

Anti-stress and nootropic activity of aqueous extract of *Piper longum* fruit, estimated by noninvasive biomarkers and Y-maze test in rodents

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Abstract

Piper longum L., also known as long pepper or pipli, belongs to family Piperaceae. Fruits of this plant have been used traditionally to treat a variety of diseases. The current study was done to evaluate anti-stress activity in rats subjected to forced swim stress one hour after daily treatment of *P. longum* extract. Urinary vanillylmandelic acid, 5-hydroxyindoleacetic acid, homo vanillic acid and ascorbic acid, estimated by HPLC and spectrophotometric methods in all groups, were selected as non invasive biomarkers. Anti-stress and nootropic activity activities of aqueous extract of *P. longum* fruit extract were estimated as locomotor and working memory in rats in a Y-maze apparatus. The *in vitro* antioxidant activity was determined based on the ability of the *P. longum* to scavenge free radicals. Daily administration of aqueous extract of *P. longum* at doses of 100, 200 and 300 mg per kg body weight one hour prior to induction of stress increased the stress-induced urinary biomarker levels in a dose-dependent manner. *P. longum* treatment showed significant dose-dependent variation in non-invasive biomarker levels in urine samples of rats taken after 24 h. Cognition, determined by working memory and locomotor activity results, were shown to be dose-dependent. The results of this study suggest anti-stress and nootropic activity effect of *P. longum* in rodents.

Key words: antioxidant activity, anti-stress activity, nootropic activity, *Piper longum*, rats, Y-maze test.

Abbreviations: HVA, homovanillic acid; VMA, vanillylmandelic acid; 5-HIAA, 5-hydroxy indole acetic acid.

Introduction

Homeostasis regulates the physiological actions in the body, and depends on the stress and antioxidant levels in the cells. Nowadays, stress is involved in the major portion of alterations of physiological actions, leading to pathogenesis (Thorpe et al. 2004). Day-to-day life has stressful events in both working and non working individuals and it is a hallmark for pathogenesis of a variety of diseases and disorders (Bruce et al. 2007), such as diabetes mellitus, hypertension, depression, anxiety, immunosuppressant, vascular disorders, male infertility, cognitive dysfunction, peptic ulcer ulcerative colitis, atherosclerosis, cancer, ageing, arthritis, Alzheimer's disease, liver disease etc (Ajay et al. 2009; Aher et al. 2011). Further overload of stress increases free radicals, produces damage to neuronal receptors and a variety of tissues. Free radical scavenging agents may have great potential in ameliorating these disease/disorders (Mark et al. 2000).

Anti-stress agents decrease stress (Patcharee et al. 2012). Considering the debut of adaptogens, many plants

have been explored due to their anti-stress and renovating properties in conventional medicines (Alexander et al. 2010). Many plant extracts and plant products are known to have promising anti-stress and antioxidant activities (Gupta et al. 2008; Lakshmi et al. 2009; Alok et al. 2010; Vinod et al. 2012). Preliminary studies on *Ocimum sanctum*, *Bacopa monniera*, *Vitis vinnifera*, and *Withania somnifera* unfolded an enormous area of research that has been carried out on plants.

Piper longum L., in the family Piperaceae, is also known as long pepper or pipli, it is widely cultivated in low elevation regions in India. The fruits of this plant have been used traditionally to treat a variety of diseases. Reported activities with affirmative effects on human health are antibacterial activity, hypocholesterolaemic activity, antidepressant activity, hepatoprotective, antiinflammatory activity, bioavailability enhancement, antioxidant activity, larvicidal activity, radio protective activity, cardioprotective activity, antifertility activity (Suresh et al. 2011), immunomodulatory and antitumour activity (Sunila et al. 2004).

The anti-stress activity of *P. longum* was evaluated *in vivo* in normal and stress induced rats. The *in vitro* antioxidant activity was considered as anti-stress potential. Nootropic activity was evaluated by using Y-maze method in rats.

