Exploring the Possible Mode of Anxiolytic Action of a Polyherbal Formulation in Animal Models of Anxiety

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ABSTRACT :

The polyherbal formulation, PH-1, containing fresh aqueous extracts of Valeriana and, Boswellia spp. as major constituents and, Plumbago, Acorus spp. as minor constituents was investigated to explore the anti-anxiolytic activity and, its mode of action through competitive GABA receptor bindings with different antagonists of known nature e.g., flumazenil, picrotoxin and bicuculine. The open field, staircase test and, mirror chamber tests of animal models of anxiety were employed at 100, 200, 400 and 800 mg/kg dose levels. The formulation exhibited significant dose dependent anxiolytic activity through oral administration for varying periods of time in different paradigms of increased time-spent in mirror chamber, in central platform of open filed and, the enhanced climbing of steps in staircase tests. The 200 and, 400 mg/kg doses were highly effective. The formulation seems to be acting through GABAA-benzodiazepine binding site as evident from the blockage of PH-1 action at 400 mg/kg dose level by the flumazenil, a known GABAA-benzodiazepine receptor antagonist, and, picrotoxin, a known GABAA receptor-chloride channel complex antagonist. However, the blockade of anxiolytic activity by bicuculine through GABAA-GABA binding receptor antagonist was not significant which also confirmed the mode of action through activation of benzodiazepine receptor and, facilitation of benzodiazepine interdependent chloride channel complex.

Keywords: Polyherbal formulation, Anti-anxiety; Flumazenil, Bicuculine, Picrotoxin; GABA.

INTRODUCTION:

The anxiety is managed through several synthetic molecules which come with the burden of adverse and unwanted effects [1]. On the other hand, formulations obtained from natural sources seems to have least pharmacological risks and, claim to cure much the same way as their synthetic counterparts. Among some of the natural drugs employed for this purpose are St. John's wort, Ginseng, Ginkgo Biloba, Kava, Saw Palmetto, Euphorbia Hirta, Valerian and, Convolvulus.

Ayurveda, the traditional healing system utilizing phytomedicines, considers plant as complex mixtures of several components and, assumes that these can provide desired biological action. It also considers that a feebly active plant when compounded with other components or, made pat of a group of plants, can lead to potentiated therapeutic action wherein other plant and, components reinforce the prevalent biological action and, may interact synergistically to neutralize and, counteract any potential side effects that may have occurred because of the single plant use or, which may get developed afterwards after the combination of plants usage. Therefore, several plants with desired biological activities and, as varied undesirable activities suppressants are selected, so that the final componential mixture or, the polyherbal formulation will have a concentrated desired bio-action capacity with the undesired activities being fully suppressed, diluted, under-check or, altogether absent [2,3]. The present study investigates the use of a polyherbal preparation, termed PH-1, consisting of aqueous extracts of leaves of *Valeriana wallichii* (60%), *Plumbago zylanica* (15 %), *Boswellia serrata* (20 %) *and, Acorus calamus* (5%), obtained from the herbalist practitioner. The aqueous extracts were authenticated through presence of marker compounds Valerinic acid, Plumbagin and, Boswellic acid by HPLC. Herein, an attempt has been made to understand the possible mode of action of the combined extracts, PH-1, and, finding a comparison of the extent of the anxiolytic activity of this polyherbal formulation.

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MATERIALS AND METHODS

Animals

The experiments were performed on male Swiss albino mice (25-30 g). The animals were group housed in colony cages at ambient temperature of 25 ± 10 C and 45 to 55 % relative humidity with a 12 hr/12 hr light/ dark cycle. They had free access of food and water *ad libitum*. The experiments were carried out between 0900 to 1400 hrs and animals were acclimatized for one week. All the experiments were conducted in a sound proof dimly lit room designed for psychopharmacological studies. The experimental protocols were approved by the Institutional Animal Ethics Committee and, were in accordance with the guidelines of the Committee for the Purpose of Control and Supervision on Experiments on Animals.

Drugs and Chemicals

Diazepam was prepared in water for injection through i.p administration.

