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RESEARCH ARTICLE

ANXIOLYTIC EFFECTS OF HYDROALCOHOL EXTRACT OF *FERONIA LIMONIA* (WOOD APPLE) FRUIT IN MICE MODEL OF ANXIETY

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ABSTRACT

The anxiolytic effect of hydroalcohol extract of *Feronia limonia* (HEF) was investigated in the present study. The extract was orally administered to the Balb/C mice for a period of 7 days at the graded dose of 50, 100 and 200 mg/kg, bd.wt. Mice treated with 100 and 200 mg/kg bd.wt extract, significantly increased the time spent by the mice in open arm in elevated plus maze test (EPM), increased the square crossing in open field test (OFT) and increased the numbers of punished lick and shocks received in Vogels conflict test (VCT). Treatment with HEF also reduced the corticosterone levels in plasma, and GABA in brain tissues of mice. Over all the peak anxiolytic effects was obtained at the dose of 100 mg/kg bd.wt in mice. Thus, the present findings suggest the use of FLH as a potential herbal anxiolytic agent.

INTRODUCTION

Anxiety is a psychiatric disorder affecting millions of people worldwide every year. Stress is one of the primary causes for initiation of anxiety, depression and other disorders. Anxiety sometimes can aggravate the diseases such as cardiovascular neuropsychiatric conditions in suffering persons (Chatterjee *et al*., 2013). There are several anxiolytic drugs currently available in the market for treating anxiety as well as mood related disorders. Most of the drugs affect the central nervous system directly or indirectly and modulate the levels of neurotransmitters such as adrenaline, serotonin and dopamines. Benzodiazepines drugs (diazepam, alprazolam, chlorazepate, lorazepam, oxazepam) work on $GABA_A$ receptors whereas Buspirones mediate effects through serotonin ($5\text{-}HT_{1A}$) receptor. Though the selective serotonin reuptake inhibitors (SSRIs) are very effective in its pharmacological action but its usage is limited by the associated side effects of insomnia, drowsiness, agitation, headache, fatigue etc.

As an alternative therapy to the existing pharmacological drugs, herbal based natural phytocompounds are being in the research domain for the last several decades as they are with least side effects, more-tolerated and sometimes better efficacious (Doron *et al*., 2012). Plants such as *Salvia elegans* (Herrera-Ruiz *et al*., 2006), Drymaria *cordata* (Barua *et al*., 2009) *Crinum zeylanicum* (Yahaya *et al*., 2013) *Passiflora* edulis (Deng *et al*., 2010, Li *et al*., 2011) *Panax quinquefolium* (Wei *et al*., 2007) *Ziziphus jujube* (Peng *et al*., 2000) *Stachys lavandulifolia* (Rabbani *et al*., 2003) *Centella asiatica* (Wanasuntronwong *et al*., 2012) *Coriandrum sativum* (Emamghoreishi *et al*., 2005) etc have been proven to posses anxiety-like effects in animal models. *Feronia limonia* L (Syn. *Feronia elephantum*, *Limonia acidissima*), commonly known as Wood apple, belongs to Rutaceae family and bears a hard-shelled seeded berry fruit with brown coloured sweet pulp. The plant possesses therapeutic properties including anti-cancerous, hypoglycemic, hepatoprotective etc (Gupta *et al*., 2009). The fruit is also used for treating dysentry, diarrhoea and several other diseases. Fruit contains polyphenolic acids, flavonoids, tannins, phytosterols, coumarins saponins,and triterpenoids.

The present study was undertaken to investigate the anxiolytic-like effect of hydroalcohol extract of *Ferroni limonia* fruit in mice model of anxiety.

MATERIALS AND METHODS

Chemicals

All chemicals were obtained from Merck (Darmstadt, Germany). Solvents used in chromatography methods were HPLC-grade. Diazepam (purity: not less than 98.0%) was obtained from Sigma.

