Behavioural and neurochemical evaluation of Perment® an herbal formulation in chronic unpredictable mild stress induced depressive model

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Perment®, a polyherbal Ayurvedic formulation that contains equal parts of *Clitoria ternatea* Linn., *Withania somnifera* Dun., *Asparagus racemosus* Linn., *Bacopa monniera* Linn., is used clinically as mood elevators. The aim of the present study was to explore the behavioural effects and to understand possible mode of action of Perment® in stress induced depressive model. Chronic unpredictable mild stress (CUMS) was used to induce depression in rats. Open field exploratory behaviour, elevated plus maze, social interaction and behavioural despair tests were used to assess behaviour. Using standard protocols plasma noradrenaline, serotonin, corticosterone and brain/adrenal corticosterone levels were measured to support the behavioural effects of Perment®. Exposure to CUMS for 21 days caused anxiety and depression in rats, as indicated by significant decrease in locomotor activity in the open field exploratory behaviour test and increased immobility period in the behavioural despair test. Perment® predominantly exhibited antidepressant action than anxiolytic activity. Further Perment® increased the plasma noradrenaline and serotonin levels in stressed rats. No significant alteration in the brain corticosterone level in stressed rats was observed with Perment® treatment. However the adrenal corticosterone level is decreased with Perment®. It can be concluded that the Perment® formulation exhibited synergistic activity, has a significant antidepressant and anxiolytic activity, which may be mediated through adrenergic and serotonergic system activation. Currently the formulation is clinically used as anxiolytic but the present results suggest that the formulation can also be indicated in patients affected with depression.

**Keywords**: Anxiety, Chronic mild stress, Corticosterone, Depression, Noradrenaline, Perment®, Serotonin

Perment® is a polyherbal Ayurvedic formulation that contains equal parts (125 mg) of *Clitoria ternatea* Linn. (Fabaceae), *Withania somnifera* Dun. (Solanaceae), *Asparagus racemosus* Linn. (Asparagaceae) and *Bacopa monniera* Linn. (Scrophulariaceae) is used clinically for psychiatric illness. *Clitoria ternatea* exhibits nootropic, anxiolytic, anti-convulsant and anti-depressant activities in rodent models. The observed behavioural effects of *Clitoria ternatea* were attributed to serotonergic and cholinerigic system activation1. *Withania somnifera* has adaptogenic, nootropic and anxiolytic properties and the major phytoconstituent withanolides correlated to this effect2. It also exhibits neuroprotective activity and contributed in synapse reconstitution3. *Asparagus racemosus*, controls the excitotoxicity and free radical generation1. *Bacopa monniera*, a Medhya Rasayana herb generally known as a brain tonic, enhances memory, improves insomnia, nervousness, tremors, irritability and also produces significant anxiolytic and anti-stress effect5,6. Most of the Ayurvedic preparations are dispensed in combination of different herbs. The advantages of polyherbal formulations are that they may have synergistic action or prevent co-morbidities or may possess minimal toxicity. Hence the present study is designed to validate the polyherbal formulation Perment® for its biological activity and explore the possible mode of action in stress induced depressive condition. Chronic unpredictable mild stress (CUMS) model was used to induce depression in rats. Neurochemical and corticosteroid levels were measured to support the behavioural effects of Perment®. By understanding the mode of action of the formulation it is possible to further explore its therapeutic use related to CNS.

**Materials and Methods**

All the plants were authenticated by a Botanist from Botanical Survey of India and for future references voucher specimens were preserved as
herbarium in AVN Industry, Madurai. Perment® is a 500 mg polyherbal capsule formulation containing aqueous extract of C. ternatea, W. somnifera, A. racemosus and B. monniera each 125.00 mg. The formulation is a greenish white powder and passes the official monograph tests.

**HPTLC standardization**—Individual herbs were standardized before formulating the capsule. The formulation was standardized using the toluene: ethyl acetate: methanol solvents in 7:2:1 ratio and the fingerprint of the herbal preparation were kept as reference. Rf value of the formulation assessed at UV 254 nm (ranging Rf 0.07-0.56) and UV 366 nm (ranging Rf 0.08-0.64).

