

## Enzymatic and non-enzymatic antioxidants in selected *Piper* species

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*Piper* species, commonly used in diet and traditional medicine were assessed for their antioxidant potential. Catalase activity was predominated in *Piper longum*, followed by *Piper cubeba*, green pepper, *Piper brachystachyum* and *Piper nigrum*. *P. nigrum* was richest in glutathione peroxidase and glucose-6-phosphate dehydrogenase, green pepper was richest in peroxidase and vitamin C while vitamin E was more in *P. longum* and *P. nigrum*. *P. brachystachyum* and *P. longum* were rich sources of vitamin A. All the *Piper* species had GSH content of around 1 to 2 nM/g tissue. The antioxidant components of *Piper* species constitute a very efficient system in scavenging a wide variety of reactive oxygen species. Antioxidant potential of *Piper* species was further confirmed by their ability to curtail *in vitro* lipid peroxidation by around 30-50% with concomitant increase in GSH content.

Reactive Oxygen Species (ROS) are generated from leakage of electrons onto oxygen from mitochondrial electron transport chain, microsomal cytochrome P<sub>450</sub> and their electron donating enzymes and other systems<sup>1,2</sup>. For useful purposes, ROS (e.g., O<sub>2</sub><sup>•-</sup>, HOCl, and H<sub>2</sub>O<sub>2</sub>) are produced from activated phagocytes<sup>3</sup>. Inactivation and removal of ROS depend on reactions involving the antioxidative defense system. The endogenous antioxidant defense includes enzymatic (e.g. superoxide dismutase, catalase, peroxidase etc.) and non-enzymatic (e.g., ascorbic acid,  $\alpha$ -tocopherol, glutathione etc.) systems<sup>4</sup>.

Oxidative stress is a state of imbalance between generation of reactive oxygen species (ROS) like hydroxyl and superoxide radicals, and the level of antioxidant defense system. Oxidative stress results in the damage of biopolymers including nucleic acids, proteins, polyunsaturated fatty acids and carbohydrates. Lipid peroxidation (LP) is oxidative deterioration of polyunsaturated lipids and it involves ROS and transition metal ions<sup>5</sup>. It is a molecular mechanism of cell injury leading to generation of peroxides and lipid hydroperoxides which can decompose to yield a wide range of cytotoxic products, most of which are alde-

hydes, like malondialdehyde (MDA), 4-hydroxynonenal (HNE) etc.

ROS and free radical mediated processes have also been implicated in the pathogenesis of a variety of diseases like Alzheimer's disease, Parkinson's disease, atherosclerosis, cancer, liver damage, rheumatoid arthritis, immunological incompetence, neurodegenerative disorders<sup>6,7</sup> etc. Nutritional antioxidant deficiency also leads to oxidative stress<sup>8</sup>, which signifies the identification of natural antioxidative agents present in diets consumed by the human population. Pepper is widely incorporated in the diet of Asian and Western countries and it is also an important constituent of more than 150 Ayurvedic formulations. Piperine, a well identified alkaloid of *Piper* species, is reported to have a antimicrobial and hypoglycemic effects<sup>9</sup>. While *Piper* species are reported to have wide spectrum of biological activity, its antioxidant potential has not been established so far, making it important and interesting to study the antioxidant potential of *Piper* species.

The main objectives of the present study is to: (1) establish the antioxidant potential of the *Piper* species by evaluating both enzymatic and non-enzymatic antioxidants in selected *Piper* species; and (2) study the efficacy of *Piper* species in preventing *in vitro* lipid peroxidation using liver mitochondria as a model system.

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## Materials and Methods

**Preparation of the pepper extract**—The *Piper* species—*Piper nigrum*, *Piper cubeba*, *Piper longum* and *Piper brachystachyum* were obtained in the dried form from the ayurvedic drug shops. Green pepper, a fleshy form of *P. nigrum* was obtained freshly from the local vegetable market, and stored under refrigerated condition. The pepper species were weighed separately and homogenised with 50% ethanol at a concentration of 1g in 2ml. The extracts were centrifuged at 10,000 rpm for 10 min and the supernatants were kept under refrigerated condition and used for biochemical estimations within 4 hr.