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Preparation of extract

Ripened fruits of wood apple were purchased from the local market in Mysore. The pulp along with seeds were oven dried at 45°C for overnight, and then the dried materials were powdered using a blender. The extract was prepared using 70 % hydroalcohol in an orbital shaker for overnight. The solvent was flash-evaporated followed by lyophilisation.

Animal experiment

Male Balb/c mice (20-25 g) were maintained under a standard laboratory conditions in accordance with the guidelines of National Institute of Nutrition, India and approved by Institutional Animal Ethics Committee (IAEC). These mice had free access to laboratory feed and tap water *ad libitum*.

Behavioural studies

a) Elevated-plus maze test (EPM)

This is the most widely used model for evaluating anxiolytic activity of any drugs/phytocompounds (Lister, 1987). The apparatus has two open-arms (35 cm x 5 cm) and two closed-arms that are extending from the common platform of 5cm x 5cm. This structure was elevated to 60 cm from the floor level. Mice were divided randomly into 5 groups consisting 6 animal each namely, Group I- Normal Control, Group II -50 mg extract, Group III-100 mg extract, Group IV-200 mg extract and Group V- Diazepalm (a standard anxiolyte). Mice were treated daily for 7 days with extract or standard (single dose) 1 hr prior to the EPM test. Each mouse was placed at the centre of the apparatus facing one of the open arms, and the behaviour of the mice was video recorded for 5 min using ANY-maze software (ver.4.98, Stoelting Co., USA). The time animal spent in the open arm is used for measuring the anxiety-like behaviour.

b) Open field test (OFT)

This test is used for measuring the spontaneous locomotor activity of the mice. The apparatus consist of 24 identical squares surrounded by Perspex walls. Each mouse was placed in the centre of the apparatus and its behavior was video recorded for a total of 5 min by measuring the total number of line crossings by the software.

c) Vogels conflict test

The plexiglass apparatus with the dimension of 50 x 12x 40 cm has grid floor of stainless steel and contained a drinking water bottle with water (Columbus, USA). The test was carried out as per the methodology described by Vogel *et al*., 1971 with slight modifications. An electric shock of 0.4 mA was applied between the grid floor and the drinking spout for 1 second during the experiment. The first shock was given after 30 s of drinking and thereafter at every 20^{th} lick for the subsequent test period of 3 min. The number of shock received by each animal was recorded. Animals were deprived of water for 24 hrs before the test.

Cortisol level

The plasma cortisol level was estimated using EIA kit as per the protocol given by Cayman chemical company, USA.

Determination of neurotransmitter levels and GABA by HPLC

Monoamine neurotransmitters (5-HT, NA and DA) were determined in homogenate of whole brain tissues by HPLC-Electrochemical detection (Waters 2465 model). In brief, the brain tissue were homogenized in 0.4 M perchloric acid containing 5 mM sodium bisuphite, 0.04 mM EDTA and then centrifuged at 15,000 rpm for 15 min at 4°C (Alburges *et al*., 1993). The separation was done on a C18 RP-18 column (150 mm x 4.6 mm, 5 µm). The mobile phase consisting of 17.6% methanol, 82.4% water containing $EDTA_2Na$ (0.0876 mM), triethylamine (1.5 mM), DL-camphorsulphonic acid (9 mM), disodium phosphate (20 mM) and citrate (9 mM) at a flow rate of 0.7 ml/min was used in isocratic elution mode at 25°C. The measurement was done at electropotentials of a glassy carbon electrode +650 mV versus Ag/AgCl reference electrode. The neurotransmitters were identified based on the retention time of the standards.

For GABA estimation a derivatising agent was used (Smith and sharp, 1994). 22mg o-pthaldehyde in 0.5 ml of ethanol was mixed with 0.5 ml of 1M sodium sulphite and 0.9 ml of 0.1m tetraborate buffer and then the pH was adjusted to 10.4 with NaOH. The required quantity of derivatising agent was mixed with standard GABA (100 nM/L) for 10 min at room temperature before injected into HPLC.

Statistical analysis

Data are shown as mean ± SD. The statistical analysis of the results was carried out with a SPSS 11.0 program. Comparison between groups was analyzed by Tukey's honestly significant difference (HSD) method and the *p* values less than 0.05 were considered as significantly different.

