Original Articles

Experimental study of various central nervous system effects of eugenol in mice and rats

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Abstract

Background: Eugenol is an essential component of many medicinal herbs. It is a member of the allylbenzene class of chemical compounds. Since years, it is being used in dental practice to relieve pain arising from pulpitis and dentinal hypersensitivity. There are few reports of anticonvulsant effect but further effects are less reported. Lack of comprehensive studies and data of eugenol on the central nervous system effects in animal models thus necessitates further research activities. Objective: To observe and evaluate various neuropharmacological effects like antinociceptive effect, anticonvulsant effect, effect on motor co-ordination, pentobarbital induced sleeping time and anxiolytic effect of eugenol in mice and rats. Methods: It was a quantitative experimental study done in the laboratory setting of the department of Clinical Pharmacology and Therapeutics. For each test, respective animal models were used. Animals were divided into three groups of six each, group I as control, group II as standard control and group III as test group. Results: Significant effects were observed in analgesic, anticonvulsant and sedative model whereas no significant effect as compared to control was observed in test of motor co-ordination and in behavioral models. Conclusion: This study shows eugenol to possess analgesic, anticonvulsant and sedative effect whereas it didn’t have any effect on models of anxiety. Various target sites have been implicated but this study doesn’t conclude a plausible mechanism behind all these observed effects.

Keywords: Eugenol, neuropharmacological effects

Introduction

Medicinal herbs constitute the cornerstone of traditional medicinal practice worldwide. The majority of the population in developing countries remains dependent on them for health care. 1 Eugenol is a constituent of many medicinal herbs. Eugenol, an allyl chain substituted guaiacol (2-methoxyphenol), is a clear to pale yellow oily liquid and is a member of the allylbenzene class of chemical compounds, slightly soluble in water and soluble in organic solvents. 2,3 It is extracted from essential oils especially from clove oil, nutmeg, bay-leaf and cinnamon 2 and is also derived from variety of other plant sources including Eugenia caryophyllus, Myristica fragrans, and Laurus nobilis Linn. For years, it has been used in dental practice to relieve pain arising from a variety of sources like pulpitis and dentinal hypersensitivity. 4 It also has pronounced antiseptic properties and is used in perfumeries, flavorings and in medicine for this purpose. 3 Eugenol is also reported to possess anticonvulsant, anti-inflammatory, antistress, antioxidant, anesthetic, antimicrobial, antiaggregatory activity and muscle relaxant properties. 5,6 In addition, a study has shown that it can be used in the treatment of vaginal candidiasis as well. 10

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With regard to mechanism of action of eugenol, there are no conclusive studies; however, it has been shown to inhibit sodium and calcium currents in dental afferent neurons suggesting a plausible mechanism for its analgesic effect.\textsuperscript{11-13} In addition to this, it is evident that both the calcium and sodium channels are involved in overall regulation of cellular and neuronal excitability.\textsuperscript{14} Whether the same mechanism accounts for its other CNS effects still remains obscure.

The aim of our study was to study the broad neuropharmacological effects of eugenol. Hence our study explores the various CNS effects of eugenol with emphasis on antinociceptive effect, anticonvulsant effect, effect on motor co-ordination, pentobarbital induced sedation and anxiolytic effects using respective animal models.

Methods
A quantitative experimental study in mice and rats was done in the laboratory setting of the department of Clinical Pharmacology and Therapeutics, BP Koirala Institute of Health Sciences, Dharan, Nepal.

Drugs and chemicals
Eugenol (Loba Chemie, India); indomethacin (Articid, Sun Pharmaceuticals, India); phenytoin (M-Toin, Medopharm, India); valproate (Encorate, Sun Pharmaceuticals, India); Diazepam (Calmpose, Ranbaxy, India); Pentylenetetrazole (Sigma Chemicals, USA); pentobarbital (Loba Chemie, India); morphine (Martindale).

