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Eugenol and liposome-based nanocarriers loaded with eugenol protect against anxiolytic disorder via down regulation of neurokinin-1 receptors in mice

Fahad Jibran Siyal[1,2], Zahida Memon[3], Rehan Ahmed Siddiqui[4*], Zara Aslam[5], Uzair Nisar[1], Rehan Imad[4] and Muhammad Raza Shah[5]

[1]Department of Pharmacology, Faculty of Pharmacy, Ziauddin University, Karachi
[2]Institute of Pharmacy, Shaheed Mohtarma Benazir Bhutto Medical University, Larkana
[3]Department of Pharmacology, Ziauddin Medical College, Ziauddin University, Karachi
[4]Department of Research, Ziauddin University, Karachi
[5]HEJ Research Institute of Chemistry, International Center for Chemical and Biological Sciences, University of Karachi, Karachi

Abstract: Anxiety disorder is a psychiatric disorder characterized by extreme fear or worry. It is highly prevalent worldwide which affects daily life and is also an enormous health burden. Neurokinin 1 receptor (NK1R) is a G protein coupled receptor, expressed in both central and peripheral nervous system, involved in affective behaviors. NK1R has established role in anxiety and it is also an important target for pathogenesis of anxiety disorder. Therefore, it has been hypothesized in previous studies that the blockades of NK1R may have antidepressant and anxiolytic effects. The present study deals with the molecular mechanism of protective activity of eugenol against anxiolytic disorder. A pre-clinical animal study was performed on 42 BALB/c mice. Animals were given stress through conventional restrain model. The mRNA expression of NK1R was analyzed by real time RT-PCR. Moreover, the NK1R protein expression was also examined by immunohistochemistry in whole brain and mean density was calculated. The mRNA and protein expressions were found to be increased in animals given anxiety as compared to the normal control. Whereas, the expressions were decreased in the animals treated with eugenol and its liposome-based nanocarriers in a dose dependent manner. However, the results were better in animals treated with nanocarriers as compared to the compound alone. It is concluded that the eugenol and its liposome-based nanocarriers exert anxiolytic activity by down-regulating NK1R protein expression in mice.

Keywords: Eugenol, anxiety, liposome, nanocarriers, anxiolytic activity, NK1R protein.

INTRODUCTION

Anxiety is termed as emotional response to potential future danger or threat, eliciting symptoms of negative affective, behavioral, cognitive components and somatic along with a continuum based on duration and intensity. When anxiety becomes uncontrollable, maladaptive and permanent, it is considered as pathological which can affect daily life. Most important feature is fear which can be adoptive and critical for survival (Perusini and Fanselow, 2015; Parsafar and Davis, 2018). Anxiety is associated with apprehension, sustained arousal and vigilance, which are mediated by multiple areas of brain such as central amygdala, basolateral amygdala, medial prefrontal cortex and the bed of nucleus of stria terminalis. Anxiety is prevalent and enormous health burden (Botta *et al*., 2015; Tovote *et al*., 2015). Evidence from previous studies suggests that central amygdala microcircuits are important for anxiety as well as fear (Tovote *et al*., 2015). In the chronic anxiety disorder animal models; there is altered tonic inhibition within central amygdale circuits (Botta *et al*., 2015). Stress also represents important influences on emotion, behavior and cognition. It can also induce analgesia in rodents and human and can affect sensory perceptions, also it adversely affects motor performances in both rodent and human. Anxiety can also modulate motor activity in the open field test (Selvan *et al*., 2016). Neurokinin 1 receptor (NK1R) or substance P is a member of tachykinin family and also acts as modulator or neurotransmitters in the mammalian peripheral and central nervous system (Ständer and Yosipovitch, 2019). NK1R belongs to G protein coupled receptor family and their function is to activate signal transduction pathway within the cells (Garcia-Recio and Gascón, 2015). Previous studies have suggested role of NK1R in post-traumatic stress disorder (PSTD) and also it has role in stress related disorders and anxiety. Substance P modulates stress in the amygdala by its action on Neurokinin 1 receptor (Frick *et al*., 2016). NK1 is highly distributed in midbrain, brain stem areas and forebrain including area implicated in the stress modulation, mood response and anxiety, for instance caudate putamen, cingulated cortex, hippocampus, nucleus aacumbens septum, various hypothalamic areas, amygdala, locus coeruleus, dorsal raphe nucleus and periaqueductal gray (Ebner *et al*., 2008).

**Corresponding author:* e-mail: rehan.siddiqui@zu.edu.pk

Eugenol (4-allyl-2-methoxyphenol) is the main component of tulsi and clove oil, derived from *Myristica fragrans, Oscimum sanctum Linn* and *Eugenia caryophyllus*. It is used to relive pain in dental care, also flavoring agent in beverages, sweets, baked foods and frozen dairy. In traditional medicine it is used in cholic, flatulence and chronic diarrhea and other GIT disorders. FDA considers it as safe (Selvan *et al*., 2004). It is clear to pale yellow oily liquid soluble in organic solvents and slightly soluble in water (Sharma *et al*., 2012). It has also effect on reversing long term and short term memory (Halder *et al*., 2011).

Nanotechnology and its advancement has revolutionized 20th century and it deals with study of very small particles. It causes breakdown of large particles into very small particles. It deals with materials having size ranging from 0.1 to 100 nm; its working relies on nanometer scale length from 1 to 100 nm and therefore it can be used in many applications for the creation of nano devices and nanoparticles. Nanomedicine can improve drug bioavailability. Molecular targeting is also done by nano-robots which are engineered devices. (Anna Pratima Nikalje *et al*., 2015). Liposome based nanocarrier compared to the other nanoparticles has advantage of crossing blood brain barrier. The main objective of this study was to evaluate the molecular mechanism of eugenol and its liposome-based nanocarrier (EUG vesicles) against anxiety disorder in mice.

