International Journal of Pharmacology and Clinical Research (IJPCR)

ISSN:2521-2206

IJPCR |Volume 1 | Issue 2 | July – Dec- 2017
www.ijpcr.net

Research Article *Clinical Research*

Evaluation of anti-anxiety activity of ethanolic extract of *rhus mysorensis* on *wistar* rats

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ABSTRACT

The aim of this study is to evaluate the preliminary phytochemical screening and anti-anxiety activity of the ethanolic extract of areal parts of *Rhus mysorensis* in rat models. The ethanolic extract of *Rhus mysorensis* was prepared using 97% ethanol 72hrs by maceration. The oral dose of 200 and 400 mg/kg of the extract was evaluated for the activity in elevated plus maze and open field models. The obtained results have shown that the *Rhus mysorensis* ethanolic extract shown the presence of flavonoids, alkaloids, terpenoids, tannins and steroids and also shown the significant anti-anxiety activity on *wistar* rats on dose dependent manner. based on the results it is concluded that the ethanolic extract of areal parts of *Rhus mysorensis* is having significant anti-anxiety activity on *wistar* rats. Further investigation is required by using the purified compound to evaluate and isolation of the phytochemicals responsible for the anti-anxiety activity of ethanolic extract of *Rhus mysorensis.*

Keywords: Rhus mysorensis, Anti-anxiety, Diazepam, Ethanol, *Wistar* rats.

INTRODUCTION

Stress is a leading precursor to diseases like anxiety and depression. Cohen, Kessler, & Underwood Gordon (1995), define stress as an, "environmental demand that exceeds the adaptive capacity of an organism, resulting in psychological and biological changes that may place a person at risk of disease". Conditions associated with long term stress also include diabetes, high blood pressure, cardiac arrest, stroke anorexia, obsessive compulsive disorder, alcohol and drug abuse, and hyperthyroidism1 Anxiety and depression can have cognitive, affective, physiological, and behavioural implications. Examining the effects of anxiety; at a cognitive level, stimuli or situations can be construed as threatening; affectively a person may feel apprehensive, tense or uneasy; and physiologically, autonomic arousal prepares the body for flight or fight and in extreme case freezing or immobility [2].

According to a survey conducted by Harvard Medical School in 1997; between 1990 and 1997, the American population made around 627 million visits to alternative medical practitioners, spending an estimated $27 billion of their own money on alternative therapies and medicines [1]. Another reason for the shift towards alternative therapies would be a general disillusionment with

conventional medicine due to the numerous side effects, their ineffectiveness to treat several chronic diseases, microbial resistance, and the high cost of new drugs [3].

Traditionally well-known plant Rhus mysorensis belonging to the family of Anacardiaceae, used in treatment of diabetis [4]. Young shoots made into paste, and applied externally on spots to treat psoriasis5 and pharmacologically used identified as hepatoprotective activity [6, 7]. The plant is aromatic, often gregarious shrub commonly distributed in North West India to the peninsular India. with a thin brown bark and spiny branches. Leaves are divided into 3 leaflets. Leaflets are deeply toothed, or lobed, the middle one 1-1.5 in long, the lateral ones smaller. The leaflets are nearly stalkless. Flowers are small, white, or greenish, borne in panicles at the end of branches or in leaf axils. Sepal is small, 4-5-parted, persisting even in fruit. Petals are 5, ovate, falling off early. Disk fleshy, obscurely 5-lobed. Ovary 1-celled; styles 3. Fruit is a small, dry, compressed drupe, 3 mm in diameter. The wood which is hard, reddish-yellow, close-grained and heavy, is only used for fuel, and the branches for fencing fields [7]

MATERIALS AND METHODS

Animals and housing

The rats were housed in pairs in standard rodent cages consisted of a plastic tray like bottom covered in dry non-treated wood shavings for bedding, and a wire cage top with a food hopper seated through a hole in the roof of the cage. The rats were kept under a 12-h reverse light cycle, in a temperature controlled environment of (22 ±1°C). Food and water were available at libitum, and water was replenished daily.

Preparation of the extracts

The course powdered of aerial parts of the plant was macerated in 97% ethanol for 72 hours to give the ethanolic extract. The filtrates were concentrated and evaporated to dryness under reduced pressure at 40^0C. The percentage yield was calculated. The dry extract was stored in a refrigerator at 4^0 C until use for the proposed experiment [8].

Phytochemical screening

Preliminary Phytochemical screening of the extract was subjected for the presence of flavonoids, alkaloids, phenolics, carotenoids, carbohydrates and glycosides [9].