Materials and methods

Drugs and chemicals

EDTA, NaCN, Nitro blue tetrazolium, deoxy-D-ribose, ferric chloride, sodium dodecyl sulphate, ascorbic acid, Tris HCl buffer, sodium nitro prusside, Griess reagent were purchased from Sisco Research Laboratories Pvt. Ltd (Mumbai, India). Vanillylmandelic acid (VMA), homovanillic acid (HVA) and 5-hydroxy indole acetic acid (5-HIAA) were purchased from Sigma Chemicals Co., U.S.A. Acetonitrile (HPLC grade) was obtained from Qualigens, methanol (HPLC grade) from SRL, hydrochloric acid, ortho phosphoric acid, potassium dihydrogen phosphate all from Merck. All other chemicals and reagents used were of analytical grade.

Preparation of extract

Piper longum was obtained from the Laila Implex Laboratory (batch number: L8121139, product code: C/AU/PILO-01) and was authenticated. Alcoholic extract was qualitatively analyzed for the presence of various phytochemical constituents. Preliminary phytochemical screening of the aqueous extract showed the presence of alkaloids, tannins and terpenoids.

Acute toxicity study

Piper longum at different doses (500 to 2000 mg per kg) was administered to mice by oral feeding tube, which were observed for gross behavioral, neurological, autonomic and toxic effects according to OECD guidelines. No mortality was observed within 24 h of dose of 2000 mg per kg.

Evaluation of *in vitro* antioxidant activity

In vitro antioxidant activity of was determined by riboflavin photo reduction method (superoxide anion; Yoshiaki et al. 2012), thiobarbituric acid method (inhibition of lipid peroxidation; Sachin et al. 2011), diazotization of nitrite with sulphanilamide and subsequent coupling with naphthalene diamine dihydrochloride (nitric oxide scavenging activity; Filomena et al. 2011) and deoxyribose degradation method (hydroxyl radical; Perumal et al. 2007). All analyses were performed in triplicate.

Animals

Albino wistar rats (150 to 200 g) of either sex were procured from the National Institute of Nutrition, Hyderabad, India. Animals were maintained under standard laboratory conditions relative humidity of $50 \pm 15\%$, temperature 22 ± 2 °C and 12/12 h dark/light cycle. Rats were fed with commercial pellet diet (Rayons Biotechnologies Pvt Ltd, India) and water was provided ad libitum. The experimental

protocol has been approved by the institutional animal ethics committee and by the Animal Regulatory Body of the Government (Regd. No. 516/01/A/CPCSEA).

Anti-stress activity

Rats were divided into four groups (I, II, III and IV), each containing four animals (two male and two female). After forced swimming stress, a 24-h urine sample from each group was collected into two different beakers, one containing 5 mL of 10% oxalic acid for the determination of ascorbic acid by spectrophotometric method at 550 nm and the other containing 0.5 mL of 6 N hydrochloric acid for the determination of metabolites of noradrenaline (VMA), 5-hydroxytryptamine or serotonin (5HIAA) and dopamine (HVA) by HPLC. The experimental procedure was divided into four parts.

In the first part of the experiment, 24-h urine samples were collected from all four groups, these were subjected to analysis on the same day for metabolites and ascorbic acid. In the second part, animals in each group, except the first group, were subjected to fresh water swimming individually in a cylindrical vessel of height 60 cm and diameter 40 cm containing water at room temperature (28 °C) until they became exhausted. Water depth was always maintained at 30 cm. After inducing stress, 24-h urine samples were collected and were analyzed on the same day. In the third part of the experiment, five groups were used. Groups III, IV and V were administered orally with *P. longum* extract at daily doses of 100, 200 and 300 mg per kg body weight respectively. Group I served as control and Group II served as a stress control, after recovery. 24-h urine samples were collected and the levels of both metabolites and ascorbic acid were determined on the same day, and also after recovery from the drug effect on the fourth day. In the final part of the experiment, the treatment given to the groups of animals was as follows:

Group I: control animals administered with distilled water orally at a dose of 0.1 mL per 100 g body weight (b.w.);

Group II: animals administered orally with *P. longum* (dissolved in distilled water) at a dose of 100 mg per kg b.w.;

Group III: animals administered orally with *P. longum* (dissolved in distilled water) at a dose of 200 mg per kg b.w.,

Group IV: animals administered orally with *P. longum* (dissolved in distilled water) at a dose of 300 mg per kg b.w.