Flumazenil, picrotoxin and bicuculine were purchased from Sigma Chemical Co.; St. Louis, USA and, polyherbal formulation was prepared as a concentrated mixture of aqueous extracts of the plant material as semi-solid. Flumazenil, bicuculine and, picrotoxin were suspended in 2 % DMSO-water mixture, 0.1 N HCl and, ethanol-water mixture respectively and, were administered through i.p route. Control animals were given saline at a dose of 10 ml/ kg B. W. of mice. All the other chemicals used in the study were of analytical grade.

Method of administration

In all the experimental models, the antagonists and herbal formulations were administered once daily for 15 days. On 15th day, experiment was performed one hour after the last dose of PH-1 and, half an hour in case of diazepam. In groups where antagonist was combined, antagonist drug was given half an hour before the respective treatments.

Experimental design

Acute toxicity test

Acute toxicity of the preparation was determined using female albino mice. The animals were fasted 3 hrs prior to the experiment. Animals were observed for 48 hrs for any mortality following administration of the different doses of PH-1 as per the guidelines. They were also observed for any behavioral or, gross morphological changes.

Stair case test in mice

The staircase apparatus was made up of plywood and consisted of five identical steps with 2.5 cm high, 10 cm wide and 7.5 cm deep surrounded by a glass wall of 12.5 cm height (Vogel, 2000). At the end of treatment period animals were placed singly on the floor of a transparent box facing away from the steps. The numbers of steps climbed and number of rearing over a period of 5 min were recorded. A step is considered to be climbed if the animal places all its four limbs on the step. After exploration by each animal the steps were cleaned thoroughly with 5% alcohol to eliminate potential cues such as fecal pellets and urination left by the previous test animal. All animals were tested only once [4].

Mirror Chamber Test

The mirror chamber consisted of a mirrored cube which is open on one side and is placed inside an open top square wooden box. The mirrored cube is constructed of five pieces of mirrored glass with one mirrored side and an opposite side painted dark brown measuring 30 cm on all sides. The three mirrored side panes, a top pane, and the floor pane faced the interior of the cube. The container wooden box was 40 X 40 X 30.5 cm. The mirrored cube was placed in the center of this container so as to form a 5 cm passage around the mirror chamber. A mirror was also placed on the container wall so that it faced the single open side of the mirrored chamber. Except for this one mirrored portion on the container wall, all portions of the container walls were dark brown. Mice were placed individually in the chamber of mirrors and scored once. At the start of the experiment, the mouse was placed in a single, fixed starting point in a corner of the chamber. The animal was allowed to explore the chamber for 5 min and the following parameters were recorded, (1) Latency to enter the chamber (the time for the first entry into the chamber), (2) Number of entries, (3) Total time spent in the chamber The criterion for entry into the chamber was all four feet being placed on the floor panel of the mirrored chamber. After exploration by each animal the floor was cleaned thoroughly with 5% alcohol to eliminate potential cues such as fecal pellets and urination left by the previous test animal [5-7].

Open field test

The open field was made up of wood with dimensions 45 cm × 45 cm with a wall 30 cm high, the floor was divided into equal sixteen squares, four in the centre and twelve in the periphery. The square in the periphery (adjacent to the wall) is designated as the peripheral zone (safe zone) and the squares in the centre are designated as the central zone (vulnerable zone, open space or anxiogenic zone). The time spent in the "safe" peripheral zone is thought to be proportionate to the level of anxiety in the animal [8]. This test uses the animal's repugnance for the central zone to quantify anxiety behavior. Apparatus was placed in a sound attenuated room, 48 cm above the floor and, was cleaned thoroughly with 5% alcohol before placing each animal for observation to eliminate the possible bias due the odor left by the previous animal. Animal was placed in the centre of the apparatus facing one of the sides at the start of experiment and behavior was recorded for 5 min[9]. During this period central and, peripheral crossings, vertical rearing (number of times the animal stood on its hind legs), grooming, defecation, total locomotion (number of units entered on the floor), defecation units (number of bolls) and Immobility times were recorded.

Statistical analysis

All the data obtained from the experiments were subjected

to the statistical analysis by using Graph pad prism v 5.01 (Demo version) and Instat 3.