MATERIALS AND METHODS

Materials and instruments used for the synthesis of eugenol loaded liposomes

Eugenol (EUG, 99%), cholesterol (CH, 99%) and phosphate buffered saline (PBS, pH 7.4) were purchased from Sigma Aldrich (St Louis, MO, USA). Lecithin from soybean (SL, 94%) was acquired from Tokyo Chemistry Industry (Tokyo, Japan). HPLC grade chloroform and methanol were used without further purification. Shimadzu double beam UV-1800 spectrophotometer having a path length of 1 cm was used for the UV-Visible spectroscopic analysis. Bruker vector 22 FT-IR spectrometer was used to obtain fourier transform infrared (FT-IR) spectrum by using KBr disk method at room temperature between the range of 4000-400 cm-1. Atomic Force Microscope Agilent 5500 was used for the morphological analysis of eugenol loaded liposomal nanocarriers. Zetasizer Nano ZSP (Malvern) was used to obtain the mean size, polydispersity index (PDI) and zeta potential of the samples. Rotavapor R-210 (BUCHI®) was used for rotary evaporation of organic solvents. Minispin Plus (Eppendorf®) centrifuge was used for centrifugation of samples. VCX 750 Sonicator (Vibra-Cell™) was utilized for probe sonication.

Preparations of eugenol loaded liposomes

Eugenol loaded liposomal nanocarriers (EUG vesicles) were prepared by thin film hydration method followed by sonication (Mohammadi *et al.*, 2014). Lecithin (120 mg), cholesterol (60 mg) and eugenol (60 mg) were dissolved in 30 ml of mixed solvent system containing chloroform and methanol (6:4, v/v). Rotary evaporator was used for the removal of organic solvents which led to the formation of thin film. Obtained thin film was further dried under slow nitrogen flow to ensure complete removal of trace residual organic solvents. Hydration of thin film was carried out by adding 10 mL of distilled water in flask and then rotary evaporator (without vacuum) was used to form multilamellar vesicles (MLVs). For the reduction of vesicles size, sample was subjected to probe sonication in an ice bath for 5 min (5 cycles/min sonication and 4 min rest intermittently for cooling of the sample). Eugenol loaded liposomal nanocarriers (EUG vesicles) were pelleted down by centrifugation (10,000 rpm for 15 min) and washed with PBS (pH 7.4) for five times. The supernatant was collected and analyzed by using UV-Visible spectrophotometer for the evaluation of amount of unencapsulated drug. The encapsulation efficiency of eugenol loaded liposomal nanocarriers (EUG vesicles) was calculated by the given formula (Cui *et al*., 2006).

$$\text{Drug Loading efficiency} = \frac{\text{Total amount of drug - Free drug in the supernatant}}{\text{Total amount of drug}} \times 100$$

Animal studies and treatment regime

42 Male BALB/c mice weighing 25-30gm were obtained from the Animal House of Liaquat National Hospital, Karachi. Mice were maintained and kept in the animal house facility of Faculty of Pharmacy, Ziauddin University. All animal experimentations were performed by following international guidelines (Institure of Laboratory Animal Resources, US, 1989) and institutional protocols approved by Animal Ethics Committee of Ziauddin University (Approval Protocol No. 2019-004). Animals were given free access to water and rodent chow in their conventional cages kept at 22–23 °C with 12-h light–dark cycle. Prior to the start of experiment, animals were acclimatized with the experimenter and the environment for few days and animals were divided into following groups i.e. Group I: Animals were given Normal Saline, *per oral.* Group II: Animals were given chronic restraint stress and Normal Saline, *per oral.* Group III: Animals were given chronic restraint stress and Eugenol, 50 mg/kg, *per oral.* Group IV: Animals were given chronic restraint stress and Eugenol, 100 mg/kg, *per oral.* Group V: Animals were given chronic restraint stress and EUG-Ves, 15 mg/kg, *per oral.* Group VI: Animals were given chronic restraint stress and EUG-Ves, 30 mg/kg, *per oral.* Group VII: Animals were given chronic restraint stress and Diazepam, 01 mg/kg, *per oral.* Animals were treated with the test compounds for five days. At the end of the experiment all animals were sacrificed humanly by cervical dislocation and whole brain was dissected out for further studies.

Table 1: Particle size, polydispersity index (PDI), zeta potential and drug loading efficiency of eugenol loaded liposomal formulation (EUG vesicles).

Sample	Composition (SL: cholesterol: EUG)	Drug loading efficiency (LE%)	Mean vesicles diameter (nm)	Poly dispersity index (PDI)	Zeta potential (mV)
Eugenol loaded liposomal formulation	2:1:1	83.5±0.5	91.2	0.213	-42.7

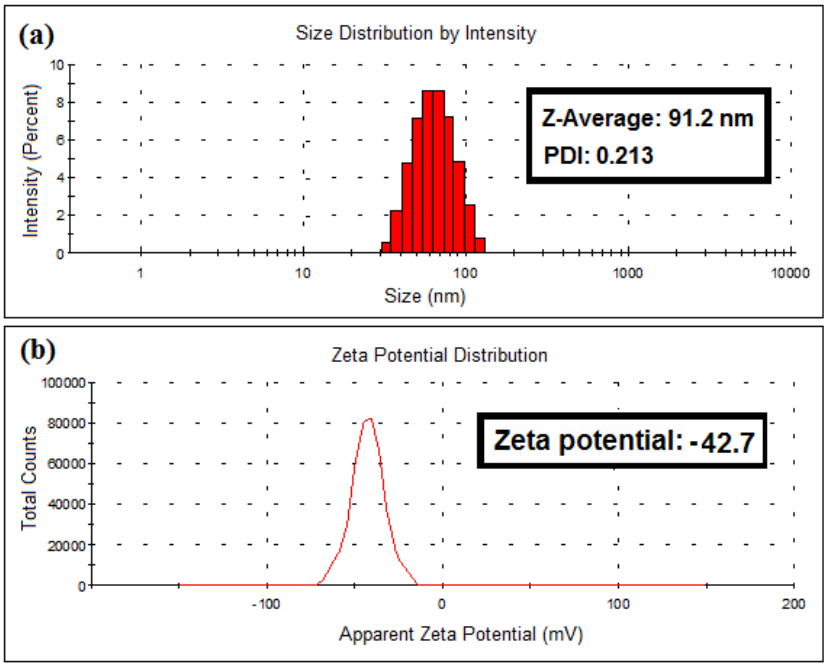

Fig. 1: Zeta sizer histogram of eugenol loaded liposomal nanocarriers (EUG vesicles) showing average particle size distribution (a) and zeta potential graph (b)