RESULTS

a) Effect of extract on elevated-plus-maze test (EPM)

The oral administration of extract (HEF) did not result in increased time spent by the mice in open arms of EPM at the lower dose of 50 mg/kg (54.5 ± 6.7 sec) comparing with the vehicle control (44.2 ± 5.3 sec) however, it was increased by >2-fold for the medium dose (100 mg/kg) and 1.5 fold for the higher dose (200 mg/kg) significantly ($p<0.05$, Fig 1). The response was found to be in an inverted U-shape over the doses. The standard drug (diazepam, 2.5 mg/kg) resulted in a pronounced 3 fold increase in time spent by the mice. This increased time spent and entries into open arms reflect the lesser aversion to the open space caused by the anxiolytic action of the extract as like as the standard.

b) Effect of extract on locomotor activity by Open field test (OFT)

Figure 2 shows the exploratory activity of the mice by OFT test. Total square crossing were not significantly altered by any treatment as vehicle control.

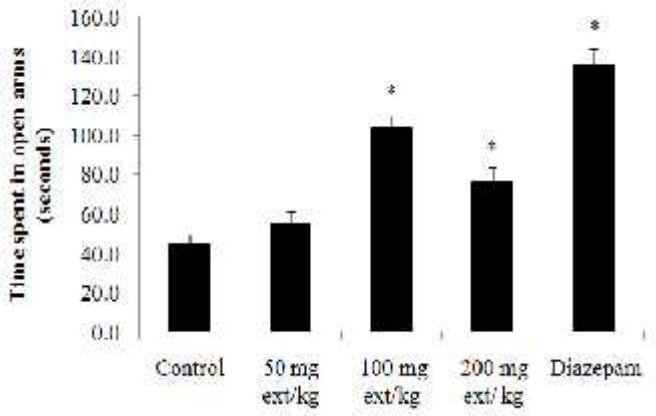


Fig. 1. Effect of extract on the time spent by mice in elevated-plus-maze test. Bars represent mean values ± S.E.M. * *P* < 0.05 vs control

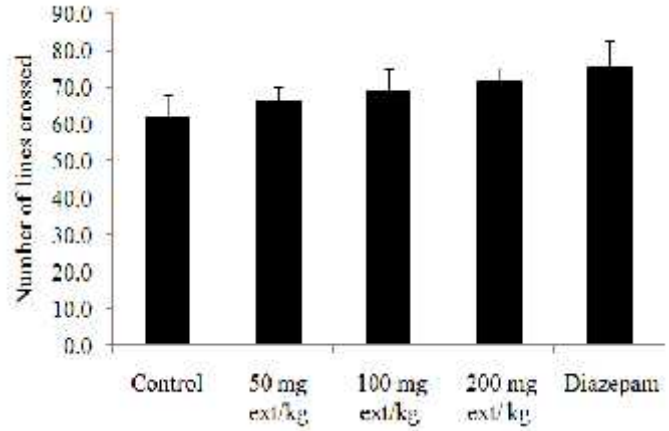
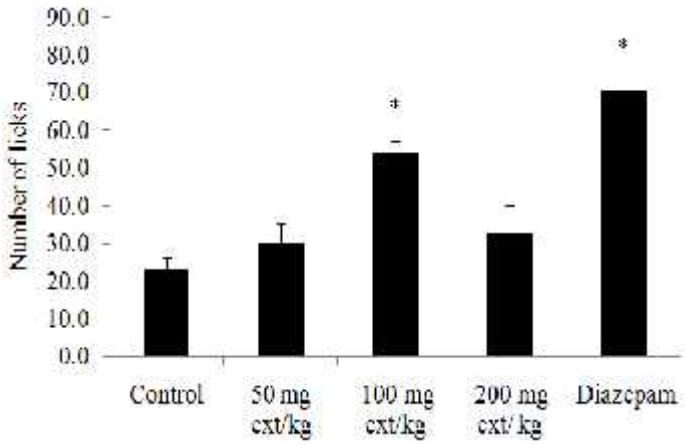


Fig. 2. Effect of extract on squares crossed in open field test. Bars represent mean values ± S.E.M. * *P* < 0.05 vs control

c) Effect of extract on Vogels conflict test (VCT)

Mice treated with HEF extract increased the numbers of punished licks endured and subsequent number of shocks accepted (Figure 3) in an inverted-U shaped pattern for all the doses (Figure 3).



