.31 s ($p < 0.001$) (Table 3). The Mean for Close arm entries for Normal group was 6.33 ± 1.03 s. The mean values for Eug 50+ANX was 8 ± 1.41 s ($p < 0.001$), for EUG 100+ ANX it was 8.66 ± 1.50 s ($p < 0.001$), regarding Eug-Lypo 15+ANX it was 8.33 ± 1.63 s ($p < 0.001$), for Eug-Lypo 30+ANX it was 8.66 ± 1.96 s ($p < 0.001$) when compared to anxiety it was 3.33 ± 0.51 s ($p < 0.001$), and diazepam was used as reference drug having mean value of 7.33 ± 1.50 s ($p < 0.001$) (Table 4).

Table 1
Elevated Plus Maze showing Mean and SD regarding open arm

Open Arm	Normal	Anxiety	Eug 50 + ANX	Eug 100 + ANX	Eug-lypo 15+ ANX	Eug-lypo 30+ ANX	Diazepam
Mean (seconds)	190.83	50.16	195.5	199.66	196	202	191.66
Standard Deviation (SD)	44.10	52.90	23.40	23.20	12.79	20.77	44.88
P value (Paired t-test)		<0.001	<0.001	<0.001	<0.001	<0.001	<0.001

Table 2
Elevated Plus Maze showing Mean and SD regarding close arm

Close Arm	Normal	Anxiety	Eug 50+ ANX	Eug 100+ANX	Eug-lypo 15+ ANX	Eug-lypo 30+ANX	Diazepam
Mean (seconds)	101.66	217	84.16	82	87.66	81	93.16
Standard Deviation (SD)	40.93	60.53	27.89	23.91	8.80	15.21	44.64
P value (Paired t- test)		<0.01	<0.01	<0.01	<0.01	<0.01	<0.01

Table 3
Mean and SD regarding open arm entries in Elevated Plus Maze

Open Arm (entries)	Normal	Anxiety	Eug 50 + ANX	Eug100 + ANX	Eug-lypo 15+ ANX	Eug-lypo 30+ ANX	Diazepam
Mean (seconds)	9.33	4.33	10.5	11.33	10.33	12	10.16
Standard Deviation (SD)	1.50	0.51	1.76	2.25	1.75	2.36	2.316607
P value (Paired t- test)		P<0.001	P<0.001	P<0.001	P<0.001	P<0.001	P<0.001

Table 4
Mean and SD regarding close arm entries in Elevated Plus Maze.

Close Arm (entries)	Normal	Anxiety	Eug 50 + ANX	Eug 100 + ANX	Eug-lypo 15+ ANX	Eug-lypo 30+ ANX	Diazepam
Mean (seconds)	6.33	3.33	8	8.66	8.33	8.66	7.333333
Standard Deviation (SD)	1.03	0.51	1.41	1.50	1.63	1.96	1.505545
P value (Paired t-test)		<0.001	<0.001	<0.001	<0.001	<0.001	<0.001

- OPEN FIELD TEST:** The Figure 3 represents the Mean for Open field (Time in centre) of Normal group was 80.83 ± 6.43 seconds (s) (Mean $\pm$ SD). The mean for Eug 50+ANX was 69.33 ± 13.35 s ($p < 0.05$). The open arm entry mean for EUG 100+ ANX was 71 ± 3.52 s ($p < 0.04$), for Eug-Lypo 15+ANX it was 68.83 ± 9.94 s ($p < 0.05$) regarding Eug-Lypo 30+ANX it was 107.16 ± 25.27 s ($p < 0.01$), when compared with anxiety which group it was 43.83 ± 23.48 s ($p < 0.02$). Diazepam was used as reference drug with mean 73.33 ± 17.73 s ($p < 0.04$) (Table 5). The mean for open filed (Time in periphery) for Normal group was 219.16 ± 6.43 s. the mean regarding Eug 50+ANX was 230.66 ± 13.35 s ($p < 0.05$), for EUG 100+ ANX it was 229 ± 3.52 s ($p < 0.04$), regarding Eug-Lypo 15+ANX it was 231.16 ± 9.94 s ($p < 0.05$) and for Eug-Lypo 30+ANX it was 192.83 ± 25.27 s ($p < 0.01$) when compared with anxiety group it was 256.16 ± 23.48 s ($p < 0.02$). Diazepam was used as reference drug with mean 226.66 ± 17.73 s ($p < 0.04$) (Table 6).

Table 5
Mean and SD of centre in open field

Time in centre	Normal	Anxiety	Eug 50+ANX	EUG 100+ ANX	Eug-Lypo 15+ANX	Eug-Lypo 30+ANX	Diazepam
Mean (Seconds)	80.83	43.83	69.33	71	68.83	107.16	73.33
SD	6.43	23.48	13.35	3.52	9.94	25.27	17.73
P value (Paired t-test)		<0.02	<0.05	<0.04	<0.05	<0.01	<0.04

Table 6
Mean and SD for time in periphery in open field

Time in Periphery	Normal	Anxiety	Eug 50+ANX	EUG 100+ANX	Eug-Lypo 15+ANX	Eug-Lypo 30+ANX	Diazepam
Mean (seconds)	219.16	256.16	230.66	229	231.16	192.83	226.66
SD	6.43	23.48	13.35	3.52	9.94	25.27	17.73
P value (Paired t-test)		<0.02	<0.05	<0.04	<0.05	<0.01	<0.04

Discussion

Our result suggests that with the help of conventional restrain stress animal model we were able to induce anxiety in the BALB/c mice. Each mouse was exposed to restraint and placed in a 50-ml conical tube (ventilated). They were restrained for five consecutive nights through period of each day i.e. restraint stress, from 17: 00 pm to 09:00 am and one hour before conventional restrain stress they were given test compounds which is eugenol and its liposomal based nano carriers at different doses and then behavioral studies were carried out and by elevated plus maze test we found out that eugenol and its liposomal based nano carrier at different doses reduces anxiety by increasing the stay of mice in open arm for longer duration when compared with closed arms and also increase in the open arm entries when compared with close arm entries. The results of elevated plus maze indicate decrease in anxiety after receiving tests compounds because mice normally like to explore open arms when compared with anxiety group which was induced by conventional restrain stress method and our results were also statistical significant.

