

Effect of Storage Conditions on Free Radical Scavenging Activities of Crude Plant Material of *Piper longum*

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Article Info	Summary
<p>Article History</p> <p>Received : 28-03-2011 Revised : 23-04-2011 Accepted : 23-04-2011</p> <p>*Corresponding Author</p> <p>Tel : +91-2025653680 Fax : +91-2025651542 Email: psri94@yahoo.com</p>	<p>Crude plant material of <i>Piper longum</i> was used for stability studies and kept in stability chambers. The effect of the stored samples on free radical scavenging activity was examined by DPPH, ABTS radical scavenging assays and Deoxyribose degradation assay. Various parameters used for the stability studies were 40°C and 75% relative humidity in case of accelerated studies and 30°C and 65% relative humidity in case of long term studies as per ICH guidelines. Real time studies samples were stored at ambient temperature. Samples were taken out at periodic intervals and suspended in Tris-HCl buffer (10Mm, pH 6.5) to check the total phenolic content and free radical scavenging activity. Outcome of the antioxidant assays indicated that the samples of long term studies showed better antioxidant activity and higher total phenolic content than the samples of accelerated and real time studies.</p>
<p>©ScholarJournals, SSR</p>	<p>Key Words: Pippali; Accelerated study; Long term study; Real time study; Free radical scavenging activity Abbreviations: PL: <i>Piper longum</i>; AS: Accelerated study; LS: Long term study; RT: Real time study; TPC: Total phenolic content</p>

Introduction

Since ancient time medicinal plants have been considered as main ingredients in Ayurvedic formulations for the treatment of various diseases and they have never lost their importance with emergence of modern science [1-2]. Plants possess pharmacological properties due to the presence of bioactive molecules [3] and the stability of these molecules decides the potential nature of plant to be use as drug. In standard Ayurvedic texts it is mentioned that 'Veerya kalawadhi' is a certain period within which the drug is to be used in the preparation of medicine. At present very few species are cultivated systematically and majority of them are collected from wild. Most of the medicinal plants are seasonal and therefore, they are stored in large store houses and used as per the requirement. Storage of plant material for long period can change the quantity of active biomolecules and potency of the plant material. Hence, stability and potency of the plant materials must be checked before using the crude drugs in the formulations.

Piper longum commonly known as 'Pippali' in Hindi or 'long pepper' in English belongs to family Piperaceae has been found in north-eastern and southern parts of India and Srilanka. It has been used as a household remedy in treating respiratory disorder. Several biological activities of *P. longum* extract have been reported, including antiamebic, anti-giardial, immunostimulatory, anti-ulcer, antioxidative and anti-inflammatory properties [4-8]. The major chemical constituents of the plant are volatile oil, resin and alkaloids viz.

piperine, piperlongumine, piperlonguminine etc. [9]. Fruit of pippali is an important constituent of 'Trikatu' formulation [10, 11]. Methanolic extract [12], hot ethyl acetate and cold hexane: water extracts [13] of dried fruit of *P. longum* were found to possess significant *in vitro* antioxidant potential and hepatoprotective activity. Ayurvedic system of medicine recommends *P. longum* for treating cardiac disorders [14].

Stability is a time during which a drug retains its chemical integrity and labelled potency within the specified limits. Stability studies provide information about storage conditions where plant material or product is intended to retain its efficacy within specified limits. Moreover, it is the most vital factor which determines whether the plant material can be used for the pharmaceutical product. The stability is affected by physical factors such as hydrolysis, oxidation, isomerization and polymerization etc. The stability testing involves the examining of quality and potency of the plant material at suitable time intervals. Stability studies of natural product are difficult as compared to pharmaceuticals because there are complex mixtures of various types of unlimited constituents. Different types of strategies such as marker compounds and metabolic fingerprint profiling may be applied to assess the stability of natural products. Stability studies of some plants are reported in literature [15, 16]. Although *P. longum* is used in Ayurvedic formulations but its stability study has not been carried out so far. In this context we are reporting for the first time the stability study of *P. longum* and effect of storage conditions on TPC

and free radical scavenging activity. This may provide beneficial information to natural product industry about the storage of raw material of the *P. longum*.

Materials and Methods

Chemical and reagents

Ammonium persulphate, 2,2'-azino-bis(3-ethylbenzthiazoline-6-sulphonic acid) diammonium salt (ABTS) and 2,2-diphenyl-1-picrylhydrazyl (DPPH) were procured from Fluka-Sigma Aldrich (USA). Ascorbic acid was procured from Molychem, India. All other chemicals unless otherwise mentioned were procured from Merck (Germany). Shimadzu UV-2501 PC spectrophotometer (Tokyo, Japan) was used for colorimetric analysis. Thermolab stability chambers were used for stability studies.