After 30 min the animals were subjected to stress and 24-h urine samples were collected to study the effect of herbal preparation on stress-induced biochemical changes. The analysis was conducted on the same day. Analytical methods for the measurement of VMA, 5-HIAA, HVA in urine have been improved remarkably with application of HPLC (Seegal et al. 1986; Hartleb et al. 1993; Mehta et al. 2012). In the present study reverse phase simultaneous method for the determination of VMA, 5-HIAA, HVA was developed and calibrated. The number of replicate measurements per treatment was four.

Table 1. Free-radical scavenging effect of *Piper longum* extract in comparison to that of ascorbic acid by *in vitro* antioxidant method. The data are expressed as IC₅₀ (µg mL⁻¹)

Compounds	Superoxide	Hydroxyl radical	Lipid peroxidation	Nitric oxide
<i>Piper longum</i>	87.4	204.5	235.0	229.7
Ascorbic acid	73.8	185.0	229.4	184.9

Nootropic activity

The nootropic activity effect of *P. longum* was evaluated by using the Y-maze test. Albino wistar rats were divided into five groups each containing six animals. Group I served as negative control, Group II served as positive control, Groups III, IV and V received orally *P. longum* 100, 200 and 300 mg per kg respectively for 7 days. After 30 min of administration of last dose of extract (7th day), all groups received scopolamine (2 mg per kg, i.p.) except Group I.

Y-maze test

The Y-maze consisted of three arms (30 cm length, 8 cm width and 15 cm height) with an angle of 120° between each two arms. The arms were randomly designated start arm (S), novel arm (N) and familiar arm (F). During the training, rats were placed in the start arm in the first trail. Rats were allowed to explore the start arm and familiar arm which was opened, whereas the novel arm was closed. In the second trail rats were allowed to explore the three arms; the number and order of arm entries for total 6 min duration was recorded. Total number of arm entries indicates the locomotor activity and successive entries into the three arms on overlapping triplet sets (SFN, FNS, and NFS etc) were used to calculate the spontaneous alteration behavior. The cognitive behavior and working memory was calculated by the formula (Robert et al. 2004):

$$\% \text{ Alterations} = \frac{\text{Number of positive alterations made}}{\text{Total number of arm entries} - 2} \times 100.$$

For the Y-maze test, six animals per treatment were used.

Statistical analysis

The results were expressed as means ± SEM. Statistical analysis was conducted using one way ANOVA followed by Tukey's post hoc test; $p < 0.01$ was considered statistically significant.

Table 2. Effect of stress and *Piper longum* extract on the total amount of metabolites (µg) excreted in 24-h rat urine. Data was expressed as mean ± SEM, n = 4. ^aStatistically significant difference when compared to control group, $p < 0.01$. ^bStatistically significant difference when compared to stress-induced group, $p < 0.01$