Conventional Restrain Model

In this method each mouse was exposed to restraint and placed in a conical tube (ventilated) 50-ml. They were restrained for five consecutive nights through period of each day i.e. restraint stress, from 17:00 to 09:00. These restraint tubes were washed and sterilized between each restraint cycles. Subsequently mice in the tubes were not having any contact with food & water throughout this time period, likewise food- and water-deprived, but not restrained, mice were used as control animals (Jin *et al.*, 2013).

Expression Analysis of NK1R using real-time RT-PCR

The real time RT-PCR studies were performed according to the procedure described by Siddiqui *et al.*, 2019. The primer sequence used in this studies were; NK1R (sense, TCCTCCTGCCACTGTTCTTG; antisense, CCACTCAG CCCTTTGTCTCA) and GAPDH (sense, GTATGACTC CACTCACGGCA; antisense, TCCACGACATACTCAG CACC).

Immunohistochemical expression studies of NK1R protein

In the first step, the slides were deparaffinized in xylene, then rehydrated in graded isopropyl alcohol (70%, 90% and 100 %) and were kept in water (deionized) for fifteen minutes. Then, slides were placed on the rack and additional water was removed then PBS was added onto the tissue sections for five minutes. PBS was removed and tissues were covered by blocking solution. Slides were then kept in wet chamber and that were then incubated at 42ºC for fifteen to twenty minutes. In the meantime, primary and secondary (antibodies) were diluted by blocking solution with ratio of 1:100 in vials and centrifuged at 10,000 RPM for ten minutes for avoiding antibodies clumps. After blocking, tissue sections were incubated with primary antibodies at 42ºC in incubator for forty-five minutes. Slides were washed 03 times with PBS for five minutes each. Secondary antibodies were then put on the tissue sections and incubated at forty-two (42ºC) in incubator for forty-five minutes. After incubation with secondary antibodies, slides were washed thoroughly with PBS 03 times for five mins each. DAB solution was applied to the tissues. Nuclei were stained with hematoxylin for 3 minutes. In the end, the slides were mounted with DPX mounting media and examined under Nikon Ts2R-FL inverted microscope. The images were captured and mean density (gray scale value) was calculated using Nikon Elements-D software.

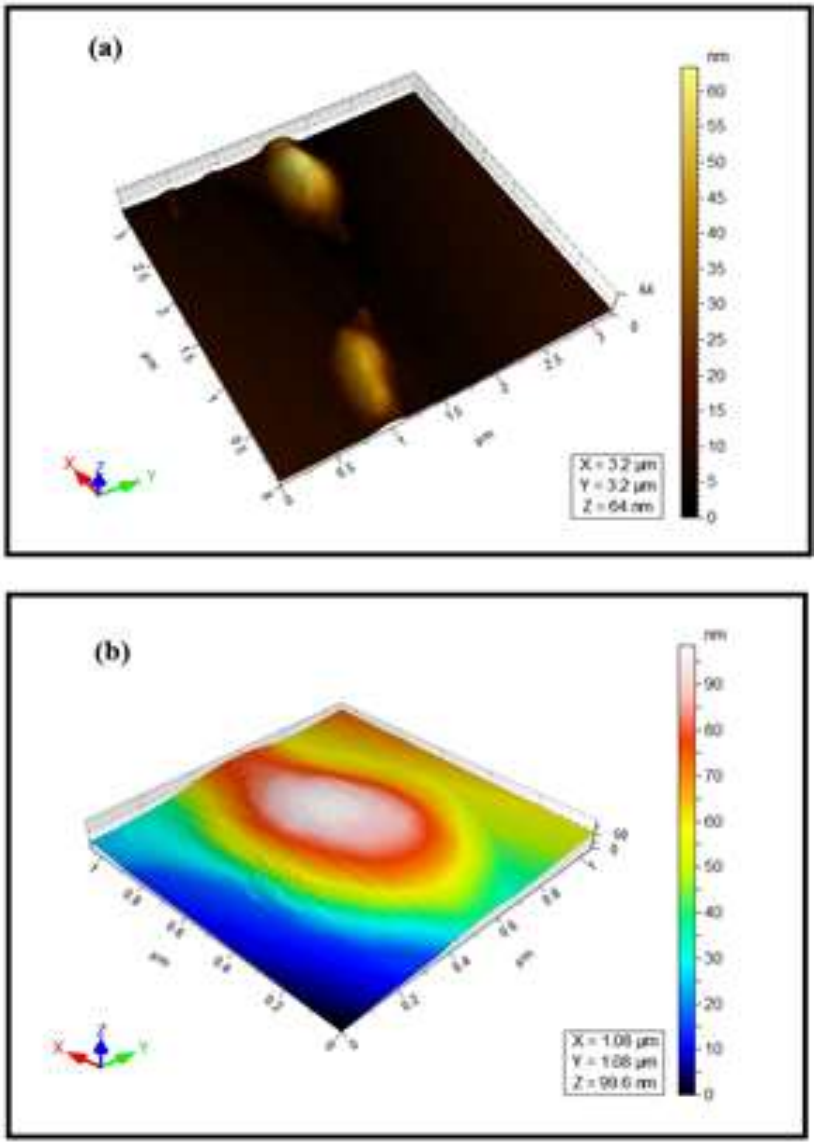

Fig. 2: Atomic force microscopic topographical images of eugenol loaded liposomal nanocarriers (EUG vesicles) showing morphology of multiple EUG vesicles (a) and single EUG vesicle (b)

STATISTICAL ANALYSIS

Data was analyzed by SPSS version 21 software. Numerical variables were represented as means ± SEM. Comparison between groups were performed using paired *t*-test followed by one way-ANOVA and *p*-value < or = 0.05 was considered statistically significant.