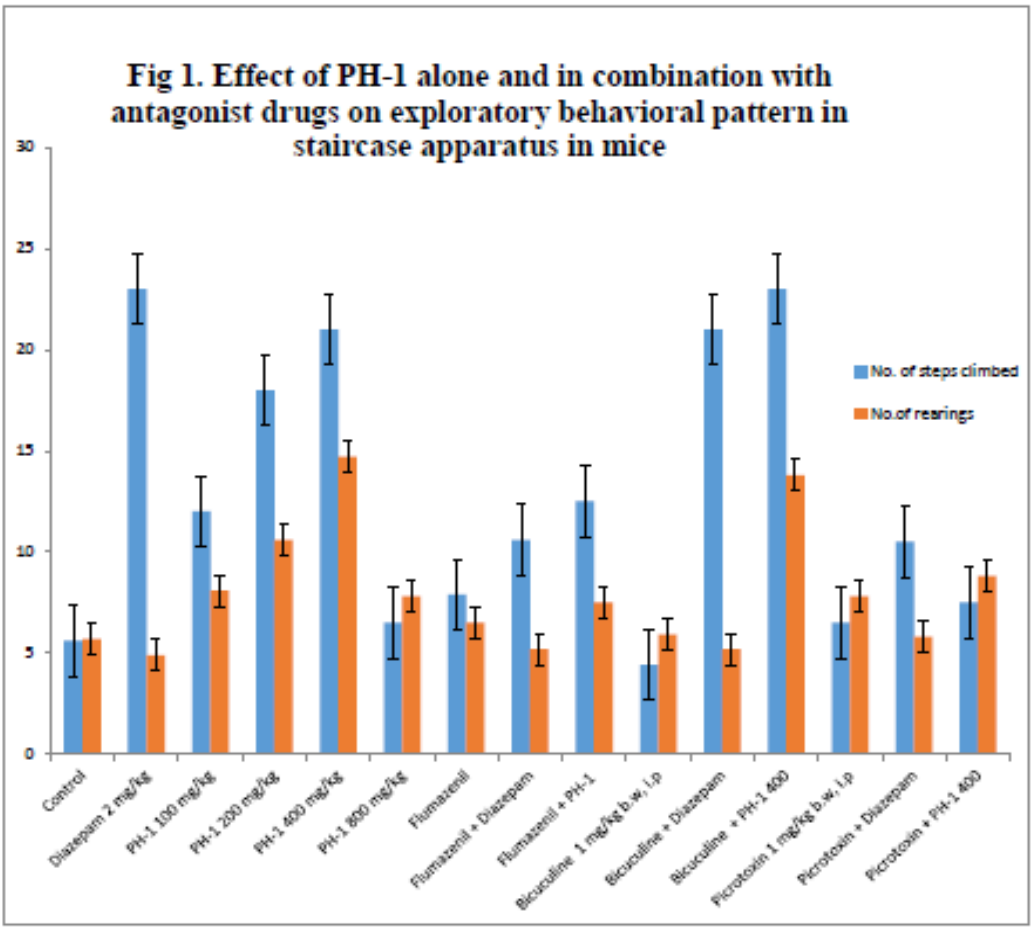
RESULTS

Staircase apparatus test

Mice treated with PH-1 (200, 400 and 600 mg/kg), showed significant increase in number of steps climbed and rearing dose dependency when compared to control. However, PH-1 at 800 mg/kg showed decrease in number of steps climbed as compared to control. The mice treated with PH-1 (200, 400 and 600 mg/kg), showed significant increase in number of steps climbed and, rearing dose dependently when compared to control. However, PH-1 at 800 mg/kg showed decrease in number of steps climbed as compared to control. Flumazenil and, picrotoxin significantly blocked the effect of PH-1. However, Bicuculine pretreatment did not alter the outcome of test formulation, PH-1 (Fig 1).

Mirror chamber test

Treatment with PH-1 resulted in decrease in latency to enter the chamber and also enhanced the number of entries when compared to control and, it was comparable to standard diazepam. The mice treated with PH-1 showed significant increase in the number of mice's entry and, time spent in the chamber according to dose manipulation as compared to the control. Flumazenil and picrotoxin significantly blocked the effects of PH-1 on number of entries and time spent in the chamber (table 1).