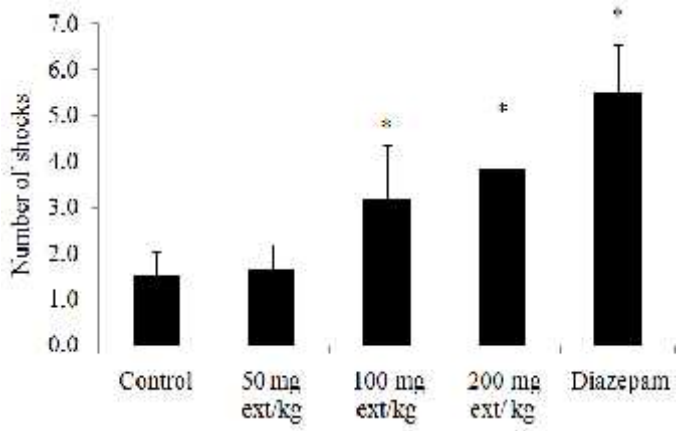


Fig. 3. Effect of extract on vogel conflict test in terms of number of licks and shocks received. Bars represent mean values ± S.E.M. * *P* < 0.05 vs control

d) Effect of extract on corticosterone levels in plasma

As shown in Figure 4, the extract significantly reduced the level of corticosterone at the medium and higher doses in comparison to control mice ($p<0.05$).

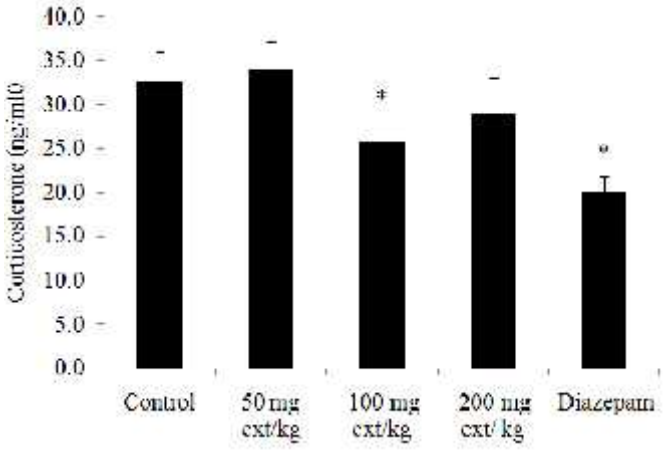


Fig. 4. Effect of extract on plasma levels of corticosterone. Bars represent mean values ± S.E.M. * *P* < 0.05 vs control

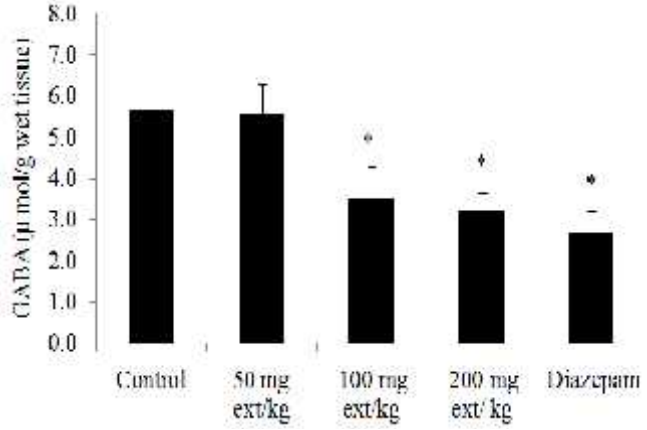


Fig. 5. Effect of extract on GABA levels in brains tissues. Bars represent mean values ± S.E.M. * *P* < 0.05 vs control

e) Effect of extract on neurotransmitters level in brain

The HEF extract showed a reduction in GABA levels in whole brain homogenate of mice treated with 100 and 200 mg/kg bd.wt compared to vehicle control (5.7 µmol/g). However, no difference was observed among all the groups with regard to the concentration of neurotransmitters such as noradrenaline, dopamine and serotonin in brain tissues (Table 1).

