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Short Communication

# Evaluation of Anxiolytic Activity of *Pulsatilla nigricans* L. in Wistar Rats by using Elevated Plus Maze and Hole Board Paradigms

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**Authors' Contribution**
**RS executed the research. IY supervised and guided in planning the research. SM did statistical analysis. TBF assisted in compiling the data. SJK helped in lab work. SS and RF helped in writing the manuscript.**

**Key words**
***Pulsatilla nigricans*, Anxiolytic, Elevated-plus maze, Hole-board paradigms**

## ABSTRACT

*Pulsatilla nigricans* Linn has traditionally been used in various disorders related to both male and female and proves a great remedy for ovaralgia, ovaritis, epididymitis, orchitis nervousness, sadness, mild restlessness and mental disorder. This investigation is designed to evaluate acute toxicity and anti-anxiety activity of ethanol extract of *Pulsatilla nigricans* leaves. The acute oral toxicity shows that, plant extract up to 5000 mg/kg produces no signs and symptoms related to toxicity and also no mortality was observed. Antianxiety activity of *Pulsatilla nigricans* was examined by using elevated plus-maze paradigm and hole-board paradigm in rats at two different doses i.e. 250 and 500 mg/kg, and significant anxiolytic effects were observed as compare to control.

*Pulsatilla nigricans* belongs to family Ranunculaceae, and it is reported that nearly about 70 species of pulsatilla have been discovered (Jackson *et al*., 1946). *Pulsatilla nigricans* is also commonly named as pasque flower, wind flower, and is native to Russia, Turkey, France, Germany, Sweden, Southern England, Denmark and Asia (Felter and Lloyd, 1983). The plant of *Pulsatilla nigricans* is covered with white soft velvety hairs, the flower is dark violet-brown in color (Kumar *et al*., 2008).

Anxiety is affecting 1/8$^{th}$ of the world's total population and have become a huge area of interest in field of research (Eisenberg *et al*., 1998). Anxiety can be the cause of several disorders such as nervousness, fear, worrying, etc. It can be indicated in initial psychiatric disorders like obsessive compulsive disorder, panic disorder, post-traumatic stress disorder and generalized anxiety or could be a part or response to, any medical diseases (Lango *et al*., 2011; Pilkington *et al*., 2006).

Anxiety disorders affect people of almost all age groups because of stressful life style. However wide range of drugs has been introduced to treat anxiety disorder like benzodiazepines but have a narrow margin of safety and can develop dependence. This has boosted many researchers to search new compounds specially herbal medicines with less undesirable side effects (Griffiths *et al*., 1987; Davidson *et al*., 1997; Bell *et al*., 2011).

Different studies evaluated behavioral effects in different animal models by using various homeopathic preparations like *Nux vomica*, *Argentum nitricum* and many other (Bellavite *et al*., 2009). In this study, we evaluated the anxiolytic activity of *Pulsatilla nigricans* by using rat behavioral paradigms.

## *Materials and methods*

Protocols of our study were approved by Animal Ethical Committee of Hamdard University. *Pulsatilla nigricans* was bought from Mektum Homoeo Pharma, Pakistan. The identification test of the plant material was conducted by Dr. Huma Sharif, Department of Pharmacognocy, Jinnah Sindh Medical University, Karachi (voucher no. 02)

For extraction, 2 kg dried crushed leaves of the plant were grounded by Ayurvedic grinder (SSK 06) machine and then macerated in 70% of ethanol for about 7 days and then filtered. The alcoholic extract was concentrated by using Rotary Evaporator (Buchi B- 169 Vacuum system, Switzerland)

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**Table I. Effect of EEPN on gross behavior of rats.**

| Observations | Control | EEPN 5 mg/kg | EEPN 50 mg/kg | EEPN 300 mg/kg | EEPN 2000 mg/kg | EEPN 5000 mg/kg |
|---|---|---|---|---|---|---|
| Hair loss | --- | --- | --- | --- | --- | --- |
| Behavioral pattern | --- | --- | --- | --- | --- | --- |
| Drowsy | --- | --- | --- | --- | +++ | +++ |
| Diarrhea | --- | --- | --- | --- | --- | --- |
| Tremors | --- | --- | --- | --- | --- | --- |
| Salivation | --- | --- | --- | --- | --- | --- |

*+++, observed effect; ---, normal effect.

at 30–40 °C, and stored at 4°C in an air tight container.