Toxicity Studies]

The limit test (2000mg/kg) of ethanolic extract of Rhus mysorensis was performed as per the OECD guidelines, the Animals were observed individually at least once during the first 30 min after dosing, periodically during the first 24 h and daily thereafter for a period of 14 days for changes in skin and fur, eyes and mucous membrane (nasal) and also respiratory rate, salivation, lacrimation, urinary incontinence and defecation and central nervous system (tremors and convulsion) changes and Mortality was determined over a period of 2 weeks [10]. We did not find any changes including morbidity and mortality in rats.

Study design

Rats were randomly assigned to one of 4 groups, 2 treated groups, and 1 control and 1 standard group. The control groups consisted of vehicle control and diazepam control treatment groups. Each group was tested in each of 2 animal tests of anxiety, the elevated plus-maze (EPM), the open-field (OF) test.

APPARATUS

Tests procedure

Each group of rats were tested on different days to prevent all the rats being exposed to the test groups. Each group of rats were placed in the experimental room 24 hours before testing began.

Testing was conducted in the laboratory lights on, during the nocturnal phase of the animal's circadian cycle. All the rats were placed in a restraint tube and weighed half an hour prior to testing. Rats in the vehicle control and diazepam groups received an i.p. injection whilst in the restraint tube and all animals were returned to their home cages prior to testing. The behavioural tests were conducted 48 hours apart in the following

order: EPM, OF. Each group of rats remained in the experimental room until all the tests were completed. The 48-hour period between tests consisted of 24hr rest period plus a 24hr exposure period for the test groups; the control groups effectively receiving a 48hr rest period.

Elevated plus maze model

The EMP consisted of a wooden maze in the shape of a plus sign raised 50cm from the floor. The maze arms where 50x10cm in length with closed (protected) arms enclosed with sides 40cm in height with open roof. The open end of the closed arms and similarly the open (unprotected) arms were positioned opposite each other extending outward from a central platform 10x10cm². In addition to the normal laboratory lighting, 100W anglepoise lamps were utilized to illuminate each open arm. The lamps were positioned in such a way as to leave the closed arms in shadow.

The rats (200–250g body weight) are housed in pairs for 10 days prior to testing in the apparatus. During this time the rats are handled by the investigator on alternate days to reduce stress. Groups consist of 6 rats for each dose. Thirty min after i.p. administration of the test drug or the standard, the rat is placed in the center of the maze, facing one of the enclosed arms. During a 5 min test period the following measures are taken: the number of entries into and time spent in the open and enclosed arms; the total number of arm entries. The procedure is conducted preferably in a sound attenuated room. The scored behaviours included, the frequency of head-dips and stretch-attends. Stretch-attend were further differentiated as protected or unprotected depending on where they occurred in the maze. Protected stretch-attends occurred in the centre square or close arm of the maze, unprotected stretch-attends occurring in the open arm of the EPM [11].

Open field Model

Open-field activity and social behaviour were tested in a 100x100x600cm square Perspex box lined on the outside with black PVC plastic. A 5x5 grid was taped to the floor of the open field dividing the arena into 25 squares (20x20cm²), effectively creating 3 concentric zones, the central (Zone1), middle (Zone2) and outer (Zone3) zones. The outer zone was situated proximal to the wall of the open-field (see Figure 2). The floor of the arena was covered with clear PVC for ease of cleaning. In addition to the normal laboratory lighting, 100W anglepoise lamps were placed on opposite side of the OF, positioned to remove any shadows.

Spatiotemporal measures in the OF included the frequency of entries into each zone and the time spent in each of the 3 zones. Behavioural measures included the total frequency and duration of immobility, grooming, sniffing and rearing. Furthermore, the total number of line crossings and the total mobility were also measured. Increased locomotion and exploration of the more central zones (1&2) of the arena represent an anxiolytic response in the OF. An increase in exploratory behaviour, i.e. rears and sniffs and a decrease in immobility and grooming are also indicative of an anxiolytic response in the OF [12, 13].

Statistical analysis

When ANOVAs were significant post-hoc tests were conducted to further investigate the effect. Dunnett's t-tests were used to compare the treatment groups to the control, whilst Bonferroni corrected t-tests were used to compare between the treatment groups when required.