RESULTS

Characterization of Liposome-based nanocarriers loaded with eugenol

Size distribution of nanocarriers plays a vital role in the transportation and delivery of the encapsulated drug to the targeting site. Zeta sizer analysis was performed for the measurement of the average particle size distribution of eugenol loaded liposomal nanocarriers (EUG vesicles) (Nguyen *et al*., 2017). The results are shown in fig. 1(a). The histogram shows EUG vesicles have mean diameter of 91.2 nm. Poly dispersity index (PDI) determines the uniformity of the sample. The PDI of the sample was found to be 0.213 which indicate the narrow distribution of EUG vesicles. Zeta potential indicates the stability of the nanocarriers by determining the surface charge density of the EUG vesicles. The zeta potential of the sample was found to be -42.7 which indicated highly stable formulation due to the strong repulsion among EUG vesicles (fig. 1(b)). Drug loading capacity of liposomal nanocarriers depends on the chemical composition and interaction of drug with lipid based membranes. Cholesterol also plays a vital role for the improvement of drug loading efficiency by acting as

stabilizing agent in vesicle formation. Drug loading efficiency was estimated by calculating the amount of unencapsulated drug in the supernatant after complete washing of the EUG vesicles and using the calibration curve of eugenol. The results indicated that the EUG vesicles were able to encapsulate high amount of eugenol (83.5 %). The results of drug loading efficiency, size zeta and zeta potential are summarized in table 1 below.

AFM analysis was performed for the morphological evaluation of EUG vesicles. The results are given in fig. 2. The AFM images indicated the spherical and round shaped vesicles formed in the formulation. Fig. 2(a) represents multiple EUG liposomal vesicles having uniform shape while fig. 2(b) represented single EUG vesicle. These AFM results confirmed the formation of well intact and highly uniform eugenol loaded liposomal nanocarriers.

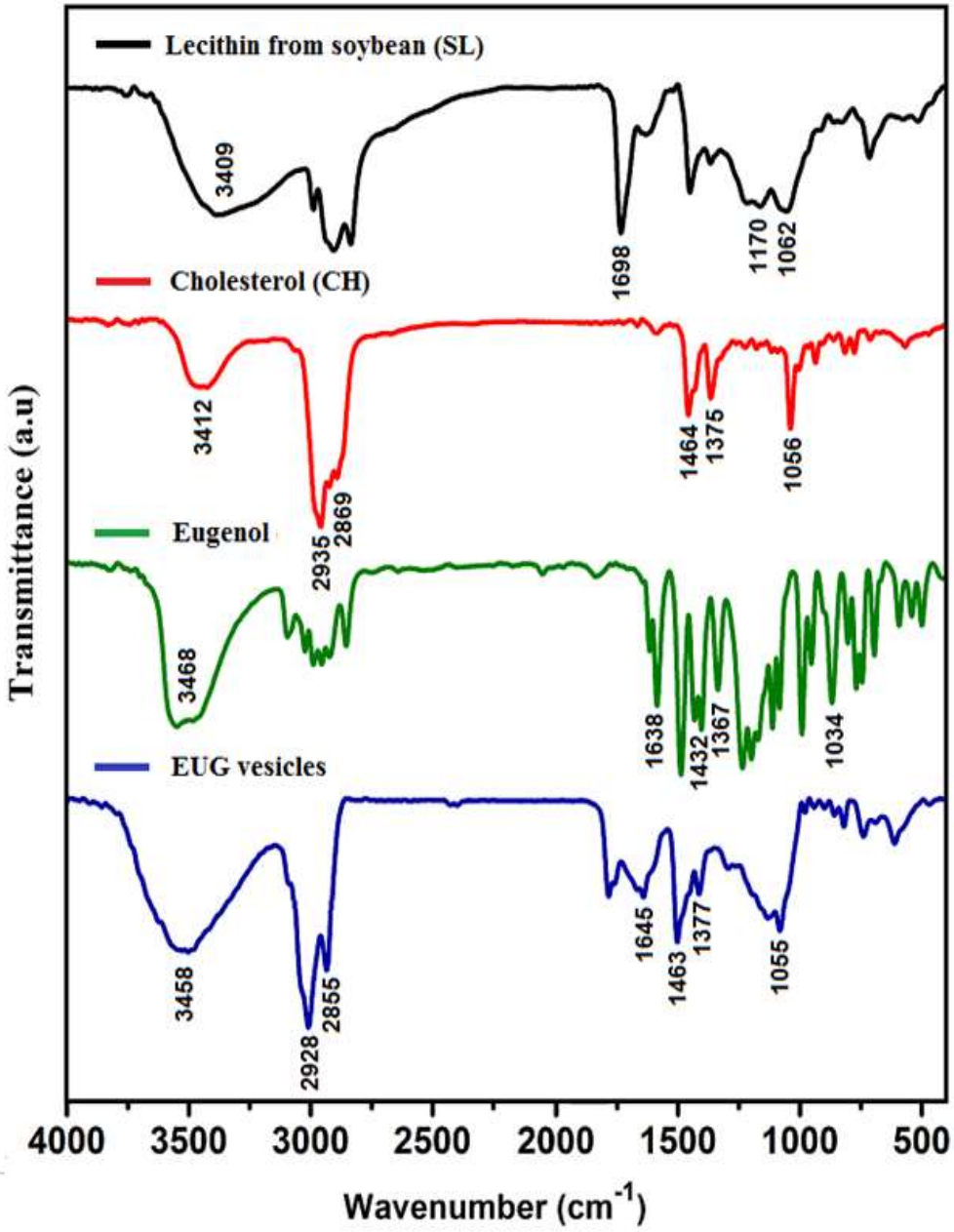

Fig. 3: Comparative FTIR spectra of soy lecithin (SL) (black trace), cholesterol (CH) (red trace), eugenol (EUG) (green trace) and eugenol loaded liposomal nanocarriers (EUG vesicles) (blue trace)