Plant material

Fruit of *P. longum* were collected from Western Ghat in June 2010. It was authenticated by Dr. A. C. Desai of IDRA&L, Pune. Voucher specimen is deposited in IDRA&L *vide* voucher no. 04PL/2010-2011. It was cleaned, shade dried and processed for further studies.

Stability studies of crude plant drugs

Accelerated and long term studies were performed according to the International Committee Harmonization guidelines [17]. Samples were stored in Thermolab stability chambers. The conditions maintained were 30°C and 65% humidity for long term studies (LS) and 40°C and 75% humidity for accelerated studies (AS). Samples were also kept at ambient temperature for real time studies (RT). Plant material was stored in fixed quantity for stability studies and was taken out periodically at the interval of 1,2,3---6 months and at 3, 6 months for accelerated and long term studies respectively. Real time studies samples were also taken out at 3 and 6 months for the analysis.

Extraction

Dried fruit of *P. longum* (PL) were taken out from stability chambers at periodic intervals and powdered. One gram of each sample was suspended in 10 ml of Tris-HCl buffer (10mM, pH 6.5) and kept at 4°C for 24 hours. Real time study samples (1 g) stored at ambient temperature were also powdered and suspended in 10 ml of Tris-HCl buffer (10 mM, pH 6.5). Supernatant obtained after 24 hours was used for further study.

Total phenolic content of Pippali aqueous extract

The total phenolic content of the aqueous fruit extract of pippali was determined using the method of Macdonald *et al.* [18] with modifications. Ethanolic solution of gallic acid (20 μ l; 0.50-0.500mg ml⁻¹) was mixed with 100 μ l of Folin Ciocalteu reagent (diluted 10 fold), sodium carbonate (300 μ l, 2M) and incubated at room temperature for 2 h. Absorbance of resulting mixture was measured at 765nm and the calibration curve was drawn. Aqueous fruit extracts of *P. longum* (20 μ l) were mixed with the above reagents in same order followed by incubation at room temperature for 2 h. Absorbance of test samples was measured at 765nm. Samples were analysed in triplicates. The total phenolic content in the extract in term of gallic acid equivalents (GAE) was calculated [19] by the following formula: $T = (C \times V)/M$; where T = total phenolic content, milligram GAE per gram plant extract, C = the

concentration of gallic acid established from the calibration curve, milligram per millilitre; V = the volume of extract, millilitre; M = the wt of aqueous plant extract, gram.

Antioxidant Activity

P. longum aqueous extracts of concentration (100mg in 1 ml Tris-HCl buffer 10mM pH 6.5) were screened for various free radical scavenging assays.

DPPH assay

DPPH radical scavenging activity was assessed according to the method of Shimada *et al.* [20]. PL extracts (50 μ l, 100 mg mL⁻¹) in Tris -HCl buffer were mixed with 1 ml of 0.05 M acetate buffer, 0.95 ml methanol and 500 μ l of 0.5 mM solution of DPPH in methanol. Blank contained 50 μ l of the PL extract of same concentration without DPPH, while the control was without test sample. The mixture was shaken immediately after addition of DPPH and allowed to stand at room temperature in dark. Decrease in absorbance at 517 nm was measured at 0 min and after every 30 min until the reaction reached a plateau. Samples were analyzed in triplicates. The inhibitory percentage of DPPH was calculated [21] as per the formula: Scavenging effect (%) = $[(A_0 - (A - A_b))/A_0] \times 100$; where, A_0 = Absorbance of control; A = Absorbance of sample and A_b = Absorbance of blank.

ABTS radical cation decolorisation assay

ABTS assay [22] was used to evaluate the ABTS radical scavenging ability of the test sample. ABTS radical cations were generated by reacting ABTS solution (7 mM, 3 ml) in water with ammonium persulphate (2.45 mM, 15 ml) in water. The mixture was allowed to stand in dark at room temperature for 16 h before use. PL extract (250 μ l, 100 mg mL⁻¹) in Tris - HCl buffer was added to 150 μ l of ABTS solution and final volume was made up to 500 μ l with methanol. Control was prepared by adding methanol (350 μ l) to ABTS solution (150 μ l) while blank was without ABTS solution. All the samples were analyzed in triplicates. Absorbance was read at 745 nm and the percentage inhibition was calculated using the formula: % Inhibition = $[(A_0 - (A - A_b))/A_0] \times 100$; where, A_0 = absorbance of control; A = absorbance of test solution and A_b = absorbance of blank.