Wistar rats (150-250 g) of both sexes were obtained from Animal House of Hamdard University, Karachi. All animals were kept in controlled environmental condition: 25 ± 2°C temperature, animals were allowed to access food and drinking water. All the experiments were conducted between 8 am to 4 pm. as per approved protocols given by Animals Ethics Committee.

Acute oral toxicity study was done according to guidelines given by Organization of Economic Corporation Development (OECD) No. 423 (OECD guideline 423, 2001). EEPN was given orally through gavage at doses of 5, 50, 300, 2000 and 5000 mg/kg body weight to female rats ($n$=3) and the percentage mortality and toxicity was recorded for a period of 24 h, the rats were observed for any gross behavioral change, convulsions, sedation, salivation, urination and defecation and body weight (Vogel, 2002). Due to no signs of toxicity and mortality, *Pulsatilla nigricans* was further evaluated for pharmacological activity at $1/10^{th}$ and $1/20^{th}$ dose of higher dose i.e. 5000 mg/kg.

Wistar rats in the weight range of 200–250 g were randomly divided into four groups of seven animals each. Group I and group II were marked as control and standard groups and given vehicle and diazepam via oral route (1 mg/kg) respectively. Other two groups were marked as treated groups, kept on ethanol extract of *Pulsatilla nigricans* (EEPN) given via oral gavage in doses of 250 and 500 mg/kg respectively.

The following two rat behavioral paradigms were used for anxiolytic activity:

(i) *Elevated plus-maze paradigm (EPM):* EPM comprises of four arms i.e. two open arms and two closed arms, closed arms are lined with side walls having an open roof, with the plus-maze elevated (50 cm) from the floor supplementary (Supplementary Fig. 1) used to evaluate anxiolytic behavior in rats (Vaz *et al*., 2009; Kumar and Sharma, 2005). The rats of all groups were placed individually in the middle of the EPM with its face facing towards the open arm for about five minutes, during which different behavior of the rats were recorded: (i) the number of entries of rat in open arms, (ii) average time spent by the rat in open arms. The maze was cleaned with 70% ethanol swab after each experiment.

(ii) *Hole-board paradigm (HBP)*: The hole-board apparatus consisted of a chamber with 16 holes evenly distributed on the floor supplementary (Supplementary Fig. 2). The apparatus was elevated to a height of 28 cm from the ground. Latency time of first head dip and counts of head dips were recorded during the 5 min observation in each group (Brown and Nemes, 2008). The apparatus was thoroughly cleaned by ethanol swab after each experiment.

The results have been expressed as mean ± standard error of mean (S.E.M.). The treated and standard groups were compared with control by one - way ANOVA along with Tukey HSD post hoc analysis. The results were considered significant at $p<0.05$.

*Results*

Tables I and II show that oral administration of EEPN up to 5000 mg/kg did not produces any kind of toxic effect and did not produces significant changes in behavior, salivation, defecation and loss of hair. No death was observed and the extract of *Pulsatilla nigricans* was found to be safe at the given administered dose. The dose levels were selected for anxiolytic study after evaluation of acute oral toxicity study, $1/10^{th}$ i.e. 500 mg/kg body weight and $1/20^{th}$ i.e. 250 mg/kg of the max dose (5000 mg/kg body weight).

**Table II. Effect of EEPN on mortality of rats in acute oral toxicity study.**

| Dose | No. of Animals | Mortality | | |
|---|---|---|---|---|
| | | 24 hour | $7^{th}$ day | $14^{th}$ day |
| EEPN 5 mg/kg | 03 | 0/3 | 0/3 | 0/3 |
| EEPN 50 mg/kg | 03 | 0/3 | 0/3 | 0/3 |
| EEPN 300 mg/kg | 03 | 0/3 | 0/3 | 0/3 |
| EEPN 2000 mg/kg | 03 | 0/3 | 0/3 | 0/3 |
| EEPN 5000 mg/kg | 03 | 0/3 | 0/3 | 0/3 |

Tables III and IV show the effect of EEPN and diazepam on rats behavior in Elevated plus maze and Hole board paradigms respectively, indicates that the plant extract exhibit significant effect on different parameters.

**Table III. Effect of EEPN and diazepam on rats in elevated plus maze paradigm.**

| Groups | Mean number of entries in open arm | Mean time spent in open arm |
|---|---|---|
| Control | 6.1 ± 0.4 | 49.0 ± 8.3 |
| Diazepam | 8.1 ± 0.3** | 149.0 ± 21.7** |
| EEPN 250 mg/kg | 7.9 ± 0.4* | 131.6 ± 23.5* |
| EEPN 500 mg/kg | 7.7 ± 0.4* | 130.7 ± 18.7* |

n=7; values are mean±SEM *P≤0.05 significant and **P≤0.005 considered highly significant as compared to control; one way ANOVA followed Tukey HSD post hoc analysis.