**Biochemical estimations**—The activities of the antioxidant enzymes namely superoxide dismutase<sup>10</sup>, catalase<sup>11</sup>, peroxidase<sup>12</sup>, glutathione peroxidase<sup>13</sup>, glucose-6-phosphate dehydrogenase<sup>14</sup>, ascorbate oxidase<sup>15</sup>, and the non enzymatic antioxidants viz., glutathione<sup>16</sup>, ascorbic acid<sup>17</sup>, vitamin A<sup>17</sup> and E<sup>17</sup> were determined in the pepper extracts by suitable colorimetric procedures. The values are expressed as mean  $\pm$  SD. The data were statistically analysed by the method of Snedecor and Cochran<sup>18</sup>.

**Isolation of goat liver mitochondria**—The goat liver mitochondria was prepared according to the method of Li *et al.*<sup>19</sup>. Lipid peroxidation level in the mitochondria was measured as thiobarbituric acid reactive substances (TBARS) and diene conjugates, using the method of Buege and Aust<sup>20</sup>. *In vitro* lipid peroxidation was induced by the addition of 1.5mM FeSO<sub>4</sub>.

## Results and Discussion

The energetic benefit of aerobic metabolism is associated with the generation of reactive oxygen species which are implicated in variety of diseased conditions<sup>21</sup>. Diet contains several substances that are capable of scavenging ROS directly or indirectly by promoting mechanism which enhance detoxification<sup>22</sup>. Strong evidence suggests that consumption of fruits and vegetables results in decreased incidence of all types of cancer<sup>23</sup>. They are known to contain variety of non-enzymatic antioxidants, namely carotenoids, tocopherols, ascorbic acid and plant polyphenols, which exert their antimutagenic activity, even after subjected to the cooking process. Pepper which is widely included in the diet is evaluated for its antioxidant property in the present study.

**Enzymatic antioxidants in *Piper* species**—The level of antioxidant enzymes assessed in different *Piper* species are collectively represented in Table 1. The highest SOD activity was found in *P. longum* (23.79 units/mg protein) compared to the other species included in the present study implicating that this source could be exploited for commercial purification of SOD. SOD's are reported widely in plant sources and superoxide scavenging effect of fresh juice and methanolic extract of *Emilia sonchifolia* leaves was reported<sup>24</sup>. Alcoholic extract of *Hypericum perforatum* also showed reasonable superoxide anion scavenging activity<sup>25</sup>. Currently, purified SOD is therapeutically used in the treatment of antioxidative and anti-inflammatory diseases.

Table 1—Enzymatic antioxidants in *Piper* species  
[Values are means of 3 replicates in 2 repetitive experiments]

Sample	SOD		Catalase		Peroxidase		Glutathione peroxidase		Glucose-6-P dehydrogenase		Ascorbate oxidase	
	Units/mg protein	Units/g tissue	Units/mg protein	Units/g tissue	Units/mg protein	Units/g tissue	Units/mg protein	Units/g tissue	Units/mg protein	Units/g tissue	Units/mg protein	Units/g tissue
P1	9.14 <sup>c</sup>	9.96	3.72 <sup>a</sup>	4.05	31.57 <sup>d</sup>	34.40	1772.2 <sup>c</sup>	1931.6	54.45 <sup>c</sup>	59.30	0.014 <sup>a</sup>	0.015
P2	3.49 <sup>a</sup>	19.90	22.30 <sup>b</sup>	128.00	0.99 <sup>a</sup>	5.66	281.9 <sup>a</sup>	1612.5	17.61 <sup>c</sup>	100.70	0.056 <sup>c</sup>	0.320
P3	23.79 <sup>c</sup>	29.73	83.19 <sup>c</sup>	103.90	1.80 <sup>b</sup>	2.25	941.1 <sup>c</sup>	1176.4	6.07 <sup>a</sup>	7.58	0.123 <sup>d</sup>	0.154
P4	4.67 <sup>d</sup>	19.90	48.39 <sup>d</sup>	82.70	7.49 <sup>e</sup>	12.80	828.7 <sup>b</sup>	1417.1	12.21 <sup>b</sup>	20.80	0.032 <sup>b</sup>	0.054
P5	5.65 <sup>b</sup>	10.00	39.72 <sup>c</sup>	70.30	75.61 <sup>c</sup>	133.80	1285.7 <sup>d</sup>	2275.7	24.40 <sup>c</sup>	43.10	0.057 <sup>c</sup>	0.100
	SED = 0.05		SED = 0.19		SED = 1.94		SED = 0.0019		SED = 0.24		SED = 0.28	
	LSD(5%) = 0.10		LSD(5%) = 0.39		LSD(5%) = 4		LSD(5%) = 0.004		LSD(5%) = 0.50		LSD(5%) = 0.57	
	LSD(1%) = 0.13		LSD(1%) = 0.53		LSD(1%) = 5.41		LSD(1%) = 0.006		LSD(1%) = 0.68		LSD(1%) = 0.77	
	1 Unit = inhibition of 50% nitrite formation		1 Unit = 1μmole of H <sub>2</sub> O <sub>2</sub> consumed/min		1 Unit = 1μmole of pyrogallol oxidized/min		1 Unit = 1μg of GSH utilized/min		1 Unit = increase in O.D. of 0.01/min		1 Unit = 0.01 O.D. change/min	