Deoxyribose degradation assay

Scavenging of hydroxyl radical was measured by employing the method of Halliwell *et al.* [23] with slight modifications. Stock solutions of EDTA (1 mM), FeCl₃ (10 mM), ascorbic acid (1 mM), H₂O₂ (10 mM) and deoxyribose (10 mM) were prepared in distilled water. Reaction mixture was prepared by adding 0.05 ml of EDTA, 0.005 ml of FeCl₃, 0.05 ml of H₂O₂, 0.18 ml of deoxyribose, PL extract (500 μ l, 10 mg mL⁻¹), phosphate buffer (50 mM, pH 7.4, 165 μ l) and ascorbic acid (50 μ l). The mixture was incubated at 37°C for 1 h. The incubated mixture (250 μ l) was mixed with 250 μ l of 10% TCA, 250 μ l of 2% TBA in 0.025 M NaOH and heated at 80°C for 1 h to develop pink chromogen, which was measured at 532 nm. Control was without test sample and blank was without H₂O₂. The inhibition effect on hydroxyl radicals was calculated as follows: % Hydroxyl radical scavenging capacity = $(1 - A_s/A_c) \times 100$; where A_c = Absorbance of control and A_s = Absorbance of sample.

Results

Total phenolic content

High total phenolic content (14.60 mg GAE/g extract of plant material) was found in fresh sample. In case of

accelerated study, there was a continuous decrease in total phenolic content (TPC) but in case of long term study and real time study TPC decreased marginally. The data is shown in Table-1.

Table-1 Total phenolic content of *P. longum* during stability studies

S. No.	Stability study	Duration	TPC (mgGAE/g of plant extract)
1.	Fresh sample	0 min	14.60
2	AS	1month	11.80
3	AS	2 month	10.64
4	AS	3 month	8.28
5	AS	4 month	7.32
6	AS	5 month	5.01
7	AS	6 month	3.93
8	LS	3 month	14.28
9	LS	6 month	10.67
10	RT	3 month	12.29
11	RT	6 month	8.38

Table 2: Results of free radical scavenging activity of AS, LS and RT of *P. longum* samples

Conditions	Duration in months	DPPH assay % Inhibition	ABTS assay % Effectiveness	Deoxyribose degradation assay % Effectiveness
AS sample of PL	3	66.11	86.01	62.99
LS sample of PL	3	82.79	92.64	90.48
RT sample of PL	3	79.31	90.22	76.87
AS sample of PL	6	19.43	65.55	42.53
LS sample of PL	6	68.82	89.98	84.89
RT sample of PL	6	21.48	66.44	56.77

DPPH Assay

PL sample showed decrease in absorption at 517 nm as compared to control. The extract of PL at zero time showed 83.01 % DPPH radical scavenging activity(Figure-1). In case of AS samples the activity reduced to 79.92 % after one month and it was 69.88 % after 3 months and at 6 months the activity reduced to 22.99 %, whereas in LS samples the activity reduced to 82.79 % in 3 months and it was 68.82 % after 6 months (Table 2). Similarly the samples kept for real time studies showed reduction in activity up to 79.31 % after 3 months and the activity reduced to 21.48 % after 6 months.

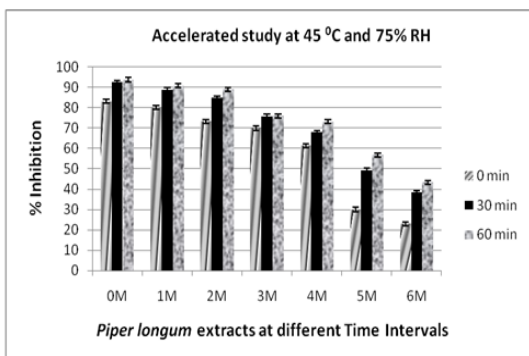


Figure 1 DPPH radical scavenging assay of *P. longum* extract at 517 nm

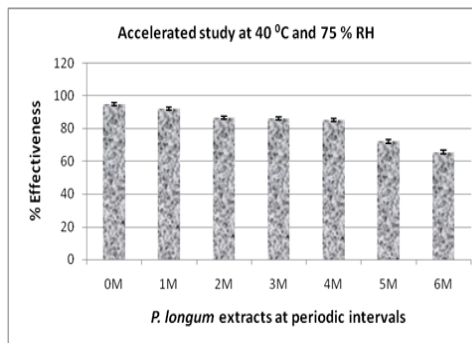


Figure 2 ABTS radical cation decolorization assay of *P. longum* extract at 745 nm