Table 1. Effect of oral administration of hydroalcohol extract of *F. limonia* on brain monoamine neurotransmitters

	Neurotransmitters level (ng/g tissue)		
Groups	Nor-adrenaline	Dopamine	Serotonin
Vehicle control	312 ±12	936 ± 37	721 ± 31
50 mg/kg bd.wt	309 ±14	915± 57	740 ±42
100 mg/kg bd.wt	316 ±10	889 ±49	722 ± 35
200 mg/kg bd.wt	321 ± 11	904 ± 52	728 ± 41
Diazepam	304 ±11	947 ± 31	707 ± 46

DISCUSSION

In the present study, three different doses (50, 100, 200 mg/kg bd. wt) of hydroalcohol extract of wood apple fruit was evaluated for its anxiolytic activity using elevated plus maze test (EPM). It is a well established, reliable and frequently used model for testing anti-anxiety of drugs. In the elevated-plus maze test, anxiolytic compounds reduce the natural animal aversion to the open arms and promote the exploration of the field therefore there is an increase in locomotion. Anxiolytic effect of the wood apple extract was evident from the increased time spent by mice in open arms at the medium dose and this effect was reduced a little at higher dose indicating that the anxiolytic effect appeared to have reached a pleateu at the medium dose.

The open field test (OFT) is performed to determine the emotional state of animals. Thus, it is expected that animals undergoes a kind of anxiety and fear due to exposure to novel environment that is different from its acclimatized cage. This augments the autonomic activity of the animal expressed by alteration in parameters, such as decreases in ambulation and exploration, immobilization, reduction in normal rearing and in grooming behaviour, and defecation. Diazepam, a positive control drug, at the dose that was used in this study did not reduce the locomotion. Similarly treatment with HEF did not alter the exploratory activity of the mice at all the doses. These results are in consistent with the earlier published report for the anxiolytic effect of *Carica papaya* (Kebebew and Shibeshi, 2013) of the mice.

In vogel conflict test, the conditional response of the mice is used to evaluate the neurological underpinning of the anxiety. Administration of HEF extract in our study increased the tolerance level of mice to punished licks and shock acceptance confirming the anti-anxiety activity of the extract. Most of the reported anxiolytic activity of drugs act via modulation of GABAA receptors and therefore it is the main target of clinically effective drugs as well as phytocompounds (Anuradha *et al*., 2008; Carro-Jua´ rez *et al*., 2012). mGLU7 receptor is an another target that can be explored to modulate excitatory and non-glutametergic neurotransmission (Cryan *et al*., 2003). Some neurotransmitters also particularly serotonin (5-HT) play a pivotal role in the anti-anxiety effects of drugs including benzodiazepines; however the effect is inconsistent at the lower doses (Costa *et al*., 2011). Increased level of 5-HT and decreased level of its metabolite has been shown to reverse the anxiety behaviours (Sayeed *et al*., 2014).

Some polyphenols (quercetin and kaempeforl) of plants remarkably alleviate the anxiolytic symptoms in animals (Aguirre-Hernández *et al*., 2010). The extract of wood apple contains an array of phytocompounds including andioxidant polyphenols and flavonoids which in partly might be exerting anxiolytic effects through binding BAGA receptors or through other neurotransmitter systems of serotoninergic, dopaminergic and noradrenergic. The precise mechanism underlying the effect remains to be further explored at cellular level.

Conclusion

In summary, the results obtained in this study indicate that extract of wood apple fruit has peak anxiolytic-like activity at the dose of 100mg/kg.bd.wt. Further research work is required to identify the responsible compound for such observed effect by bioactivity guided fractionation.