In a column, means followed by a common letter are not significantly different at 5% level by DMRT.  
P1 : *Piper nigrum*; P2 : *Piper brachystachyum*; P3 : *Piper longum*; P4 : *Piper cubeba*; P5 : Green pepper

The highest activity of catalase observed in *P. longum* (83 units/mg protein) coincides very well with the highest activity of SOD noted in the same species, indicating that the  $H_2O_2$  formed by SOD is effectively removed by the catalase. Plant catalases are reported to be very sensitive to environmental conditions and has a rapid turnover rate<sup>26</sup>.

Among the five species studied *P. nigrum* showed highest GSH-Px activity (1772 units/mg protein) and it is interesting to note that the highest GSH-Px activity observed in *P. nigrum* was associated with very low catalase activity observed emphasizing that GSH-Px activity plays the major role in peroxide removal in this pepper species, whereas, the highest catalase activity in *P. longum* was associated with low activity of GSH-Px indicating the role for catalase in peroxide removal in *P. longum*. In green pepper and *P. cubeba*, both catalase and GSH-Px activity were reasonable, indicating a role for both the enzymes in peroxide removal. The presence of GSH-Px was also reported in cultured plant cells<sup>27</sup>. GSH-Px isolated from *Nicotiana glauca*<sup>28</sup> has shown homology to animal GSH-Px.

The ascorbate oxidase(AO) activity in *P. longum* was 0.123 units/g tissue which could be related with the highest SOD activity noted in this species. Different forms of AO has been reported in chloroplast, mitochondrial, cytosol, peroxisomes and glyoxysomes<sup>29</sup>. In the present study, the highest AO activity in *P. longum* emphasizes the role of ascorbate system in controlling  $H_2O_2$  concentration in this species. Green pepper was found to contain highest peroxidase activity (75 units/mg protein). This observation points out the importance of peroxidase system in fresh tissue rather than in dried fruits.

The observed high activity of glucose-6-phosphate dehydrogenase in *P. nigrum* and green pepper coincides very well with high GSH-Px activities. While GSH-Px is involved in the reduction of  $H_2O_2$  and organic peroxide, a continuous flow of reducing equivalents through glutathione system necessarily has to be balanced by continuous formation of NADPH maintaining the steady state. It is established by increased glucose 6-phosphate-dehydrogenase activity noted in the above species.

*Non-enzymatic anti-oxidants in Piper species*— Apart from enzymatic antioxidants, spectrum of non-enzymatic antioxidants namely vitamin A, C, E and glutathione are important in cellular system in curtailing ROS. The levels of these antioxidants were assessed and the results are represented in Table 2.

The vitamin C level was lowest in *P. brachystachyum* (0.88  $\mu\text{g/g}$  tissue) while highest level of 2.7  $\mu\text{g/g}$  tissue was observed in *P. longum*. Ascorbic acid is reported to be associated with better scavenging activities *in vivo* than the antioxidant enzymes, because they are present both intracellularly as well as in the extracellular fluid<sup>30</sup>. As an antioxidant, it is reported that ascorbate reacts with superoxide, hydrogen peroxide or the tocopheroxyl radical to form monodehydroascorbic acid and/or didehydroascorbic acid. The oxidised forms are recycled back to ascorbic acid.

The estimated vitamin E level in different *Piper* species ranges widely from 18 to 66  $\mu\text{g/g}$  tissue (Table 2). The antioxidant properties of tocopherol are result of its ability to quench both singlet oxygen and peroxides<sup>31</sup>. Within the membrane, tocopherol is the only protective agent that can act against the toxic effects of oxygen radicals<sup>32</sup>.

The vitamin A content in different *Piper* species ranges from 16 to 70  $\mu\text{g/g}$  tissue. *Piper brachystachyum* had the highest level, whereas lowest level was observed in *P. longum*. Carotenoids exhibit a central role against cancers, cardiovascular disease, HIV infection and other age-related disorders<sup>33</sup>. Recent reports suggests that all the carotenoids except  $\beta$ -carotene are very efficient antioxidants.