**Drugs and Chemicals**—Perment® formulation was supplied by manufacturer AVN Industries, Madurai. Noradrenaline, serotonin and corticosterone standards were obtained from Sigma Aldrich, USA and all other chemicals used were of analytical reagent grade. Diazepam (Cipla, Mumbai) tablets were purchased commercially from the market.

**Drug preparation and administration**—Rats exposed to chronic unpredictable mild stress (CUMS) received po: vehicle, diazepam and test drug Perment® (75, 150 and 300 mg/kg). Dose for the Perment® was fixed based on the previous study report on the respective herbs. Drugs were as a fine suspension in 0.3% carboxy methyl cellulose and administered 1 h before the stress exposure for 21 days. Diazepam (2 mg/kg) was used as a standard to assess the stress induced behavioural alterations.

**Animals**—Wistar strain male rats (160–180 g; 40 rats) were procured from the central animal facilities of PSG Institute of Medical Sciences & Research, Peelamedu, Coimbatore and divided into 5 groups of 7 animals each for stress administration. One group of 5 rats was kept as control; no stress was administered and received the vehicle. The rats were housed in colony cages at an ambient temperature of 25±2°C and 40–65% RH, with a 12:12 h L:D cycle. The animals had free access to standard pellet chow and drinking water unless there was a restriction due to stress protocol. The study was approved by Institutional Animal Ethics Committee and the work was carried out as per CPCSEA guidelines, New Delhi.

**Induction of stress**—Stress was induced by employing CUMS protocol as per the procedure mentioned by Nirmal et al. Each stress regimen was carried out for 2 periods with the following stressors: food deprivation for 24 h, day-night reversal (0600 hrs – 1800 hrs), soiled bedding (~150 ml water per cage) for 22 h, cage tilting (~45 degree inclined) for 22 h, crowded housing (10 animals per cage) for 12 h, exposure to a novel odor (household air freshener) for 12 h, restraint stress for 20 min, cold stress 4º– 8°C and heat stress 38º– 39°C for 20 min and intermittent white noise (80dB) for 5 h for 3 periods.

**Behavioural assessment**

**Open field exploratory behaviour**—Open field test was used to study the exploratory and anxiety behaviour of rats. The open field apparatus consists of a square arena 61 × 61 cm with 40 cm high wall. The entire apparatus was painted black except for 3 mm white lines that divided the floor into 16 equal size squares. The apparatus was illuminated with a low intensity diffuse light (45 W) situated 45 cm above the floor level. Entire room, except the open field was kept dark during the experiment. Each animal was placed in the central square and observed for 5 min. The following behaviours were recorded; ambulation: the number of grid lines it crossed with all the four paws; rearing: by counting the number of times the animal stood on its hind limbs; grooming: number of times the animal made these responses viz. grooming of the face, licking/cleaning and scratching the various parts of the body, defecation: the number of fecal boli excreted during the period and immobility period. Between tests, the apparatus was cleaned with 5% alcohol.

**Elevated plus maze**—The plus maze consisted of two opposite arms (50 × 10 cm) crossed with two opposite enclosed arms of the same size dimension with 40 cm high. The arms are connected with a central square of (10 × 10 cm) to give the apparatus a plus sign appearance. The maze was kept elevated to a height of 50 cm above the floor in a dimly lit room. On testing day, the rat was placed on the central square of the plus maze, facing one of the enclosed arms. The number of entries and the time spent in open and closed arms were noted for next five minutes period. An arm entry was defined when all the four limbs were on the arm.

**Social interaction test**—The social interaction test is a valuable behavioural model and quantifies the level of social behaviour between pairs of rats and it is usually based on manual analysis of behaviour. Rats were housed singly for 5 days prior to the test. The social interaction arena was a dimly lit plexi glass
extracts were unified and shaken with ice cold 0.1N centrifugation at 2000 rpm for 10 minutes. Both MC for 15 min. The MC phase was separated by vigorous shaking and 2 ml of the water layer was extracted twice with 5 ml of methylene chloride (MC) by vigorous shaking to remove the lipids. The petroleum ether was discarded water and extracted with 5 ml petroleum ether to obtain the MC supernatant (1ml) was diluted with 2 ml of distilled 80ºC until the corticosterone assay was done.