Values are expressed as mean ± S.E.M (n = 06). ^{ns}- statistically non-significant, *<0.05, **$p < 0.01$, when compared to controld group. #$p<0.05$, ##$p < 0.01$, when compared to respective drug alone treated group.

Table 1. Effect of PH-1 alone and in combination with antagonist drugs on exploratory behavioral pattern of mice in mirror chamber following treatment for 15 days

Groups	Latency in sec	No. of entries	Time spent in the chamber
Control	247 ±12.5	2.85±0.031	13 ± 1.2
Diazepam	88±11.2	16 ±1.4	124 ±11.1
PH-1 100 mg/kg	125±13.3	07 ±0.5*	98 ±10.3*
PH-1 200 mg/kg	109±11.7	11 ±1.1*	102 ±10.5*
PH-1 400 mg/kg	90±09.1	15 ±1.7**	114 ±11.11*
PH-1 800 mg/kg	189±12.6	3.2 ±0.2 ^{ns}	53.6 ±05.5
Flumazenil	251±22.3	03 ± 0.1	23 ±1.5
Flumazenil + Diazepam	210±21.2###	04.5 ± 0.12###	51.2±2.5##
Flumazenil + PH-1	231±21.3###	3.1 + 0.01###	42.4±3.9##
Bicuculine 1 mg/kg b.w, i.p	211 ±11.6	3.45±0.02	14 ± 1.6
Bicuculine + Diazepam	83±11.7^{ns}	14 ±2.5^{ns}	131 ±8.6^{ns}
Bicuculine + PH-1 400	97±5.6^{ns}	17 ±1.6^{ns}	111 ±11.51^{ns}
Picrotoxin 1 mg/kg b.w, i.p	263 ±22.1	2.4±0.04	10 ± 3.12
Picrotoxin + Diazepam	222 ±18.5###	5.81±0.041###	16 ± 1.7###
Picrotoxin + PH-1 400	231 ±14.6###	3.34±0.045###	15 ± 2.1###

Values are expressed as mean ± S.E.M (n = 06). ^{ns}- statistically non significant, *<0.05, **$p < 0.01$, ***$p < 0.001$ when compared to controld group. #$p<0.05$, ##$p < 0.01$, ###$p < 0.001$ when compared to respective drug alone treated group.

Table: 2. PH-1 and, antagonists effects on behavioral patterns in open field test after 15 days of treatment in mice

Groups	Rearing	No. of squares Crossed	No. of fecal pellets	Grooming
Control	0.6 ± 0.04	23.60±3.28	5.1 ± 0.64	0.5 ± 0.22
Diazepam	3 ± 0.64	64.60±7.14	2.7 ± 0.26	3 ± 0.051
PH-1 100 mg/kg	0.9 ± 0.03 ns	25.8 ± 8.33 ns	5 ± 0.66 ns	1.1 ± 0.37 ns
PH-1 200 mg/kg	2 ± 0.44*	49.6 ± 8.05 ns	4.5 ± 0.61 ns	2.7 ± 0.96*
PH-1 400 mg/kg	3 ± 0.40*	57.5 ± 6.95*	3.3 ± 0.84 ns	2.9 ± 0.16*
PH-1 800 mg/kg	1 ± 0.32	33 ± 0.40	4 ± 0.060	1.9 ± 0.040
Flumazenil	2 ± 0.05	23 ± 2.40	3 ± 0.040	1.6 ± 0.060
Flumazenil + Diazepam	1 ± 0.03 ##	34.33 ± 3.30#	4.3 ± 0.03	0.6 ± 0.04###
Flumazenil + PH-1 400	1.2 ± 0.02	27.4 ± 2.43**	5.4 ± 0.40	1.2 ± 0.02
Bicuculine 1 mg/kg b.w, i.p	2.33 ± 0.04	21.15 ± 3.52	3.5 ± 0.15	2.31 ± 0.041
Bicuculine + Diazepam	3.45 ± 0.17 ns	55.55 ± 11.43	3.12 ± 0.06	2.9 ± 0.03
Bicuculine + PH-1 400	2.91 ±0.05 ns	49.14 ± 6.21 ns	4.1 ± 0.14 ns	2.8 ± 0.01 ns
Picrotoxin 1 mg/kg b.w, i.p	1.93 ± 0.06	21.15 ± 2.52	1.77 ± 0.015	1.92 ± 0.041
Picrotoxin + Diazepam	2.11 ± 0.012 ns	31.22 ± 1.33#	1.93 ± 0.06 ns	0.93 ± 0.03 #|
Picrotoxin + PH-1 400	1.95 ± 0.06 ns	41.59 ± 5.55#	2.22 ± 0.09	2.55 ± 0.02