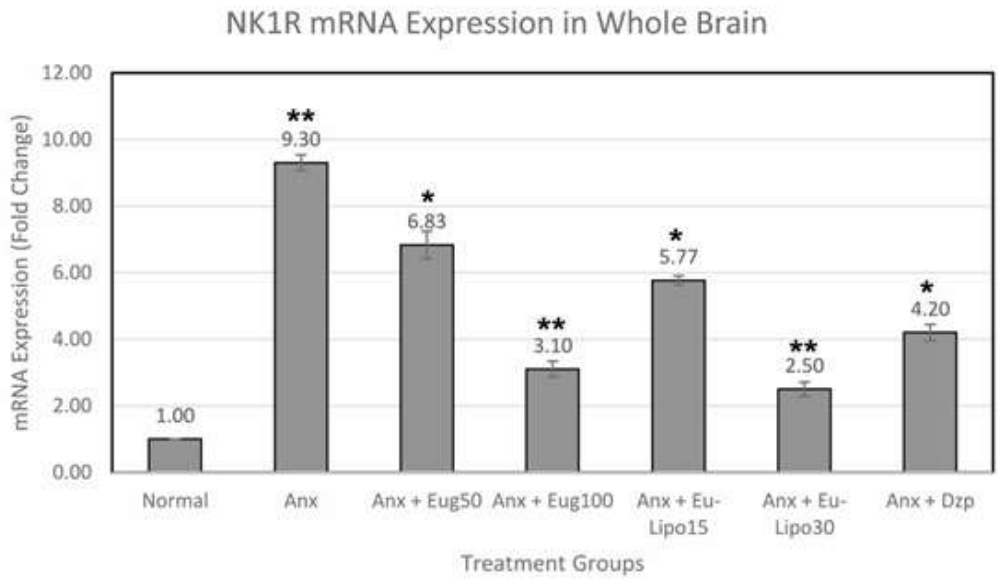

Fig. 4: mRNA expressions of NK1R gene in whole brain by real time RT-PCR in different treatment groups. (* p <0.05, ** p <0.005)

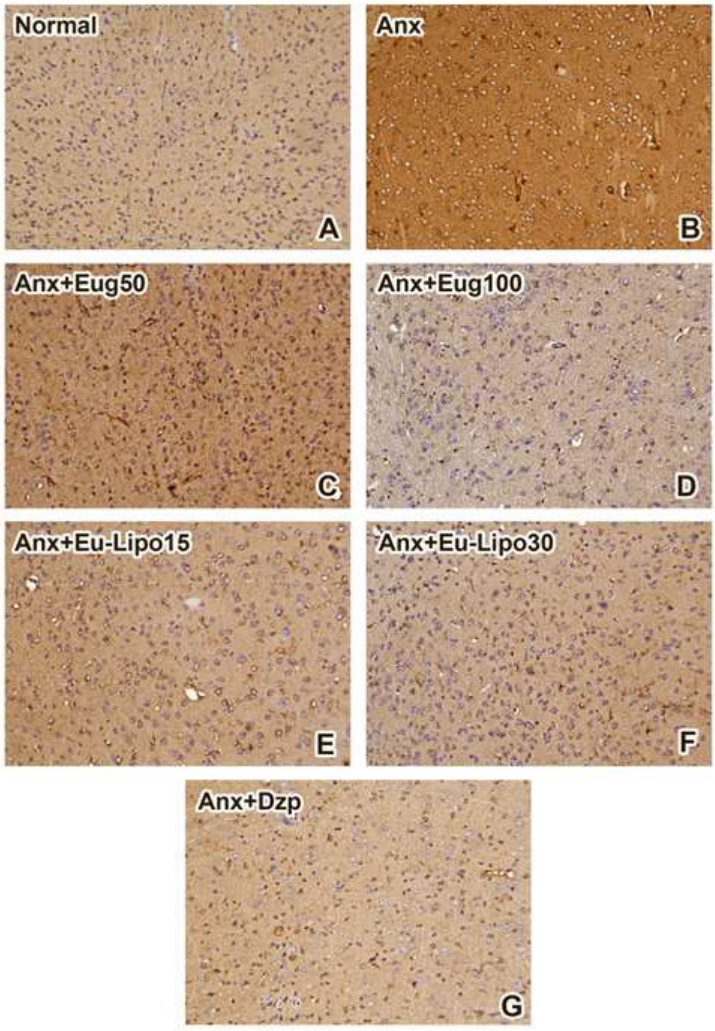

Fig. 5: Immunohistochemical expression of NK1R protein in whole brain in different treatment groups.