RESULTS

The results are presented in 2 sections, each addressing one of the two novelty evoked models of anxiety utilized in this study. Firstly, within each section a comparison will be made between the test and vehicle control, to determine whether they can be pooled. Secondly, the diazepam group will be compared to the control group to illustrate the effect of an anxiolytic on rat behaviour and establish a baseline for comparison for the anxiolytic properties of the test. The test groups will then be compared to the control to determine if they had a significant effect on rat behaviour. Finally, a comparison will be made between the effects of the test groups and the diazepam group sample size constraints prohibited the exclusion of behavioural outliers; to this extent all animals were included in the analysis. Furthermore, it should be noted that only

significant ($p<0.05$, $p<0.01$) and marginally significant ($p<0.1$) results will be presented.

ELEVATED-PLUS MAZE

Table 1 Comparison of anti-anxiety activity in test and standard groups in EPM

Behaviour	Control	Diazepam (2mg/kg)	T1	T2
Closed arm frequency	6.8±0.6	7.4±1.8	9.0±0.4**	10.0±0.6*
Closed arm duration	183.3±12.5	126.1±26.0**	178.7±11.8**	187.2±13.4**
Open arm frequency	1.2±0.3	2.9±0.8**	0.8±0.2**	1.1±0.4**
Open arm duration	18.8±6.2	40.7±10.9**	9.7±3.3**	12.5±5.3**
Centre frequency	8.3±0.7	10.7±1.9	10.2±0.4*	11.4±0.5**
Centre duration	98.4±10.1	133.2±25.9**	111.6±11.2*	100.4±9.4**
% Closed Arm	42.5±1.8	29.0±5.3**	45.0±1.4**	44.4±1.6**
% Open Arm	6.7±1.9	15.0±4.3**	4.0±1.2**	5.0±1.7**
% Centre	50.8±0.3	56.0±4.9	51.0±0.4 0	50.7±0.4*
Head-dip frequency	1.3±0.5	4.1±0.9*	.9±0.4°	0.7±0.4°
Head-dip duration	3.5±1.3	11.2±3.8**	2.3±1.2**	1.2±0.7***
Unprotected stretch-attend frequency	1.8±0.6	4.9±1.1**	1.2±0.4**	1.6±0.7**
Unprotected stretch-attend duration	11.8±4.4	15.8±4.5*	5.9±1.9*	8.5±3.3**
Protected stretch-attend frequency	5.3±0.6	4.8±0.9	5.6±0.7*	5.1±0.9**
Protected stretch-attend duration	36.0±4.6	25.8±6.4	37.4±3.9	26.6±5.6
Total entries	16.2±1.3	21.0±3.8	20.0±0.8**	22.5±0.9**

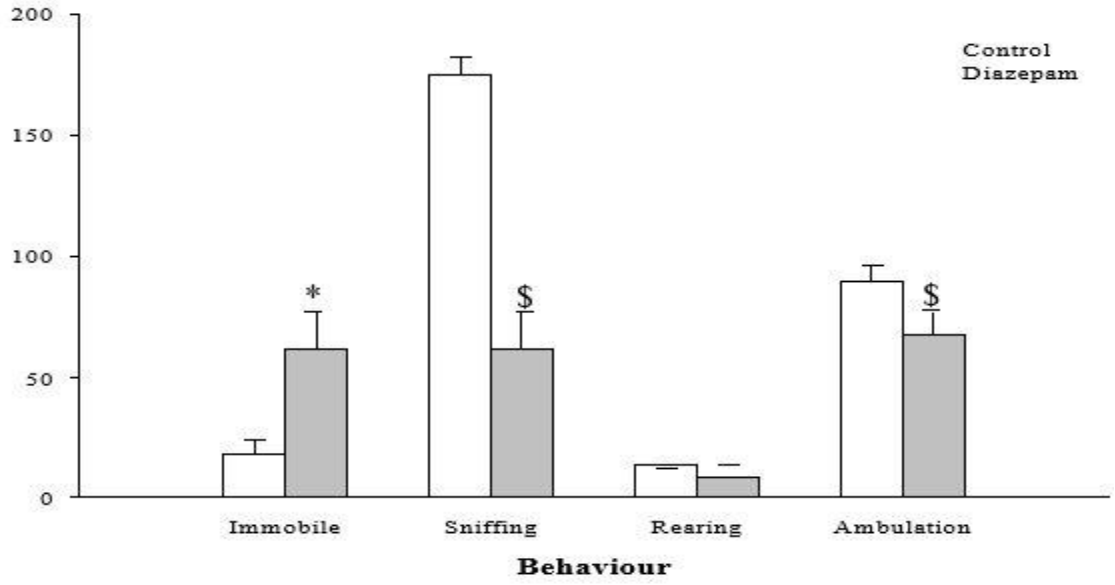
Values are expressed as mean±SEM (n=6); Statistical analysis of data was carried out by two way ANOVA followed by dunnet's test, *P<0.05, **P<0.01, ***P<0.001