In one of previous study it was found that the changes in elevated plus maze behavior were brought about by ablation of Neurokinin 1 Receptor expressing neurons in amygdala causing increase in anxiety related behaviors. The amygdala is involved in anxiety especially in association of environmental stimuli with fearful events (Phillips and LeDoux, 1992). In comparison to our study during elevated maze plus test that in case of anxiety the mice remained for majority of time in close arm and when treated with eugneol and its liposomal based nano particle in dose dependent manner they remained in open arm for majority of time as discussed in our results which marks reduction in anxiety.

With the help of Open field test we also found out that eugenol and its liposomal based nano carriers at different doses reduces anxiety because mice normally like to move across centre and during anxiety they like to move along with periphery. Hence our results are suggestive of reduction in anxiety among treated groups when compared with anxiety group which was induced by conventional restrain stress method and our results were also statistical significant.

Many herbal plants contain eugenol possessing many activities (Muller *et al.*, 2006). In also found out that Eugenol and its liposomal based nanocarrier posses anti anxiety effects in dose dependent manner.

In traditional Practices, eugenol is also effective in reversing the short and long term memory (Halder *et al.*, 2011). In our study we found out that eugenol and its liposomal based nanocarriers were useful in reducing anxiety at dose dependent manner.

In one of the study eugenol's various pharmacological effects were evaluated at 100mg/kg dose with the help of animal model. To check the antinociceptive effects three models were used Viz Tail flick test, acetic acid induced writhing tests and hot plate tests. Eugenol showed significant effect $p = 0.002$ in all of the three models and this effects was in accordance with previous studies.(Kurian *et al.*, 2006) (Müller *et al.*, 2006). In comparison to our study we used two behavioral tests of anxiety which were elevated maze plus and open field and assessed that eugenol and its liposomal based nano carrier in dose dependent manner reduced anxiety.

Conclusion

The animal model was developed to assess whether Eugenol and its liposomal based nano carrier protect anxiety disorders in mice or vice versa. Therefore with the help of behavioral studies i.e. open field test and elevated plus maze test, eugenol and its liposomal based nano particle protect anxiety disorder in the mice by increasing the entry time and stay of mice in open arms for longer duration in elevated maze plus test and in open field the anxiety was reduced after mice spent most of time in the center rather than periphery.

LIMITATIONS OF STUDY:

- Due to financial limitation, Westerns Blot couldn't be tested.
- By increasing sample size, more efficient could be obtained.
- Testing with other Anxiety models couldn't be done due unavailability of equipment's.

RECOMMENDATION: We recommend evaluating the further molecular mechanisms or pathways which causes reduction in anxiety; therefore this compound may be selected for clinical trials.

Abbreviations

SNRIs= serotonin–noradrenaline reuptake inhibitors

SSRIs= serotonin reuptake inhibitors

ANOVA= analysis of variance

Eug 50+ANX =) Animals were given Chronic restraint stress and Eugenol, 50 mg/kg

EUG 100 + ANX= Animals were given Chronic restraint stress and Eugenol 100 mg/kg,

Eug-Lypo15+ ANX = Animals were given Chronic restraint stress and EUGVes or Eug-lypo 15 mg/kg

Eug-lypo 30 + ANX = Animals were given Chronic restraint stress and EUGVes or Eug-lypo 30 mg/kg

Declarations

The author does not have any conflict of interest

Ethics approval and consent to participate: Ethical endorsement was taken from Animal Ethical Committee (AEC) of Ziauddin University (Protocol No. 2019-004).

Consent to publish: the authors give consent to publish the above mentioned study.

Availability of data and materials: can be provided if required.

Competing interests: The author doesn't have any competing interests.

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Authors' Contributions: FJS generated the idea conducted the study, RAS and ZM formulated the study. ZB and AAR helped in statistics, AH helped in literature survey.

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Figures

Figure 1

Elevated Plus Maze test representing values in seconds regarding mice traveled in open arm and close arm. * Representing statistical significance in open arm with paired t-test in open filed. (Anxiety group was compared with normal and treated group were compared with Anxiety groups). ** Representing statistical significance in close arm with paired t-test in open field. (Anxiety group was compared with normal and treated group were compared with Anxiety groups).

Figure 2

Elevated Plus Maze test representing entries by mice in open arm and close arm. * Representing statistical significance in open arm with paired t-test (Anxiety group was compared with normal and treated group were compared with Anxiety groups). ** Representing statistical significance in close arm with paired t-test (Anxiety group was compared with normal and treated group were compared with Anxiety groups).

Figure 3

Open field test representing values in seconds regarding mice traveled in centre and periphery. * Representing statistical significance in time in centre with paired t-test (Anxiety group was compared with normal and treated group were compared with Anxiety groups). ** Representing statistical significance in time in periphery with paired t-test (Anxiety group was compared with normal and treated group were compared with Anxiety groups).