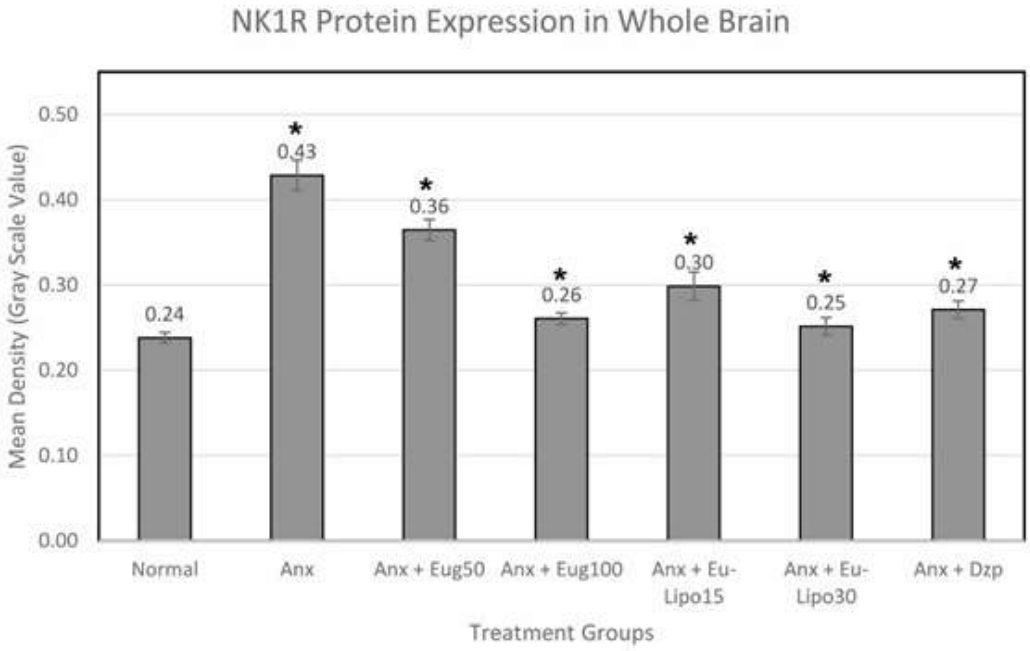

Fig. 6: Quantification of NK1R immunohistochemical images in gray scale value in different treatment groups (*p <0.001).

Drug interaction study (FTIR)

The FTIR analysis of samples was performed for the determination of effective encapsulation of eugenol in the liposomal nanocarriers. The FTIR spectra of eugenol (EUG), cholesterol (CH), soy lecithin (SL) and eugenol loaded liposomal nanocarriers (EUG vesicles) are shown in fig. 3. The FTIR spectrum of soy lecithin (SL) showed peaks at 3409 cm^{-1} and 1698 cm^{-1} which corresponds to the O-H and C=O stretching frequencies (Hidayah, 2018). Stretching vibrational peaks at 1170 cm^{-1} and 1062 cm^{-1} are due to the presence of C-O and C-C bonds present in molecule (fig. 3, black trace) [4].The FTIR spectrum of cholesterol (CH) showed broad absorption peak at 3412 cm^{-1} which corresponds to the stretching frequency of O-H group while strong absorption bands between the range of 3000-2800 cm^{-1} are due to the asymmetric and symmetric stretching vibrations of CH_2 and CH_3 groups (Gupta *et al.*, 2014). Strong band at 1464 cm^{-1} is due to the asymmetric stretching while peak at 1375 cm^{-1} corresponds to the bending vibrations of CH_2 and CH_3 groups, respectively (fig. 3, red trace). The sharp peak at 1056 cm^{-1} is due to the C-O stretching in cholesterol molecule [5]. FTIR spectrum of eugenol (EUG) showed broad peak at 3468 cm^{-1} due to the O-H stretching vibration (fig. 3, green trace). The characteristic band at 1638 cm^{-1} corresponds to the C=C group present in eugenol molecule (Silvianti *et al.*, 2017). Sharp peaks at 1432 cm^{-1} and 1367 cm^{-1} are due to the stretching vibrations of CH_2 and CH_3 groups, respectively. Peaks at 1034 cm^{-1} is due to the C-O stretching vibration (Silvianti *et al.*, 2017). The FTIR spectrum of eugenol loaded liposomal nanocarriers (EUG vesicles) showed broad peak at 3458 cm^{-1} which corresponds to the O-H stretching frequency while sharp peaks at 2928 cm^{-1} and 2855 cm^{-1} corresponds to the stretching vibrations of CH_2 and CH_3 groups. The absorption peak observed at 1638 cm^{-1} for C=C group in eugenol is shifted to higher frequency and appeared at 1645 cm^{-1} while broad peaks appeared at 1463 cm^{-1} and 1377 cm^{-1} corresponds to the stretching vibrations of CH_2 and CH_3 groups of eugenol present in EUG vesicles (fig. 3, blue trace). Peak at 1055 cm^{-1} is due to the C-O stretching vibration. The slight changes and shifting in stretching frequencies might be due to the electrostatic interaction between the pure eugenol and the eugenol loaded liposomal nanocarriers. The FTIR results conclude that the eugenol is effectively encapsulated in the liposomal vesicles.

mRNA expression of NR1R genes in whole brain

Fig. 4 demonstrates the mRNA expression of NK1R in mice brain of different groups. A significant increase (9.30 fold) in mRNA expression can be observed in anxiety group (p<0.001) as compared to the normal control. A significant decrease in the mRNA expression were found in the animals treated with the test compounds with different doses in a dose dependent manner compared to the anxiety model. In the animal group treated with eugenol at the dose of 50 mg/Kg, the mRNA expression was 6.83 fold as compared to the anxiety group (p <0.001), whereas the 3.10-fold change was observed in animals treated with eugenol at the dose of 100 mg/Kg in comparison with anxiety group (p <0.001). 5.77-fold change was seen in animals treated with EUG vesicles at the dose of 15 mg/Kg and expression level

was decreased to 2.50-fold in the animals treated with Eu-Lipo at the dose of 30 mg/Kg as compared to the anxiety group (p <0.001). The diazepam was given to the animals for reference purpose. The results showed better activity of EUG vesicles at the low doses as compared to the compound alone.