**Table IV. Effect of the EEPN and diazepam on rats in Hole Board Paradigm.**

| Groups | Latency time in seconds | No. of head dips |
|---|---|---|
| Control | 47.3 ± 4.4 | 7.3 ± 1.0 |
| Diazepam | 29.3 ± 3.7* | 14.7 ± 1.6* |
| EEPN 250 mg/kg | 31.0 ± 3.4* | 13.6 ± 1.3* |
| EEPN 500 mg/kg | 32.1 ± 3.8* | 13.4 ± 1.6* |

n=7; values are mea± SEM *P<0.05 significant as compared to control; ANOVA followed by Tukey HSD post hoc analysis.

*Discussion*

In our study, the behavioral effect of EEPN was examined using rats. The extract was also subjected to evaluate acute oral toxicity, which showed no sign and symptoms of toxicity, behavioral changes and mortality, and was found safe and non-toxic at dose up to 5000 mg/kg. Although, drowsiness was noticed at higher doses but no significant increase or decrease in weight of body was observed. Usual gain in body weight was not observed, since a decrease in this parameter would indicate the presence of toxicity (Teo *et al*., 2002). This study also proves the beneficial effect of *Pulsatilla nigricans* as anxiolytic agent. *Pulsatilla nigricans* was examined for anxiolytic activity at two different doses of 250 and 500 mg/kg.

Elevated plus maze paradigm is a standard equipment because it produces a natural stimuli, the panic and fear of balancing on a restricted and narrow, elevated platform (Dawson *et al*., 1995). Among two different doses of EEPN, it was observed that diazepam and treated doses i.e. 250 and 500 mg/kg show significantly increased in mean number of entries and mean time spent by rats in open arms with respect to control, thus producing anti-anxiety activity.

Anxiolytic activity of *Pulsatilla nigricans* at doses of 250 and 500 mg/kg was also examined by Hole Board paradigms. It has been commonly and frequently used to identify and estimate anti-anxiety activity of many drugs (Njung'e *et al*., 1991). The increase in the number of head-dips is the most major and important parameter in this model to evaluate the anti-anxiety effect (Toshiharu *et al*., 2004). In this study diazepam (1 mg/kg) and EEPN at doses of 250 and 500 mg/kg shows significant increase in the number of head dips and decrease in latency time as compared to control,

Diazepam is the prototype of benzodiazepines and gives anti-anxiety activity by its agonistic action on GABA in the CNS. Whether, *Pulsatilla* acts in a similar way, the actual mechanism is not known. It is also reported that *Pulsatilla nigricans* has spasmolytic, analgesic, anxiolytic and sedative action (Mills *et al*., 2004; Karnick, 1994; Lakshmipathy *et al*., 2012). According to previous research *Pulsatilla nigricans* has anemonin and anemonic acid. The biological effects are described related to anemonin; anemonin might produce anxiolytic effect by agonistic action on GABA which is same as benzodiazepines. However, anemonic acid is an irritant and have no pharmacological effect (Lakshmipathy *et al*., 2012). However, further studies are required to determine the anxiolytic mechanism of action of *Pulsatilla nigricans*.

*Conclusion*

In the current study *Pulsatilla nigricans* indicated anxiolytic potential in rat behavioral paradigms. The plant is commonly used in homeopathic preparations and this study provides scientific basis for anxiolytic effect in rat models however further studies are required to evaluate anxiolytic mechanistic action of the plant.

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*Statement of conflict of interest*

The author has no conflict of interest

*Approval of project and ethical clearance*

All the research protocol, procedures, euthanasia as well as ethical clearance were approved by the BASR (Board of Advanced Studies and Research), Hamdard

University, Karachi (Ref. No. HU/DRA/2017/716). The animals used in the study were handled as per specifications described in Helsinki Resolution 1964.