Animals
Experiments were performed on adult albino mice (n = 144) weighing 20-30g and wistar albino rats (n = 18) weighing 100-200g. Animals were produced in the laboratory breeding house of the department of Pharmacology. The animals were maintained under controlled room temperature (25 ± 2°C) and light and dark (12:12 hr) conditions and were given food pellets and water ad libitum. Before conducting the experiment, ethical clearance was obtained from the local Ethical Committee on Animal Research and ethical guidelines for investigations were followed in accordance with Indian National Science Academy (INSA). Each experimental test consisted of three groups of animals of six each viz. Group I (control, vehicle treated); Group II (standard control) and Group III (test drug, eugenol 100mg/kg).\textsuperscript{4}

Drug preparation
Drugs were administered intraperitoneally (i.p.) in a volume of 10ml/kg with a 27-gauge needle attached to the 1ml disposable syringe. The standard control for the analgesic effect was morphine 2.5mg/kg (hot-plate and tail-flick test) and indomethacin 20mg/kg (writhing test). Standard controls for anticonvulsant effect were phenytoin 10mg/kg (maximal electroshock seizure) and valproate sodium 300mg/kg (pentylenetetrazole induced seizure). Diazepam 5mg/kg was the standard control for effect on motor co-ordination; diazepam 3mg/kg for pentobarbital induced sleeping and diazepam 1mg/kg for anxiolytic effect (open-field and passive avoidance tests). Eugenol and indomethacin were dissolved in 0.5% Tween 80 in saline; morphine, phenytoin, valproate sodium and diazepam were dissolved in saline. Pentobarbital was dissolved in distilled water.

Experimental models
1. Antinociceptive effect
   (i) Hot plate method
   The thermal noxious stimulus was induced to mice by placing them in a hot-plate (UGO BASILE, ITALY) maintained at 53°C 10 min prior to the experiment. Drugs were injected i.p. 30 min before placing the mice in hot-plate and the reaction time (hot-plate latency) was recorded. Reaction time was taken as the period between placing the mice on hot-plate and the time when they jumped or licked their paws. A cut off time of 30 sec was used to prevent any thermal injury to the mice.\textsuperscript{15}
   (ii) Tail – flick method
   For the tail-flick method, pain was induced by giving infrared light on the tail of the mice (Tail-Flick Unit, UGO BASILE, ITALY) 5 cm away from the tip of the tail. Reaction time (tail-flick latency) was noted by observing the interval between placing the tail on the infrared light source and the withdrawal of the tail. A cut off time of 30 sec was used.\textsuperscript{16} Drugs were injected i.p. 30 min prior to the test.
   (iii) Writhing test
   At 15 min post dosing with the drugs, 1ml of 1% acetic acid was injected i.p. and the no. of abdominal contractions (writhing) produced was counted for 30 min from the time immediately after the injection of acetic acid. Antinociception was expressed as the difference in the number of abdominal contractions.
between saline treated control and animals pretreated with eugenol and indomethacin.4

2. Anticonvulsant effect
(i) Maximal Electroshock Seizure (MES) test
At 30 min post dosing, mice were subjected to MES of 50mA of alternating current from convulsimeter (Techno, India) for 0.2 sec through a pair of electrodes attached to ear. The duration of tonic hind-limb extension phase and the number of animals protected from convulsions were noted.14
(ii) Pentyltetetrazole (PTZ) induced seizures
Clonic seizures were produced in mice by injecting PTZ (50mg/kg) i.p. and the latency to first convulsions and the number of mice which exhibited seizures was noted.17 Following PTZ administration, animals were observed for a maximum period of 30 min. Reduction of jerky movements in comparison to the control was selected as the criteria to establish anticonvulsant activity of the drugs. Drugs were injected i.p. 30 min prior to the test.