Physiological observations

Plasma was separated by centrifuging the blood at 4000 rpm for 10 min. During stress condition, the period of immobility after an initial 2-3 min period of vigorous activity was recorded. A rat was considered to be immobilized when it remained floating in the water without struggling, making only minimum movements of its limbs necessary to keep its head above water. The total duration of immobility was recorded during the next 4 min of a total 6 min test. Rats were then allowed to dry in a pre-warmed enclosure (~32ºC) before being returned to their home cage. All the behavioural experiments were carried out between 0900-1400h by blind observer with live recording.

Blood and organs collection

After 24 h of the last stress administration the blood was collected from overnight fasted rats through retro orbital puncture for neurotransmitter and corticosterone estimation. Plasma was separated by centrifuging the blood at 4000 rpm for 10 min. During stress condition, activation of hypothalamic adrenal axis is observed; hence the adrenals and brain corticosterone level were estimated. The brain and adrenals were isolated, weighed and homogenized immediately with ice cold 10% KCl. The supernatant was separated by centrifugation at 5000 rpm for 10 min and stored at -80ºC until the corticosterone assay was done.

Corticosterone estimation

Plasma or the supernatant (1ml) was diluted with 2 ml of distilled water and extracted with 5 ml petroleum ether to remove the lipids. The petroleum ether was discarded and 2 ml of the water layer was extracted twice with 5 ml of methylene chloride (MC) by vigorous shaking for 15 min. The MC phase was separated by centrifugation at 2000 rpm for 10 minutes. Both MC extracts were unified and shaken with ice cold 0.1N sodium hydroxide. The water phase was immediately removed and the MC extracts was dried by addition of dry sodium sulphate. A 5 ml aliquot of the MC extracts was mixed with 5 ml of the fluorescence reagent (7 parts conc. H₂SO₄, 3 parts 96% ethanol v/v). After vigorous shaking the MC phase was removed and the fluorescence was measured with primary filters of 436 nm and secondary filters of 530-545 nm. For calibration curve concentrations of 0, 20, 50, 100 and 200 µM/ml of corticosterone were measured identically and measured 12.

Neurotransmitter estimation

Plasma noradrenaline and serotonin levels were measured using 10AT HPLC Systems (Shimadzu® LC). The mobile phase used for the study was acetonitrile: methanol: sodium acetate (pH 3.5) in the ratio of (10:10: 80% v/v). The stationary phase used was Phenomenex C₁₈ (250 × 4.6 mm id, 5µ) at the flow rate of 0.7 ml/min and detection at 275 nm. The samples were prepared with Phenomenex Strata solid phase extraction cartridge and were conditioned with methanol and water (1 ml) sequentially. To this 0.5 ml of plasma was added. The cartridge was washed with 2 ml of water. The drug was eluted from the cartridge using 0.5 ml of mobile phase. Serotonin and noradrenaline were used as standards for the preparation of calibration curve 13.

Statistical analysis

The data are expressed as mean ± SD. The data were analyzed using one way ANOVA followed by post hoc Newman Keul's multiple comparison test for individual group. The probability level of P<0.05 was considered as statistical significance.

Results

Effect of Perment® on food intake and body weight gain in CUMS rats

In CUMS administered rats, the cumulative feed intake was found to be approximately 20% more in comparison to control rats. Similar result was observed with diazepam treated CUMS rats, whereas Perment® treated rats, at all the dose levels did not alter the feed intake and it was similar to that of control rats (Fig. 1).

Stress administration for 21 days, increased approximately 5% of the body weight of vehicle treated stressed rats in comparison to 25% increase in control rats. Treatment of diazepam did not alter the body weight gain in stressed rats. However gradual body weight gain was observed with Perment® (300 mg/kg) treated rats (Fig. 2).
**Open field exploratory behaviour**—CUMS rats exhibited significant decrease ($P<0.01$) in open field activity (Table 1), as observed by decreased ambulatory behaviour, central activity, rearing and increased immobility period. Perment® (75, 150 and 300 mg/kg) treatment during CUMS period, significantly increased and decreased the ambulatory behaviour and immobility period, respectively. The central activity of the rats also significantly increased with Perment® treatment. However, grooming and rearing behaviour remains unaltered. Increased number of faecal boli excretion was observed with Perment® (300 mg/kg) treated rats. Diazepam treated rats exhibited significantly increased ambulatory behaviour central activity grooming activity and decreased immobility period in the open field.