*Supplementary material*

There is supplementary material associated with this article. Access the material online at: https://dx.doi.org/10.17582/journal.pjz/20190625060602

*References*


Bell, I.R., Howerter, A., Jackson, N., Aickin, M., Baldwin, C.M. and Bootzin, R.R., 2011. *Sleep Med.,* **12**: 505–511. https://doi.org/10.1016/j.sleep.2010.03.013

Bellavite, P., Magnani, P., Marzotto, M. and Conforti, A., 2009. *Homeopathy*, **98**: 208–227. https://doi.org/10.1016/j.homp.2009.09.005

Brown, G.R., Nemes, C. and Behav., 2008. *Process*, **78**: 442–448. https://doi.org/10.1016/j.beproc.2008.02.019

Davidson, J.R., Morrison, R.M., Shore, J., Davidson, R.T. and Bedayn, G., 1997. *Altern. Ther. Heal. Med*., **3**: 46–49. https://doi.org/10.1353/aq.1997.0043

Dawson, G.R. and Tricklebank, M.D., 1995. *Trends Pharmacol. Sci*., **16**: 33–36. https://doi.org/10.1016/S0165-6147(00)88973-7

Eisenberg, D.M., Roger, B., Davis., Susan, L., Ettner., Scott Appel., Wilkey, S., Rompay, M.V., Ronald, C., Kessie. 1998. *J. Am. med. Assoc*., **280**: 1569–1575. https://doi.org/10.1001/jama.280.18.1569

Felter, W. and Lloyd, J.U., 1898. *King's American Dispensatory.* Available at: https://www.henriettes-herb. com/eclectic/kings/index.html [Accessed 5 Jul. 2019].

Henriettes-herb.com. (n.d.). *King's American Dispensatory,* 1898. *Henriette's Herbal Homepage*. [online] Available at: https://www.henriettes-herb.com/eclectic/kings/index.html [Accessed 5 Jul. 2019].

Griffiths, R.R., Ator, N.A., Roache, J.D. and Lamb, R.J., 1987. *Psychopharmacol. Res*., **3**: 83–87. https://doi.org/10.1007/978-3-642-71288-3_10

Jackson, B.D. and Kewensis, I., 1946. *An enumeration of the genera and species of flowering plants*. Claerendon Press Oxford. pp. 660–661.

Karnick, C.R., 1994. *Pharmacopoeial standards of herbal plants*. India Sri Satguru Publ. pp. 268.

Kumar, S., Madaan, R., Farooq, A. and Sharma, A., 2008. *Pharmacogn. Rev.,* **2**:116–124.

Kumar, S. and Sharma, A., 2005. *Altern Med*., **2**:117–119. https://doi.org/10.1093/ecam/neh069

Lango, D., Fauxi, A., Kasper, D., Hauser, S., Jameson, J. and Loscalzo, J., 2011. *Principles of internal medicine*. 18th ed, McGraw Hill Publishers.

Mills, S. and Bone, K., 2004. *Essential guide to herbal safety*. Churchill: Living- stone publishers, pp. 523.

Njung'e, K. and Handley, S.L., 1991. *Pharmacol. Biochem. Behav*., **38**: 63–67. https://doi.org/10.1016/0091-3057(91)90590-X

Obici, S., Otobone, J.F., daSilvaSela, V.R., Ishida, K., DaSilva, J.C., Nakamura, C.V., Cortez, D.A.G., Audi, E.A., 2008. *J. Ethnopharmacol*., **115**: 131-139. https://doi.org/10.1016/j.jep.2007.09.013

OECD guideline 423., 2001. *Acute oral toxicity– Acute toxic class method.* Oecd Guideline for Testing of Chemicals, pp. 1–14.

Pilkington, K., Kirkwood, G., Rampes, H., Fisher, P. and Richardson, J., 2006. *Homeopathy*, **95**: 151–162. https://doi.org/10.1016/j.homp.2006.05.005

Lakshmipathy, P.R., Ruckmani, A., Venkatesan, D., Madhusudhanan, N. and Pavithra, R., 2012. *Homeopathy,* **101**:171–174. https://doi.org/10.1016/j.homp.2012.05.003

Teo, S., Stirling, D. and Thomas, S., 2002. *Toxicology*, **179**: 183–196. https://doi.org/10.1016/S0300-483X(02)00338-4

Toshiharu, S., Michihiko, I. and Shigeyuki, C., 2004. *Eur. J. Pharmacol*., **501**: 121–125.

Vaz, A., Marques, R., Campos, V., Costa, K., Viriato, E., Carlos, J. and Carvalho, T., 2009. *Int. J. High Dilut. Res*., **8**: 91–99.

Vogel, H.G., 2002. *Pharmacological assay.* Berlin, Heidelberg, New York. pp. 385.