The glutathione values in *Piper* species varied from 0.92 to 1.8 nM/g tissue. Glutathione reduced the formation of toxic lipid peroxide and hydrogen peroxide in biological system by acting as substrate for glutathione peroxidase<sup>34</sup>. GSH can function as an antioxidant in the following ways:

- It can react chemically with singlet oxygen, superoxide and hydroxyl radicals and therefore function directly as a free radical scavenger.
- GSH may stabilize membrane structure by removing acyl peroxides formed by lipid peroxidation reactions<sup>35</sup>.
- GSH is the reducing agent that recycles ascorbic acid from its oxidized to its reduced form by the enzyme dehydroascorbate reductase<sup>29</sup>.

Upon comparison of antioxidant status of green pepper (fleshy form) and *P. nigrum* (dried form) the following results were observed:

- No significant change in total activity was observed for SOD and glucose-6-phosphate dehydrogenase, whereas specific activity increased by 1.6 fold in dried form.
- Both specific activity and total activity of catalase, peroxidase and ascorbate oxidase decreased in dried

form. Only 10-25% of activity was observed in dried form when compared to fleshy form.

- The flesh form of pepper is more predominant in vitamin C and GSH while dried form is enriched with vitamin C and E.

The above observed variation in antioxidant status could be attributed to different maturation stages of pepper fruit and also could be due to sun-drying process.

*Evaluation of efficacy of antioxidant potential of Piper species*—Peroxidation of membrane system are the foremost consequences of free radical damage and the efficiency of plant extracts in inhibiting lipid peroxidation *in vitro* is a very good measure of assessment of antioxidant potential. The presence of iron in the reduced form was found to be necessary

for lipid peroxidation and this was generally achieved by the addition of ferrous salts or by adding Fe<sup>3+</sup> and reducing agents like ascorbate<sup>26,36</sup>.

To evaluate the antioxidant potential of different *Piper* extracts, liver mitochondria was selected as model system based on the following considerations:

1. It has been well established that isolated mitochondria produces H<sub>2</sub>O<sub>2</sub> and O<sub>2</sub><sup>•-</sup> in presence of compounds like NADH, antimycin A, etc<sup>37</sup>.
2. Various Fe-S proteins and NADH dehydrogenase have also been implicated as possible sites of superoxide and H<sub>2</sub>O<sub>2</sub> formation.
3. Mitochondrial membrane was found to contain large amounts of PUFA in their phospholipid which include fatty acids with 2,4,5, and 6 double bonds. The rate of peroxidation both *in vivo* and *in*

Table 2—Non-enzymatic antioxidants in *Piper* species  
[Values are means of 3 replicates in 2 repetitive experiments]

Sample	Vitamin C (µg/g tissue)	Vitamin E (µg/g tissue)	Vitamin A (µg/g tissue)	GSH (nM/g tissue)
P1	1.68 <sup>c</sup>	66.98 <sup>c</sup>	43.24 <sup>c</sup>	0.92 <sup>a</sup>
P2	0.88 <sup>a</sup>	46.30 <sup>c</sup>	69.46 <sup>c</sup>	1.03 <sup>b</sup>
P3	1.72 <sup>c</sup>	63.62 <sup>d</sup>	63.64 <sup>c</sup>	1.18 <sup>c</sup>
P4	1.37 <sup>b</sup>	18.12 <sup>a</sup>	30.93 <sup>b</sup>	1.27 <sup>d</sup>
P5	2.70 <sup>d</sup>	38.95 <sup>b</sup>	16.81 <sup>a</sup>	1.84 <sup>c</sup>
SED	= 0.05	SED = 0.39	SED = 0.27	SED = 0.094
LSD(5%)	= 0.11	LSD(5%) = 0.81	LSD(5%) = 0.56	LSD(5%) = 0.01
LSD(1%)	= 0.14	LSD(1%) = 1.09	LSD(1%) = 0.76	LSD(1%) = 0.02

In a column, means followed by a common letter are not significantly different at 5% level by DMRT.