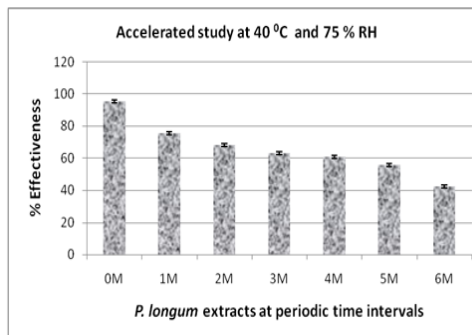


Figure 3 Deoxyribose degradation assay of *P. longum* extract at 532 nm

ABTS assay

Crude extract of PL at zero time interval showed 94.94 % inhibition of ABTS radical cation (Figure 2) In AS samples the activity reduced gradually from 91.99 % to 65.55% in 6 months duration while in case of LS the activity reduced from 92.64 % to 89.98 % within six months. In case of RT samples the activity reduced from 90.22 % to 66.44 % within a period of six months.

Deoxyribose degradation assay

Deoxyribose degradation assay for fresh sample of PL showed 95.36 % inhibition of H₂O₂ radical. The activity reduced in gradual manner from 75.62 % in one month to 62.99 % in 3 months and after 6 months the activity reduced to 42.53 % in case of AS samples whereas for LS sample, the activity reduced to 90.48 % in 3 months and there is a slight decrease up to 84.89 % in activity after 6 months. The activity of the real time study samples reduced up to 76.87 % within 3 months and it was 56.77% after 6 months.

Discussion

Oxidative stress is defined as the disturbance between pro-oxidant and antioxidant balance in favour of the increase level of the former in organs, tissues and cells. To counter this oxidative stress the natural antioxidative defence mechanism operates to detoxify or scavenge the ROS. However if the rate of production of ROS overcomes the rate of their consumption, the body need the supplementation of antioxidant from outside. Synthetic antioxidants such as BHA and BHT suffer from some disadvantage due to their toxicity at high dose [24]. It is therefore sensible to elucidate the detailed protective mechanism of human consumable medicinal plants for the biologically active compounds possessing intrinsic antioxidant activity therein. Phenols and polyphenols including flavanoids in wine, fruits and vegetables have been reported to exhibit a wide range of biological activities and their effects are mainly attributed to antioxidant properties that prevent free radical mediated cytotoxicity, lipid peroxidation and oxidation of low density lipoproteins. The ability of these compounds to scavenge ROS is well documented.

It is reported in literature that *P. longum* contains polyphenols and the quantity of polyphenols changes with extraction in different solvents [13]. The stability of the polyphenols is always important for the antioxidant activity. Temperature enhances the rate of degradation of ingredients due to increase in kinetic energy. Moisture content amplifies the rate of decomposition and makes the product susceptible to hydrolysis. In case of herbal crude material or extract it also facilitates the growth of microbes, which not only deteriorates the constituents but may produce toxic substances. Hence moisture content should be controlled in storage condition. Therefore in the present study we have stressed on change in quantity of total phenolic content of *P. longum* on storage at different conditions and their effect on free radical scavenging activity. The study indicates that total phenolic content does not change considerably in case of long term study which in turn shows its effect on free radical scavenging activity. In case of accelerated study samples, the TPC decreases rapidly due to which the antioxidant activity is also reduced in considerable amount as compared to RT and LS study samples. Study on evaluation of extracts of *Piper sarmentosum* for accelerated

stability by metabolic fingerprinting profiling also suggests that products made from this plant ought to be stored at room temperature, below 30 °C and 45 % relative humidity and excessive heating must be avoided during manufacturing process [25]. This result is in agreement with our observation.

Conclusion

The present investigation indicates that there is no major difference in total phenolic content of *P. longum* LS samples as compare to RT and AS samples. Antioxidant activity of the aqueous extract reduced slightly on keeping the plant material at different conditions but sample stored at LS conditions showed maximum total phenolic content and better antioxidant activity. Therefore, the use of either fresh sample or the sample stored at lower temperature and less humidity has been suggested for better efficacy of the plant material of pippali as a natural antioxidant.

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