Values are expressed as mean ± S.E.M (n = 06). ns- statistically non significant , *<0.05, **p < 0.01, ***p < 0.001 when compared to controld group. #p<0.05, ##p < 0.01, ###p < 0.001 when compared to respective drug alone treated group

Open field test

PH-1 caused a dose dependent increase the number of crossings, rearing and, grooming in the open field paradigm which reflected the enhanced exploratory activity and, the condition of reduced fear when compared to control. The biological effects produced by flumazenil and, picrotoxin were in line with the observations made in the study (Table 2).

DISCUSSION

Several central receptor systems have been suggested in the modulation of fear and, anxiety. The GABA receptor is one of the prominent anxiolytic activities modulating receptor site [2]. The antagonists bicuculine, flumazenil and, picrotoxin working on different sites of this receptor were chosen to find out comparative binding and, study the detailed biological interaction outcomes in comparison with the investigational polyherbal formulation, PH-1.

The results obtained with the investigational drug, PH-1, clearly indicated the anxiolytic activity profile. A dose response effect curve was constructed by using different doses of PH-1 (100, 200, 400, 600 and 800 mg/kg BW). The dose which showed prominent anxiolytic effect (400 mg/kg) was selected for further study to explore the mechanism.

The mechanisms underlying the anxiolysis produced by any drug is multifaceted, as several reports which claim to be involved in the activity, to name a few, are GABAergic, serotonergic and noradrenergic systems which are prominent ones in the brain. Add to the problem of complexity, neural regulatory mechanisms are abundant, regulating one another in the brain, or in some cases themselves. The mechanism involved in the anxiolytic-like effect of PH-1 was studied by using various antagonists for GABA receptor system e.g., flumazenil (GABAA-benzodiazepine binding site antagonist), picrotoxin (GABAA-chloride channel complex antagonist) and bicuculine (GABAA-GABA binding site antagonist).

The flumazenil produces no marked behavioral effects, but can reverse almost all the pharmacological actions of anxiolytics belonging to benzodiazepine group. In our study too Flumazenil per se caused no obvious effect in mice submitted to the staircase, mirror chamber and OFT tests. However it showed a significant antagonistic effect on the anxiolytic outcome induced by diazepam as well as by the PH-1 (400 mg/kg). This might suggest that anxiolytic effect produced by PH-1 due to in part by interaction with central benzodiazepine binding site on GABAA-receptors. Furthermore, pretreatment with the bicuculine, did not alter the effect of either of the antagonists and, thus ruling out the possibility of interaction with the GABA binding site on the GABAA receptors.

However, picrotoxin resulted in the complete blockade of effects of PH-1, supplementing the possibility of involvement of interaction with GABAA chloride channel complex along with benzodiazepine binding site. Thus it is likely that formulation's anxiolytic effect is due to the blockade of benzodiazepine site/chloride channel complex of GABA receptor system. However, a cursor glance at the literature available on some of the components of this formulation could help to uncover the possible mechanism of anxiolytic activity of PH-1. Valeriana was reported to have anxiolytic, tranquilizing, and sleep inducing effects. These effects were claimed to be produced by an interaction with central GABA receptors [10-14]. In an *in vitro* binding study to GABA receptors, Valerian was found to displace the agonist, muscimol, suggesting its binding

to these receptors. The Valerian extract was also found to contain GABA and other amino acids. Thus these studies coupled with the present findings suggested the possibility of interaction of PH-1 with the benzodiazepine binding site or, GABA-chloride channel receptor complex excluding GABA-b in its anxiolytic effect [15-18].

CONCLUSION

It can be concluded that PH-1 has promising anxiolytic activity and, works through occupation of benzodiazepine binding site and, facilitation of interdependent chloride channel complex. However, further studies are required to ascertain the action of each marker component and, polyherbal extract with varying composition of plants and marker products in these plants.

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