Treatment	Vanillylmandelic acid	5-hydroxy indole acetic acid	Homovanillic acid	Ascorbic acid
Control	30.8 ± 3.1	54.4 ± 5.0	154.2 ± 24.6	22.8 ± 1.0
Stress	108.2 ± 8.7 ^a	153.1 ± 16.1 ^a	31.1 ± 4.4 ^a	7.7 ± 0.9 ^a
Control + <i>P. longum</i>	29.9 ± 3.1 ^b	54.4 ± 5.0 ^b	173.4 ± 20.0 ^{ab}	23.2 ± 1.4 ^b
Stress + <i>P. longum</i> (100 mg kg ⁻¹)	52.0 ± 6.0 ^{ab}	97.4 ± 4.4 ^{ab}	115.1 ± 2.9 ^{ab}	17.0 ± 2.2 ^{ab}
Stress + <i>P. longum</i> (200 mg kg ⁻¹)	45.6 ± 3.7 ^{ab}	85.9 ± 11.6 ^{ab}	167.0 ± 8.2 ^{ab}	20.1 ± 1.7 ^b
Stress + <i>P. longum</i> (300 mg kg ⁻¹)	30.0 ± 2.4 ^b	56.0 ± 4.1 ^b	195.8 ± 5.2 ^{ab}	23.3 ± 1.8 ^b

Results

Preliminary phytochemical studies and antioxidant activity

Phytochemical investigation indicated the presence of alkaloids, tannins and phenols. *P. longum* showed significant free radical scavenging activity (superoxide, hydroxyl radical, lipid peroxidation, nitric oxide), when compared to standard ascorbic acid in dose dependant manner. This indicates that the aqueous extract of *P. longum* possessed potential antioxidant activity. The IC₅₀ values of superoxide, hydroxyl radical, lipid peroxidation, nitric oxide scavenging effect of *P. longum* were comparable to those of ascorbic acid and were especially high in the case of lipid peroxidation (Table 1)

Anti-stress activity

The urinary values of VMA, 5HIAA, HVA and ascorbic acid, determined in various parts of the experiment, are shown in Table 2. The levels of VMA, 5HIAA were higher and HVA, and ascorbic acid were lower, when compared to control following induction of stress. Recovery from the stress was seen on the fourth day. Daily treatment of *P. longum* extract to the animals under control conditions produced no significant variation in VMA, 5HIAA, HVA and ascorbic acid compared to normal basal levels. Recovery from the drug was seen on the fourth day. With administration of *P. longum* extract one hour prior to the induction of stress, the VMA and 5HIAA levels were significantly ($p < 0.01$) decreased and HVA, ascorbic acid were significantly ($p < 0.01$) increased, compared to stress alone.

Nootropic activity

Scopolamine-induced memory impairment was suggested by reduced number of arm entries and successive three arm visits. Oral administration of *P. longum* extract decreased

the scopolamine-induced memory interruption and increased cognitive performance and working memory in the Y-maze (Table 3). Duration of time spent in novel arm (Fig. 1), number of arm entries and successive entries into three arms were increased, whereas transfer latency to novel arm was decreased (Fig. 2), when compared with the scopolamine treated group; this indicated increased locomotion and working memory (% alteration), respectively. *P. longum* showed dose dependent memory enhancement, when compared to the scopolamine-treated group.

Discussion

Stress induces free radicals, and produces biochemical changes in all tissues, leading to different diseases and disorders. Several studies have supported the role of stress in neurodegenerative disorders by the release of biogenic amines noradrenalin, 5-hydroxytryptamine and dopamine from the central nervous system by measuring the levels in urinary metabolites of biogenic amines like VMA, 5HIAA and HVA in 24 h urinary samples (Ion et al., 1969; Satyanarayana Sreemantula et al. 2005; Anil Kumar et al. 2014). The results of the present study showed that stress increased excretion of VMA and 5HIAA and decreased the HVA and ascorbic acid levels. In the present experiment VMA, 5HIAA, HVA and ascorbic acid were used as noninvasive biomarkers for evaluation of anti-stress activity of aqueous extract of *P. longum*. Different concentrations of extract were tested by *in vitro* methods for antioxidant activity by free radical scavenging, in comparison with the standard well known antioxidant, ascorbic acid (Jayaprakasha et al. 2001; Singh et al. 2002; Münir et al. 2003). *P. longum* extract was found to possess potential antioxidant activity in a dose dependent manner. The presence of alkaloidal components like piperine and piperlongumine are known to possess antioxidant activity (Lidao et al. 2012).