**Elevated plus maze**—Vehicle treated stressed rats have made less number of open and closed arms entries and spent less and more time in open and closed arms ($P<0.01$) respectively, which indicates anxiogenic behaviour of stressed rats. Treatment of Perment® (300 mg/kg) during stress period significantly attenuated the anxiogenic behaviour as evidenced by increased and decreased time spent in open arms and closed arms respectively. At 75 and 150 mg/kg dose level, Perment® did not produce significant anxiolytic behaviour. Diazepam treatment produced anxiolytic behaviour as indicated by increased ratio levels in arm entries and time spent (Table 2).

**Social interaction test**—Stressed rats exhibited significant decrease in social interaction activity, indicating the presence of anxiety in these rats. Treatment of diazepam ($P<0.01$) and Perment® 150 mg/kg significantly increased the social interaction time in comparison to stressed rats (Table 1).

**Behavioural despair test**—Twenty one days administration of CUMS produced depression in rats as recorded by increased immobility period in Porsolt’s swim test in comparison to control rats. Diazepam and Perment® (75 and 150 mg/kg) decreased the immobility period in comparison to vehicle treated stressed rats (Table 1).

**Plasma, brain and adrenals corticosterone levels**—Stress administration increased the plasma and adrenal corticosterone levels. Brain corticosterone levels also significantly increased in comparison to control group.

![Figure 1](image1.png) Effect of Perment® on feed intake (g/day) in CUMS rats

![Figure 2](image2.png) Effect of Perment® on the body weight gain (g) in CUMS rats

<table>
<thead>
<tr>
<th>Groups (mg/kg)</th>
<th>Ambulation</th>
<th>Rearing</th>
<th>Grooming</th>
<th>Fecal pellets</th>
<th>Immobility period (s)</th>
<th>Social interaction period (s)</th>
<th>Swim test immobility period (s)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Peripheral</td>
<td>Central activity</td>
<td>Ratio</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>36.80±4.44</td>
<td>16.60±4.99</td>
<td>0.45</td>
<td>20.00±2.07</td>
<td>38.20±6.11</td>
<td>5.00±0.31</td>
<td>12.40±2.79</td>
</tr>
<tr>
<td>Stress</td>
<td>20.14±9.12</td>
<td>6.86±3.02</td>
<td>0.38</td>
<td>15.86±3.51</td>
<td>19.86±3.96</td>
<td>3.14±1.05</td>
<td>50.57±15.75</td>
</tr>
<tr>
<td>Diazepam</td>
<td>54.50±11.33</td>
<td>22.50±10.40</td>
<td>0.37</td>
<td>16.67±3.17</td>
<td>53.33±10.52</td>
<td>2.67±0.76</td>
<td>20.17±7.42</td>
</tr>
<tr>
<td>Perment (75)</td>
<td>35.86±6.47</td>
<td>6.86±3.02</td>
<td>0.38</td>
<td>15.86±3.51</td>
<td>19.86±3.96</td>
<td>3.14±1.05</td>
<td>50.57±15.75</td>
</tr>
<tr>
<td>Perment (150)</td>
<td>33.71±5.43</td>
<td>13.71±8.58</td>
<td>0.38</td>
<td>19.00±1.56</td>
<td>21.00±3.14</td>
<td>4.43±0.37</td>
<td>22.29±4.32</td>
</tr>
<tr>
<td>Perment (300)</td>
<td>34.00±5.97</td>
<td>7.43±3.73</td>
<td>0.21</td>
<td>17.71±2.42</td>
<td>35.00±4.85</td>
<td>5.71±0.47</td>
<td>26.00±3.56</td>
</tr>
</tbody>
</table>

$P$ values: $^a$ $<0.05$; $^b$ $<0.01$; $^c$ $<0.001$ in comparison to control ($^a,^b,^c$) and stress vehicle treated ($^*,^**,^***$) groups, respectively.
levels did not alter with the stress administration and Perment® treatment. Treatment with Perment® (150 and 300 mg/kg) decreased the plasma and adrenals corticosterone levels indicating anti stress property of the formulation. Similar effect was observed with diazepam treatment (Table 3).