Immunohistochemical expression of NK1R protein in whole brain

Fig. 5 demonstrates the immunohistochemical expression of NK1R protein in the whole brain of animals treated with different conditions. Fig. 5A showed the normal pattern of expression of NK1R protein, while the expression level was increased in anxiety group (fig. 5B). There is a decrease in expression of NK1R protein is seen in animals treated with eugenol at the dose of 50 mg/Kg and 100 mg/Kg (fig. 5C and D respectively) as compared to the anxiety group. We also observed the decreased NK1R protein expression in the animals treated with EUG vesicles at a relatively lower dose i.e. 15 mg/Kg and 30 mg/Kg as compared to the anxiety group (fig. 5E and F respectively). The expression level was also decreased in the animals treated with diazepam at the dose of 1 mg/Kg (fig. 5G). This group was used as a positive control. The results of IHC images was analyzed by calculating the mean density (gray scale value) of images by Nikon Elements-D software as shown in fig. 6. Through this method, the IHC results were quantified and validated.

DISCUSSION

In a previous study there was strong support for the role of NK1R expressing neurons within amygdale in the modulation of anxiety. It was also found that loss of NK1R expressing neurons in the amygdale is anxiogenic. (Gadd *et al.,* 2003; Steinberg *et al.,* 2002). In comparison to our study we are also of view that on the basis of our results, there is over expression of NK1R genes and proteins during anxiety. The systemic administration of NK1R antagonists produces anxiolytic effects in gerbil EPM and in the intra-amygdala, reducing the amount of separation-induced vocalizations in the guinea pigs with observations consistent with NK1R in mice (Varty *et al.,* 2002; Boyce *et al.,* 2001; Santarelli *et al.,* 2001). In contrast to our study eugenol and its lyposome-based nanocarriers also possess anti-anxiety effects. The amygdale is important in meditation of the conditional aspects of rewards process specially connection between NAcc and basolateral nucleus which is important in controlling the behavior by discrete and reward related stimuli (Parkinson *et al.,* 2001; Fuchs *et al.,* 2002). In NK1R expressing neurons, neurochemical identity remains unclear in amygdale but in guinea pig's basolateral nucleus it is co-localized with glutamic acid decarboxylase which is marker for GABA (Maubach *et al.,* 2001). If endogenous NK1R is involved in the stress modulation and also anxiety, then it must be released in central response to different stressors. When exposure to variety if painful, emotional and physical stresses causing altered NK1R in many regions of brain like increase in NK1R levels were found in dentate gyrus and septum after foot shock (Ebner *et al.,* 2006). In previous study with the help of immunohistochemcial studies, demonstrating a dense plexus of NK1R containing cell bodies in the terminals in this brain area (Ribeiro-da-Silva and H€ o okfelt, 2000). In association with our study by IHC we also noticed increased expression of NK1R in brain during anxiety. It was also reported that stress induced and basal release of NK1R in the medial amygdale is regulated in a different way by NK1R (Ebner and Singewald, 2005). We have also found the increased expressions of NK1R in anxiety. NK1R are not found in serotonergic cells in dorsal raphe (Froger *et al.,* 2001; Santarelli *et al.,* 2001), they are present on GABAergic and glutamatergic cells (Ma and Bleasdale, 2002; Commons and Valentino, 2002) suggestive of an indirectly influence of NK1R on 5-HT neurotransmission within this area. In this study, we found out that when the animals were treated with our test compounds i.e. eugenol and its liposome-based nanocarrier, the expression of NK1R genes and proteins were decreased, thus proving their anxiolytic activities.

CONCLUSION

It is concluded that eugenol and liposome-based nanocarrier loaded with eugenol (EUG vesicles) protect anxiety disorder in mice in a dose dependent manner. Moreover, the results were better observed in animals treated with nanocarriers compared to the eugenol alone. The underlying molecular mechanism by which the test compounds exert their activity was down-regulation of NK1R genes and proteins in mice brain. This may be one mechanism by which the test compounds showed their anxiolytic activity. Therefore, the study suggests to explore more molecular mechanisms of the test compounds to make it better drug candidate for future therapeutics.

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REFERENCES

Botta P, Demmou L, Kasugai Y, Markovic M, Xu C, Fadok J P and Rudolph U (2015). Regulating anxiety with extrasynaptic inhibition. *Nat. Neurosci.,* **18**(10): 1493-1500.

Boyce S, Smith D, Carlson E, Hewson L, Rigby M, O'Donnell R, Harrison T and Rupniak N M (2001). Intra-amygdala injection of the substance P (NK1

receptor) antagonist L-760735 inhibits neonatal vocalisations in guineapigs. *Neuropharmacol.*, **41**: 130-137.

Commons KG and Valentino RJ (2002). Cellular basis for the effects of substance P in the periaqueductal gray and dorsal raphe nucleus. *J. Comp. Neurol.*, **447**: 82-97.

Cui F, Shi K, Zhang L, Tao A and Kawashima Y (2006). Biodegradable nanoparticles loaded with insulin-phospholipid complex for oral delivery: Preparation, *in vitro* characterization and *in vivo* evaluation. *J. Contr. Rel.*, **114**(2): 242-250.

Ebner K and Singewald N (2006). The role of substance P in stress and anxiety responses. *Amino Acids*, **31**(3): 251-272.

Ebner K, Muigg P, Singewald G and Singewald N (2008). Substance P in stress and anxiety: NK-1 receptor antagonism interacts with key brain areas of the stress circuitry. *Ann. NY. Acad. Sci.*, **1144**(1): 61-73.

Ebner K and Singewald N (2005). Effects of NK1 receptor antagonist administration on basal and stress-induced substance P release in the amygdala. *J. Neurochem.*, **94**(Suppl. 2): 183.

Frick A, Åhs F, Palmquist Å M, Pissiota A, Wallenquist U, Fernandez M and Furmark T (2016). Overlapping expression of serotonin transporters and neurokinin-1 receptors in posttraumatic stress disorder: a multi-tracer PET study. *Mol. Psych.*, **21**(10): 1400-1407.