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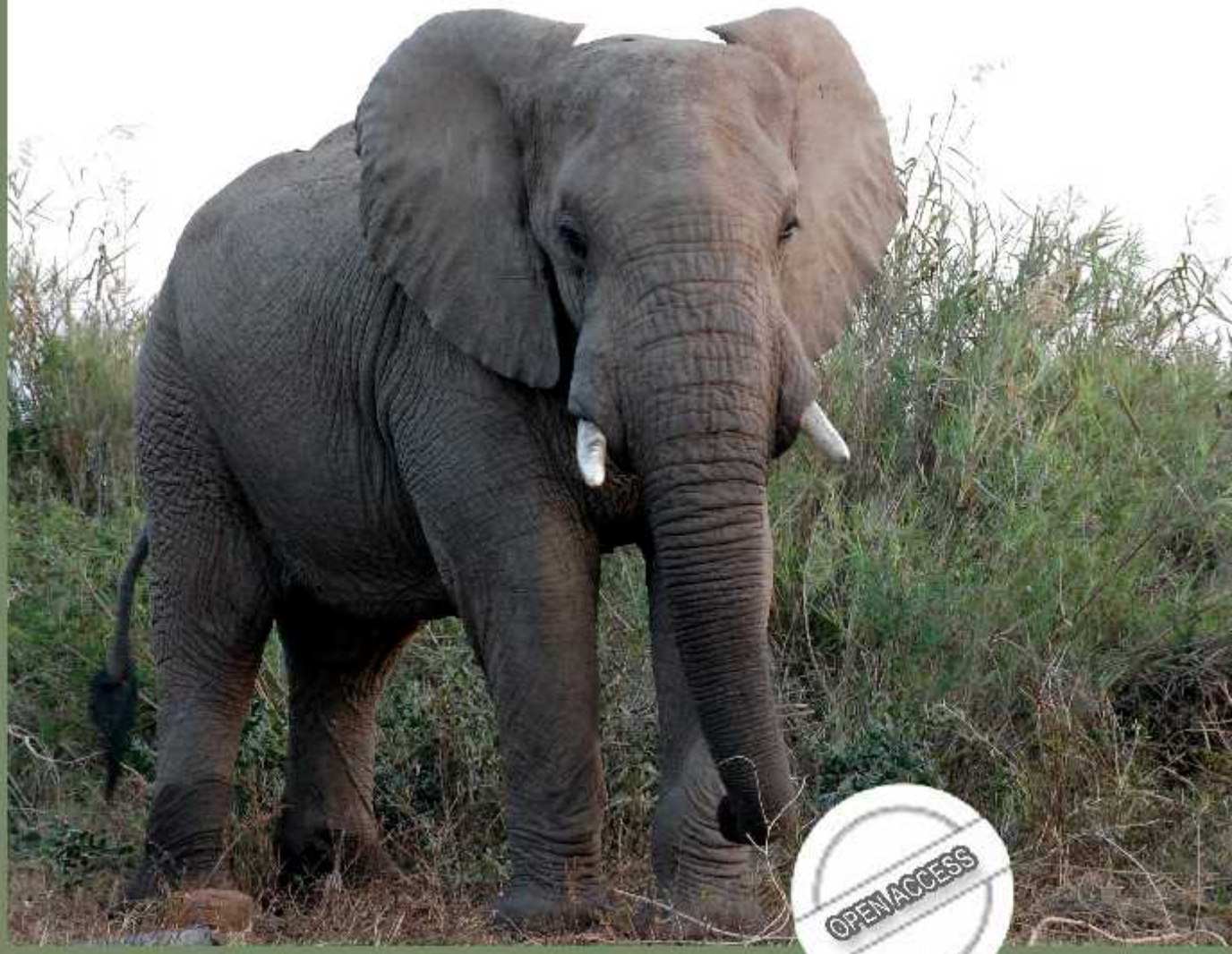

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