The extract was evaluated for anti-stress activity against

fresh water swimming stress in rats (Sushruta et al. 2012). The advantage of using estimation of urinary metabolites was its non-invasiveness and ability not to apply additional stress during sample collection. During stress, the levels of VMA and 5HIAA increased due to increased metabolism of noradrenalin (Zeinab et al. 2012), 5-hydroxytryptamine; the levels of HVA and ascorbic acid decreased due to decreased metabolism of dopamine and increased free radical scavenging activity of ascorbic acid.

The extract did not alter their levels during normal conditions on daily administration. Application of stress (fresh water swimming) increased the VMA and 5HIAA levels and decreased HVA and ascorbic acid levels, which returned back to normal level on cessation of the stress. Prior administration of extracts blocked the stress-induced changes and such effects were found to be dose dependent. The potency of the extracts in abolishing stress correlated well with their lipid peroxidation inhibitory activity seen in *in vitro* studies, indicating that the *in vivo* method could be used to model the *in vitro* antioxidant activity. Since the aqueous extracts of *P. longum* did not change the levels of metabolites in normal conditions and blocked only stress induced changes, they appear to be good anti-stress agents.

Studies indicated that acute stress deflates memory (Hyunyoung et al. 2011). Hence, the extracts, being good anti-stress agents, were considered to possess nootropic activity (Ravishankar et al. 2012). Experiments with the known antioxidants quercetin (Siva Reddy et al. 2011), and ascorbic acid (Omar et al. 2012) showed that lipid peroxidation inhibition appeared to be responsible for memory enhancing activity. So far, bacosides from *Bacopa monnieri* are available as ayurvedic remedies for memory boosting (Jobin et al. 2010) by possessing the mechanisms associated with antioxidant activity (Chowdhuri et al. 2002), by improving acetylcholine activity (Amitava et al. 2002), anti-stress activity (Kashmira et al. 2010), and increasing protein kinase activity in the hippocampus (Russo et al. 2005).

The cholinergic system plays a vital role in synaptic

Table 3. Effect of *Piper longum* on spontaneous alteration behavior and locomotor in scopolamine induced memory impairment in a Y-maze test. S, starting arm; N, novel arm; F, familiar arm. Data are means \pm SEM. ^aStatistically significant difference when compared to control group, $p < 0.01$. ^bStatistically significant difference when compared to stress-induced group, $p < 0.01$

Treatment	Alteration (%)	Number of arm visits		
		S	N	F
Control	29.10 \pm 0.80	4.16 \pm 0.59	4.33 \pm 0.45	3.50 \pm 0.77
Scopolamine 0.5 mg kg ⁻¹	17.53 \pm 0.78 ^a	2.33 \pm 0.19	1.83 \pm 0.54	2.50 \pm 0.20
<i>P. longum</i> 100 mg kg ⁻¹ + scopolamine 0.5 mg kg ⁻¹	27.01 \pm 0.57 ^{ab}	1.33 \pm 0.19	1.33 \pm 0.19	1.33 \pm 0.19
<i>P. longum</i> 200 mg kg ⁻¹ + scopolamine 0.5 mg kg ⁻¹	29.00 \pm 0.56 ^b	3.50 \pm 0.38	3.33 \pm 0.5	2.50 \pm 0.31
<i>P. longum</i> 300 mg kg ⁻¹ + scopolamine 0.5 mg kg ⁻¹	43.86 \pm 0.69 ^{ab}	3.33 \pm 0.73	2.83 \pm 0.49	2.00 \pm 0.32