3. Test of motor co-ordination
A rota-rod treadmill device (Techno, India) was used. Mice were placed on a horizontal rotating rod (16RPM) and these mice were selected for their ability to remain on the revolving bar for a 2 min period. 15 min after administration of drug, each mouse was placed on a rotating rod for 60 sec at intervals of 30 min for 2 hrs. The endurance time for each mouse on the rota-rod was noted.18

4. Pentobarbital induced sleeping time
The rats were sedated by administration of pentobarbital. 15 min after administration of drug or vehicle, all the animals received 30mg/kg i.p. of Pentobarbital. The time that elapsed between loss and recovery of righting reflex was taken as the sleeping time and recorded accordingly.19

5. Anxiolytic effect
(i) Open-field test
An 'open field apparatus’ comprising of floor space of 40cm×40cm with 30cm high walls was used. The floor area was painted black and divided into 9 equal squares by white lines.20 A mouse was placed at the centre of the field and left for 2 min. for acclimatization with the apparatus. Then after for the next 5 min. the following parameters were noted:

1. No. of squares crossed.
2. Time spent in central square
3. No. of rearing (standing on rear paws)

All the above parameters are inversely proportional to the level of anxiety. The observation was made on a closed circuit TV.
(ii) Passive avoidance test
A study chamber measuring 34cm×34cm×20cm was used through which electric shock of 20mv was delivered on contact. A shock free zone (SFZ) was provided in the centre of the chamber by placing an inverted Petri dish. Mice were initially placed on the shock free zone and they got down on the grid floor where they received an electric shock. A reduction in the natural anxiety on exposure to such a situation was implicated by decrease in the latency to step down and an increase in the no. of step down errors.21 The parameters noted during passive avoidance behavior were:
1. Step down latency (duration for which the animal stayed in SFZ).
2. Step down error (no. of attempts made to come to shock zone).
3. Total time spent in the shock zone.

Horizontal bar test – Whether the drugs at the given doses produced muscle relaxation in mice was tested using the ‘horizontal bar test’. In this, the animals were made to hang from a horizontal wire suspended between two vertical stands. Animals that failed to retain on the rod for 30 seconds were considered test-positive indicating muscle relaxation in them.

Statistical analysis
All the data were expressed as median and interquartile range. Analysis was done using Mann Whitney U test and p <0.05 was considered to be statistically significant. Number of animals protected in MES and PTZ tests was compared using Chi square test.

Results
Effect of eugenol on nociception
The effect of eugenol on acute thermal nociception was tested using Hot-plate test. Intraperitoneal administration of eugenol (100mg/kg) significantly increased the hot-plate latencies. Similarly, Tail-flick test was used to test the effect of eugenol on radiant heat nociception and eugenol significantly increased the tail-flick latency as compared to control. Also in writhing test, there was a significant decrease in the
number of writhing produced by administration of 1% acetic acid (Table 1).

**Effect of eugenol on convulsion**
In MES test, eugenol (100mg/kg) reduced the no. of convulsing animals suggestive of protective effect in mice against the maximal electroshock induced convulsion and it also significantly reduced the duration of tonic hind limb extension. Likewise, there was an increase in the latency to convulsions with the use of eugenol in PTZ induced seizure and 50% of the animals were protected (Table 1).

**Effect of eugenol on sedation**
Sedation on rats was induced by intraperitoneal administration of 30mg/kg of pentobarbital. Eugenol (100mg/kg) significantly increased the total sleep time as compared to control (Table 1).

**Table 1: Median, interquartile range and P Value of the of the observed parameters comparing Group I (control) vs Group III (100mg/kg Eugenol)**

| S.N. | Test                          | Variables                        | Median  | Inter-quartile range | P value |
|------|-------------------------------|----------------------------------|---------|-----------------------|---------|
| 1    | Hot-plate                     | Hot-plate latency                | 24.5    | 10.00 – 29.17         | 0.002*  |
| 2    | Tail-flick                    | Tail-flick latency               | 3.30    | 1.87 – 8.77           | 0.002*  |
| 3    | Acetic acid induced writhing  | No. of writhes                   | 28.50   | 20.50 – 66.75         | 0.002*  |
| 4    | MES                           | Duration of tonic hind limb extension | 5.00   | 0.00 – 10.00         | 0.009*  |
| 5    | PTZ                           | Latency to convulsion            | 1.15    | 0.00 – 3.26          | 0.004*  |
| 6    | Pentobarbital induced sleep   | Total sleep time                 | 235.20  | 124.30 – 286.85      | 0.002*  |
| 7    | Motor co-ordination in Rota-rod| Endur. time (30min)              | 41.00   | 3.75 – 60.00         | 0.004*  |
|      |                               | Endur. Time (1hr)                | 34.00   | 13.75 – 60.00        | 0.485   |
|      |                               | Endur. time (1.5hr)              | 47.00   | 31.5 – 60.00         | 0.589   |
|      |                               | Endur. Time (2hr)                | 60.00   | 51.50 – 60.00        | 0.480   |
| 8    | Open - field                  | No. of sq. crossed               | 94.00   | 76.50 – 134.50       | 1.000   |
|      |                               | Time spent in cntl. Sq.          | 4.00    | 3.00 – 6.25          | 0.589   |
|      |                               | No. of rearing                   | 24.00   | 16.50 – 41.25        | 0.485   |
| 9    | Passive - avoidance           | Step down latency                | 61.00   | 12.00 – 87.25        | 0.394   |
|      |                               | Step down error                  | 3.00    | 2.75 – 4.25          | 0.093   |
|      |                               | Time spent in sh. zone           | 9.50    | 8.00 – 39.25         | 0.589   |