Froger N, Gardier AM, Moratalla R, Alberti I, Lena I, Boni C, De Felipe C, Rupniak NM, Hunt SP, Jacquot C, Hamon M and Lanfumey L (2001). 5-hydroxytryptamine (5-HT)1A autoreceptor adaptive changes in substance P (neurokinin 1) receptor knock-out mice mimic antidepressantinduced desensitization. *J. Neurosci*., **21**: 8188-8197

Fuchs RA, Weber SM, Rice HJ and Neisewander JL (2002). Effects of excitotoxic lesions of the basolateral amygdala on cocaine-seeking behavior and cocaine conditioned place preference in rats. *Brain Res*., **929**: 15-25.

Gadd CA, Murtra P, De Felipe C and Hunt SP (2003). Neurokinin-1 receptor-expressing neurons in the amygdala modulate morphine reward and anxiety behaviors in the mouse. *J. Neurosci.*, **23**(23): 8271-8280.

Garcia-Recio S and Gascon P (2015). Biological and pharmacological aspects of the NK1-receptor. *Bio Med. Res. Int.*,

Gupta U, Singh V K, Kumar V and Khajuria Y (2014). Spectroscopic studies of cholesterol: Fourier transform infra-red and vibrational frequency analysis. *Mater. Focus*, **3**(3): 211-217.

Halder S, Mehta AK, Kar R, Mustafa M, Mediratta PK, and Sharma KK (2011). Clove oil reverses learning and memory deficits in scopolamine-treated mice. *Planta Med.*, **77**(08): 830-834.

Hidayah N (2018). The effect of papain enzyme dosage on the modification of egg-yolk lecithin emulsifier product through enzymatic hydrolysis reaction. *Int. J. Tech.*, **9**(2): 380-389.

Institute of Laboratory Animal Resources (US). Committee on Care and Use of Laboratory Animals. (1986) Guide for the care and use of laboratory animals (No. 86). US Department of Health and Human Services, Public Health Service, National Institutes of Health.

Maubach KA, Martin K, Smith DW, Hewson L, Frankshun RA, Harrison T and Seabrook GR (2001). Substance P stimulates inhibitory synaptic transmission in the guinea pig basolateral amygdala *in vitro*. *Neuropharmacol.*, **40**: 806-817.

Mohammadi M, Ghanbarzadeh B and Hamishehkar H (2014). Formulation of nanoliposomal vitamin D3 for potential application in beverage fortification. *Adv. Pharm. Bull.*, **4**(Suppl. 2): 569.

Nguyen TL, Nguyen TH and Nguyen DH (2017). Development and *in vitro* evaluation of liposomes using soy lecithin to encapsulate paclitaxel. *Int. J. Biomat.*, **2017**: Article ID 8234712.

Nikalje AP (2015). Nanotechnology and its applications in medicine. *Med. Chem.*, **5**(2): 081-089.

Parkinson JA, Crofts HS, McGuigan M, Tomic DL, Everitt BJ and Roberts AC (2001). The role of the primate amygdala in conditioned reinforcement. *J. Neurosci.*, **21**: 7770-7780.

Parsafar P and Davis EL (2018). Fear and anxiety. In H. C. Lench (Ed.), The function of emotions: When and why emotions help us. *Cham: Spr. Int. Pub.*, pp.9-23.

Perusini JN and Fanselow MS (2015). Neurobehavioral perspectives on the distinction between fear and anxiety. *Lear. Mem.*, **22**(9): 417-425.

Ribeiro-da-Silva A and HE o okfelt T (2000). Neuroanatomical localisation of substance P in the CNS and sensory neurons. *Neuropep.*, **34**: 256-271.

Santarelli L, Gobbi G, Debs PC, Sibille EL, Blier P, Hen R and Heath MJ (2001). Genetic and pharmacological disruption of neurokinin 1 receptor function decreases anxiety-related behaviors and increases serotonergic function. *Proc. Natl. Acad. Sci. USA*, **98**: 1912-1917.

Selvan P, Malathi RR and Rajan RR (2016). Effect of 4-Allyl-2-methoxyphenol (eugenol) on motor co-ordination in subacute restraint stress Induced wistar albino rats. *J. App. Pharm.*, **6**(11): 120-125.

Sharma M, Rauniar GP and Das BP (2012). Experimental study of various central nervous system effects of eugenol in mice and rats. *Health Renaiss.*, **10**(3): 208-214.

Shults CW, Quirion R, Chronwall B, Chase TN, O'Donohue TL (1984). A comparison of the anatomical distribution of substance P and substance P receptors in the rat central nervous system. *Peptides*. **5**: 1097-112.

Siddiqui RA, Simjee SU, Kabir N, Ateeq M, Shah MR and Hussain SS (2019). N-(2-hydroxyphenyl) acetamide and its gold nanoparticle conjugation prevent glycerol-induced acute kidney injury by attenuating inflammation and oxidative injury in mice. *Mol. Cell Biochem*., **450**(1-2): 43-52.

Silvianti F, Siswanta D, Aprilita NH and Kiswandono AA (2017). Adsorption characteristic of iron onto Poly [eugenol-co-(divinyl benzene)] from Aqueous solution. *J. Nat.*, **17**(2): 108-117.

Ständer S and Yosipovitch G (2019). Substance P and neurokinin 1 receptor are new targets for the treatment of chronic pruritus. *Br. J. Dermatol.*, **181**(5): 932-938.

Tovoten P, Fadok J P and Luthi A (2015). Neuronal circuits for fear and anxiety. *Nat. Rev. Neurosci.*, **16**(6): 317-331.

Varty GB, Cohen-Williams ME, Morgan CA, Pylak U, Duffy RA, Lachowicz JE and Carey GJ, Coffin VL (2002). The gerbil elevated plus-maze II: Anxiolytic-like effects of selective neurokinin NK1 receptor antagonists. *Neuropsychopharmacol.*, **27**: 371-379.