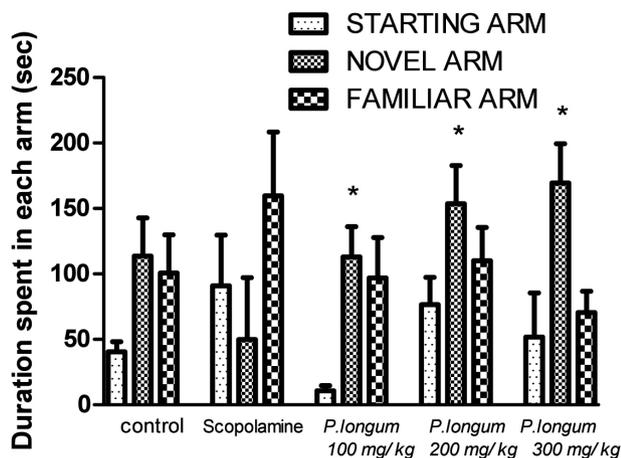


Fig. 1. Effect of *Piper longum* on duration of time (s) spent in each arm in scopolamine induced cognitive impairment. * $p < 0.01$ considered as statistically significant difference compared to scopolamine.

plasticity, learning and memory. Cholinergic agonists like donepezil, galantamine, rivastigmine and tacrine can improve memory, whereas cholinergic antagonists can impair memory. Literature reports the use of scopolamine anti-cholinergic agent using animal model for cognitive impairment and memory retrieval loss. The anti-cholinergic agent acts through decreasing superoxide dismutase, catalase and glutathione specific activities, resulting in increased malondialdehyde level. This was evaluated by the Y-maze method. Y-maze spontaneous alternation is a behavioral test for measuring the willingness of rodents to explore new environments. Rodents typically prefer to investigate a new arm of the maze rather than returning to one that was previously visited. Many parts of the brain, including the hippocampus, septum, basal forebrain, and prefrontal cortex, are involved in this task (Cognato et al. 2012). In the Y-maze, the duration of time spent in the novel arm indicates exploratory time, spontaneous alterations indicates working memory and total number of entries indicates the locomotor activity. In the present study, treatment with aqueous extract of *P. longum* resulted in significant differences in novel arm entries, duration of time spent in the novel arm, transfer latency for novel arm and spontaneous alteration behavior, compared to scopolamine treated group. Our data suggest that the extract had protective effect against scopolamine-induced memory loss, which was due to its anti-cholinesterase activity apart from antioxidant effect.

Finally, we reported that urinary biomarkers levels were decreased and memory loss is absent, in the stress and scopolamine-treated rats, respectively exposed to aqueous extract of *P. longum*, suggesting that *P. longum* possess dose dependent anti-stress and nootropic activity.

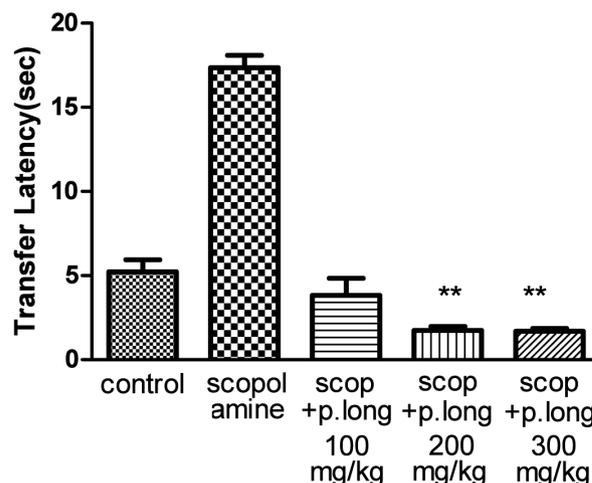


Fig. 2. Effect of *Piper longum* on transfer latency (s) in scopolamine induced cognitive impairment. ** $p < 0.01$ considered as statistically significant difference compared to scopolamine.

Conclusions

This study provided evidence for antioxidant, anti-stress and nootropic activity activities of the selected aqueous extract and that use of them by humans as nutraceuticals is beneficial and scientific. Antioxidant effect provides the mechanistic basis in relieving stress and memory by way of combating stress in both the models. Further work is needed for isolation of compounds and its pharmacological evaluation.

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