Plasma noradrenaline and serotonin levels—Plasma noradrenaline and serotonin concentration of various treatment groups are tabulated in Table 3. In comparison to control rats significant decrease in plasma noradrenaline and serotonin level were observed in stressed rats. Perment® (150 and 300 mg/kg) increased the plasma noradrenaline and serotonin levels in comparison to stressed rats, however, the reversal of serotonin level was found to be partial. Diazepam increased the plasma serotonin level in comparison to stressed rats.

Discussion

The objective of the present study was to elucidate the effect of the polyherbal formulation Perment® on behavioural, neurochemical and on stress marker cortisone levels in CUMS induced depressive model. Long term exposure to multiple stressors can cause depression in humans. Stress is a state of threatened homeostasis provoked by psychological, physiological or environmental stressors. Stressor is a stimulus either internal or external, which activates the HPA axis and the sympathetic nervous system resulting in physiological changes. CUMS depressive model is a well validated model to produce depression, and has face and predictive validity. In the present study, stressed rats exhibited anxiogenic behaviour and depression along with decreased plasma noradrenaline and serotonin with increased corticosterone level in plasma and adrenals. This indicates that the rats are in stressful condition and the alteration observed is similar to clinically related pathophysiology of depression.

Administration of Perment® (W. somnifera, A. racemosus, C. ternatea and B. monniera) during stress period restored the ambulatory behaviour of the rats and produced antidepressant property in behavioural despair test. Interestingly, in comparison to 300 mg/kg dose the lower dose levels (75 and 150 mg/kg) exhibited better behavioural performance in forced swim test. The increased ambulatory behaviour of the rats can be correlated with restoration of plasma noradrenaline level. Since hypothalamus is involved in hypothalamic pituitary adrenal (HPA) axis, there may be a chance that whole brain corticosterone level might not change. However, clarity on the plasma and tissue corticosterone level could not be achieved in the present study. Increased fecal pellets excretion with Perment® treatment groups in open field test indicates incomplete reversal of anxiety induced with

<table>
<thead>
<tr>
<th>Groups (mg/kg)</th>
<th>Open arms</th>
<th>Closed arms</th>
<th>Open arms</th>
<th>Closed arms</th>
<th>Arm entries</th>
<th>Time spent</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>2.50 ±0.43</td>
<td>6.00 ±0.68</td>
<td>33.17±2.64</td>
<td>187.30±17.47</td>
<td>0.44</td>
<td>0.19</td>
</tr>
<tr>
<td>Stress</td>
<td>0.57±0.29b</td>
<td>3.00±0.78b</td>
<td>3.29±1.86</td>
<td>209.10±6.70</td>
<td>0.14</td>
<td>0.01</td>
</tr>
<tr>
<td>Diazepam</td>
<td>3.33±0.33**</td>
<td>8.83±0.47</td>
<td>76.17±14.48**</td>
<td>82.50±11.13**</td>
<td>0.70**</td>
<td>1.10**</td>
</tr>
<tr>
<td>Perment (75)</td>
<td>0.33±0.21</td>
<td>3.33±0.49</td>
<td>2.83±1.91</td>
<td>189.00±4.19</td>
<td>0.07</td>
<td>0.01</td>
</tr>
<tr>
<td>Perment (150)</td>
<td>1.43±0.52</td>
<td>4.57±0.57</td>
<td>16.57±5.92</td>
<td>176.70±6.97</td>
<td>0.29</td>
<td>0.10</td>
</tr>
<tr>
<td>Perment (300)</td>
<td>1.43±0.43</td>
<td>3.71±0.47</td>
<td>30.86±9.67</td>
<td>157.70±11.25**</td>
<td>0.39</td>
<td>0.19</td>
</tr>
</tbody>
</table>