Many studies (Dawson & Tricklebank, 1995) have differentiated between protected and unprotected risk assessment behaviours, this distinction will only be applied to stretch-attend and not head-dip behaviour because it was difficult to establish a clear distinction between the protected stretch-attend and the protected head-dip behaviours, i.e. all protected risk assessment was considered to be stretch-attend behaviour. Compared to the control group, the diazepam group made significant more open arm entries [$t(28)=2.33$, $p<0.05$] of marginally longer duration [$t(28)=1.92$, $p<0.1$], spent marginally more time in the centre [$t(28)=1.51$, $p<0.1$] and significantly less time in the closed arm [$t(28)=2.25$, $p<0.05$] of the EPM. Analysis of the behavioural measures revealed the diazepam group made significantly more unprotected stretch-attends [$t(28)=1.49$, $p<0.01$], as well as significantly more [$t(28)=2.33$, $p<0.5$] and longer [$t(28)=2.33$, $p<0.05$] head-dips than the control group (see Fig 1). Proportionate to the total number of entries, the diazepam group made significantly less closed arm entries [$t(28)=2.39$, $p<0.5$] and more open arm entries [$t(28)=2.08$, $p<0.05$]. On the whole diazepam increased open arm entries and the time spent in the open arms and centre square of the EPM; decreased time spent in closed arms and increased the number of head-dips and unprotected stretch-attends. The ANOVAs revealed significant differences among these groups for closed arm [$F(2,37)= 3.48$, $p<0.05$], centre square [$F(2,37)= 3.72$, $p<0.05$] and total [$F(2,37)=3.80$, $p<0.05$] entries; marginally significant differences were found for the number of entries [$F(2,37)=2.27$, $p<0.1$] and time spent [$F(2,37)=2.07$, $p<0.1$] in the open arm, and the frequency [$F(2,37)=2.47$, $p<0.1$] and duration [$F(2,37)=2.12$, $p<0.1$] of unprotected stretch-attends. Dunnett's t-test comparisons of the groups indicate the T1 group made significantly more close arm entries ($p<0.05$) and marginally more centre square entries ($p<0.1$) than the control group.

Table 2 Comparison of anti-anxiety activity in test and standard groups in open field model

Behaviour	Control	Diazepam (2mg/kg)	T1	T2
Frequency zone 1	1.4±0.2	1.5±0.3	1.3±0.2	1.5±0.2
Frequency zone 2	3.2±0.5	3.1±0.8	3.3±0.4	2.6±0.6**
Frequency zone 3	2.9±0.3	2.8±0.7	3.0±0.4	2.0±0.4*
Duration zone 1	4.4±0.8	10.3±6.2	2.2±0.3	3.9±1.0
Duration zone 2	8.0±1.5	4.0±1.1*	11.0±2.5**	5.8±1.6**
Duration zone 3	287.6±1.5	285.7±6.0	286.8±2.7	290.3±2.4
Total line frequency	44.1±3.8	51.9±6.8	50.2±2.6	42.3±3.6^{aa}
Total immobile frequency	2.3±0.5	2.5±0.6*	0.9±0.5*	0.6±0.3**
Total immobile duration	18.2±6.1	62.0±13.9*	10.1±4.7°	15.8±9.3***
Total grooming frequency	0.6±0.2	1.1±0.3$	1.1±0.3	1.4±0.3**
Total grooming duration	4.4±1.4	6.9±2.0*	7.0±2.5	6.8±1.7
Total sniffing frequency	39.8±3.0	35.3±4.1*	38.4±2.8	38.5±2.6
Total sniffing duration	175.4±6.7	154.5±8.9*	150.8±5.2**	169.0±6.2
Total rearing frequency	11.1±1.3	7.0±2.1*	15.5±2.4°°	13.7±2.0°°
Total rearing duration	12.5±1.5	8.8±3.1*	21.3±3.8**	18.4±2.8$
Total ambulation	89.5±6.9	67.7±9.2*	110.8±4.6*°	90.0±7.3£

Values are expressed as mean±SEM (n=6); Statistical analysis of data was carried out by two way ANOVA followed by Dunnett's test, *P<0.05, **P<0.01, ***P<0.001