*P<0.05

**Discussion**
Our study evaluated the various neuropharmacological effects of eugenol at 100mg/kg dose using respective animal models. To assess the antinociceptive effect, three models were used viz. Hot-plate test, Tail-flick test and the acetic acid induced writhing test and eugenol had a significant effect (p = 0.002) in all the three models. This effect is in accordance with previous studies, however, this study differs from the earlier studies primarily with respect to the route of administration, the dose employed and the source of eugenol. From this study, we can say that eugenol alleviates pain of both central as well as peripheral origin. The mechanism behind the central analgesic effect cannot be elucidated; however, it can be due to inhibition of sodium and calcium currents as well as modulation of vanilloid receptor. The intensity of analgesic effect of eugenol was similar to that of indomethacin (20 mg/kg, i.p.) in acetic acid induced abdominal
accompanying motor co-ordination beyond that time which is in accordance to the results of study by Kurian et al.\textsuperscript{4} The reason behind reduction in endurance time at 30 min cannot be elucidated but nevertheless this finding also adds one more clue to the sedative effect of eugenol.

To test the anti-anxiety effect, two models were used viz. open-field test and the passive-avoidance test. The results obtained in the open-field test showed no significant differences among all the groups tested. This behavior model is used to study exploratory and motor activity i.e. locomotion. After the administration of eugenol, no alterations were observed in this behavior model. Anxiolytic drugs like diazepam increase the number of squares crossed, number of rearing and increase the time spent in the central square. Lack of such findings with eugenol suggests that it does not alter the normal CNS paths for locomotion activity and emotionality. Likewise, in passive-avoidance test, the animal avoids punishment by refraining from to a grid floor. Hence, a decrease in step-down latency, increase in step-down errors and an increase in total time spent in the shock zone indicate reduction of normal anxiety associated with exposure to a novel environment.\textsuperscript{21} In this study, there were no significant differences in the above parameters in control and the test group suggesting that eugenol doesn’t have anti-anxiety effect.

Eugenol thus acts at diverse sites that may be relevant to various neuropharmacological actions but the precise mechanism remains unclear. Relevant sites of action include voltage gated ion channels (sodium and calcium), ligand gated ion channels, vanilloid receptor, excitatory receptors of glutamate i.e. N-methyl-D-aspartate (NMDA) and the inhibitory receptors for GABA as well.

**Conclusion**

In conclusion, this study evaluates various central nervous system effects of eugenol at 100mg/kg dose using the respective animal models. Though a few isolated studies to evaluate analgesic and anticonvulsant effect of eugenol were done earlier, there was no single study like this one that tested a gamut of neuropharmacological effects in a single study with pure eugenol. This study shows eugenol to possess antinociceptive, anticonvulsant and sedative effect whereas it didn’t have any effect on models of motor co-ordination and anxiety i.e. it was
neither anxiogenic nor anxiolytic. Various target sites have been implicated but this study doesn’t conclude a plausible mechanism behind all these observed effects. Further studies are required to explain precise mechanisms implicated in such a varied response at wider range of doses and dosage combinations and to elucidate potential therapeutic utility of eugenol.

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