P values: a, b*<0.05; b**, c**<0.01 in comparison to control (a, b) and stress vehicle treated (*****) groups, respectively

<table>
<thead>
<tr>
<th>Groups (mg/kg)</th>
<th>Noradrenaline</th>
<th>Serotonin</th>
<th>Corticosterone Brain</th>
<th>Adrenals</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>10.66±1.52</td>
<td>21.33±3.21</td>
<td>3.12±1.50</td>
<td>6.14±2.61</td>
</tr>
<tr>
<td>Stress</td>
<td>4.00±2.00b</td>
<td>12.00±1.00b</td>
<td>7.52±2.80c</td>
<td>5.82±2.11</td>
</tr>
<tr>
<td>Diazepam</td>
<td>5.00±2.00c</td>
<td>16.33±1.53a</td>
<td>3.45±0.86**</td>
<td>6.25±2.00</td>
</tr>
<tr>
<td>Perment (75)</td>
<td>5.00±1.73a</td>
<td>13.00±1.00c</td>
<td>5.25±1.60</td>
<td>6.88±3.50</td>
</tr>
<tr>
<td>Perment (150)</td>
<td>10.00±2.00b</td>
<td>16.33±1.55b</td>
<td>4.33±1.10**</td>
<td>7.01±2.50</td>
</tr>
<tr>
<td>Perment (300)</td>
<td>9.00±2.00c</td>
<td>17.00±1.00b</td>
<td>4.45±0.98**</td>
<td>5.95±3.11</td>
</tr>
</tbody>
</table>

P values: a, b*<0.05; b**, c**<0.01, c**<0.001 in comparison to control (a, b, c) and stress vehicle treated (*****) groups, respectively

†Adrenal samples were pulled together and treated as single sample for corticosterone treatment.
stress application. Earlier, *W. somnifera* root extract (50 mg/kg) has been reported to have adaptogenic property in chronic stress model in rats\(^1\) and the active principle withanolide\(^1\)^, glycwithanolides\(^2\) were correlated to the adaptogenic and antidepressant activity which support the present findings. Similarly, *B. monniera* reversed the stress induced plasma corticosterone, noradrenaline, serotonin and dopamine levels in cortex and hippocampus regions of the brain, which are more vulnerable to stressful conditions\(^2\). *A. racemosus* (100, 200 and 400 mg/kg) and *B. monniera* extract (20 and 40 mg/kg) were reported to have antidepressant property in the forced swim test and learned helplessness behaviour\(^2\)^. Methanolic extract of *C. ternatae* exhibited anxiolytic and antidepressant property in stress conditions through serotonergic and cholinergic system activation rather than dopaminergic and noradrenergic systems\(^1\). In the present study Perment\(^\circledR\) exhibited dual activity and produced significant antidepressant property. At this juncture it can be stated that the *W. somnifera* and *B. monniera* would have contributed to adaptogenic as well as behavioural alterations, whereas *C. ternatae* and *A. racemosus* would have attenuated the stress induced depression. The predominant antidepressant property observed with the formulation may be due to the model selection\(^1\)^.

It has been well documented that, activation of HPA axis during stressful condition can alter the biochemical as well as neurochemical activity along with increased corticosteroid levels\(^2\). The Perment\(^\circledR\) did not influence the body weight gain and feed intake as equivalent to unstressed rats during stressful period indicates that the formulation could not contribute much to the biochemical/metabolic regulations to maintain the normal physiological functions during the course of stress administration. Though serotonin levels were restored with Perment\(^\circledR\) treated group, food intake did not alter and it is maintained during CUMS period. Earlier reports indicate that in invertebrates, serotonergic neurons can enhance the feed intake directly by acting on muscles involved in consuming food\(^2\)^.\(^2\)^.

The results of the present study conclude that the Perment\(^\circledR\) formulation exhibited significant antidepressant activity rather than anxiolytic action. Currently the formulation is clinically used as anxiolytic but present results suggest that the formulation can preferably be indicated in patients affected with depression.

References