Finally, in order to make a comparison between the anxiolytic effects of diazepam and the TESTs on rat behaviour in the OF, independent group t-tests were conducted. Only the behavioural measures where the diazepam demonstrated significant differences compared to the control rats were included in this analysis. Compared to the diazepam group, the T1 group spent significantly more time in Zone2 [t(18)= 2.49, p<0.05], spent significantly less time immobile [t(18)=3.55, p<0.01], made significantly more rears [t(18)=2.66, p<0.05] and spent significantly more time ambulation/exploring the arena [t(18)=4.19, p<0.01]. Similarly, the T2 group spent significantly less time immobile [t(18)=2.77, p<0.05], made significantly more rears [t(18)=2.36, p<0.05] and spent marginally more time ambulation/exploring the open-field [t(18)=1.90, p<0.1]. On the whole it

appears T1 and T2 increased the time spent in Zone2, and both Test groups reduced immobility, increased rearing and increased the overall ambulation/ exploration of the test arena compared to diazepam. These data are summarized in table 2.

DISCUSSION

The overall aim of the thesis was to evaluate the effects of Rhus mysorensis in two different tests of anxiety. The anxiolytic diazepam was used as a positive control. Following exposure to T1, rats showed fewer episodes of immobility, increased the time spent sniffing and rearing, and the time ambulating/exploring the OF. The increase in total entries suggests that T1 had a simulating effect in the EPM. The increase in locomotion and exploratory behaviour suggests T1 had and anxiolytic effect in OF. T2 produced a decrease in the frequency and duration of immobility, increased the frequency of grooming and increased the time spent rearing in the OF.

The increase in total entries also indicates the simulating effect of T2 in the EPM. The increase of grooming frequency could indicate that T2 had an anxiogenic effect in the OF. However, when compared to a decrease in immobility and the increase in time spent rearing, it appears that T2 had an anxiolytic effect in the OF. Exposure to of Rhus mysorensis produced an increase in centre square and total entries in the EPM. The oil combination increases the number of entries into Zone2 and Zone3 and the time spent in Zone2 in the OF. Furthermore, the combination produced an in decrease in immobility frequency and duration and an increase in the frequency and duration of rears.

The Rhus mysorensis also produced an increase in the frequency of line crossing and the time spent ambulating around the arena. With regards to diazepam, the results of this study are consisted with other research which indicate diazepam can have an anxiogenic effect on rat behaviour in the OF test14. A plausible explanation for the differing results could be that the different models of anxiety are tapping into different levels or types of anxiety, i.e. stress, fear or worry. Like diazepam, there appears to be a conflict between the effect of the Rhus mysorensis observed in the EPM and the effects of the Rhus mysorensis in the OF a tests. Research suggest that poor correlation15 and inconsistent drug effects across different animal models of anxiety and depression indicates that there are other mechanisms at work than just generalised anxiety, i.e. different animal models could be taping different aspect of anxiety. One explanation for these behavioural inconsistencies could be the difference between trait (a motive or acquired behavioural disposition that predisposes an individual to perceive a wide range of objectively non-dangerous circumstances as threatening) and state anxiety (characterized by subjective, consciously perceived feelings of apprehension and tension, accompanied by or associated with activation or arousal of the autonomic nervous system45 It has been suggested that lack of test/re-test stability illustrates that the elevated plus-maze is a better measure of state anxiety because novel exploration of the open arms is not stable but reduces with successive testing.

The present study demonstrates the addition of T1 had a potentiating effect on the anxiolytic properties of T2 in the OF because it caused an increase in the number of entries into Zone2 and Zone3, and the number of line crossings. Furthermore, the addition of T1 to T2 increased the time spent in Zone2. As such, this study indicates that further research is necessary to determine the importance of the unique composition of Rhus mysorensis on their overall effectiveness

The diazepam is considered the gold standard for the evaluation of anxiolytic effects in animal models of anxiety and depression, differences between the effect of diazepam and the Rhus mysorensis in this study suggest further research is needed to determine if the anxiolytic profiles of Rhus mysorensis are best compared to SSRIs or amphetamines rather than diazepam. As outlined earlier, lack of conformity in the research methodologies has produced conflicting conclusions as to the anxiolytic and anti-depressant properties of Rhus mysorensis.

CONCLUSION

Medicinal plants have been offered as a plausible alternative to conventional treatment approaches for anxiety and depression; however

there is insufficient empirical evidence to support their efficacy. However, there is little consensus about the appropriate testing methods and which of the myriad of psychotropic drug associated with the treatment of anxiety or depression are more appropriate as a positive control. Furthermore, many of the more familiar medicinal plants have been investigated however they are not necessarily the most appropriate oils for the treatment of human depression and or anxiety. Based on the results Rhus mysorensis showed a significant anti-anxiety property further studies are carried out to identify the phytochemical candidate showing anti-anxiety activity.

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