P1: *Piper nigrum*; P2: *Piper brachystachyum*; P3: *Piper longum*; P4: *Piper cubeba*; P5: Green pepper

Table 3—Effect of pepper extracts on Lipid peroxidation and reduced GSH content in the goat liver mitochondria  
[Values are means of 3 replicates in 2 repetitive experiments. Figures in parentheses are % inhibition of lipid peroxidation and thiol oxidation by the different pepper extracts]

Experimental group	TBARS* (µM/mg protein)	Conjugated dienes* (µmole/mg protein)	GSH** (µM/mg protein)
<b>Group I</b> Liver mitochondria	0.25 ± 0.001	57.725 ± 0.476	6.42 ± 0.32
<b>Group II</b> Liver mitochondria + 1.5 mM FeSO <sub>4</sub>	2.09 ± 0.013	318.16 ± 0.354	3.17 ± 0.10
<b>Group III</b> Liver mitochondria + 1.5 mM FeSO <sub>4</sub> + pepper extract			
<i>Piper nigrum</i>	1.480 ± 0.002 (29%)	124.18 ± 0.274 (61%)	5.27 ± 0.26 (66%)
<i>Piper brachystachyum</i>	1.235 ± 0.002 (41%)	125.23 ± 0.216 (60%)	5.24 ± 0.29 (65%)
<i>Piper longum</i>	1.700 ± 0.060 (50%)	149.42 ± 0.346 (50%)	4.87 ± 0.27 (54%)
<i>Piper cubeba</i>	0.860 ± 0.002 (59%)	188.17 ± 0.460 (40%)	5.01 ± 0.19 (58%)
Green pepper	1.010 ± 0.040 (50%)	157.36 ± 0.302 (50%)	4.93 ± 0.15 (55%)

\* The levels of TBARS and conjugated dienes were compared between Group II and Group III

\*\* GSH level was compared between Group I and Group III

*vitro* increase with increasing number of double bonds in fatty acids<sup>37</sup>.

4. Damage to mitochondria due to lipid peroxidation can have profound effect on the cell. Lipid peroxidation correlated well with swelling and finally with lysis and disintegration of mitochondria<sup>37</sup>.

Lipid peroxidation was induced in the model system by incubating the liver mitochondria in the presence of 1.5mM FeSO<sub>4</sub> for 30 min with and without different *Piper* extracts. In earlier studies for anti-lipidperoxidative property, the ED<sub>50</sub> value of 58 µg was reported for vitamin E<sup>25</sup>. Hence in the present study, the ethanolic extracts of *Piper* species were concentrated so as to give around 60µg of vitamin E in 0.5ml of the concentrated extract.

*Effect of Piper extracts on lipid peroxidation of liver mitochondria*—The effect of ethanolic extracts of different *Piper* species on *in vitro* lipid peroxidation of liver mitochondria was assessed by estimating both TBARS and diene conjugates. The results were represented in Table 3.

*Piper cubeba* was found to be more effective in curtailing lipid peroxidation by 59%, while the extent of inhibition was around 50% for *P. longum* and green pepper and around 41% for *P. brachystachyum*. *P. nigrum* was found to be less effective with only 29% inhibition. The observed high levels of TBARS in the model system in the presence of *P. nigrum* extract would be contributed by the inherent piperine content of the pepper extract. Khajuria *et al.*<sup>38</sup> also reported increased TBARS of rat intestinal mucosa and epithelial cells upon piperine treatment although conjugated diene levels were not altered.

The conjugated diene (initial peroxidative product and accurate indicator of lipid peroxidation) levels were also assessed in the present study. The extent of inhibition of lipid peroxidation exerted by different pepper extracts were ranged from 40-60%, the lowest being contributed by *P. cubeba* and highest by *P. nigrum* and *P. brachystachyum*. The lowest antioxidant efficacy of *P. cubeba* is correlated with the lowest level of vitamin E (18.12µg/g tissue) in this species, compared to that of vitamin E in *P. nigrum* (66.98 µg/g tissue).

The inhibition of *in vitro* lipid peroxidation by the pepper extracts observed in the present study can be attributed to the presence of known antioxidants like vitamin C, E, A and glutathione, and also the various enzymic antioxidants in the extracts. The observed effects could also be due to the presence of other

secondary metabolites in *Piper* species. The piperine content has been reported to vary from 1.9 to 3.1% (moisture free base), depending on fruit maturation stages. It is interesting to note that piperine, a principle alkaloid of *Piper* species increased the serum response i.e., the serum β-carotene level when it is supplemented with oral β-carotene<sup>39</sup>.

*Effect of Pepper extracts on GSH content during lipid peroxidation*—In the liver mitochondria, upon the induction of lipid peroxidation with Fe<sup>2+</sup>, GSH level decreased significantly by 50%, whereas when mitochondria was preincubated with pepper extracts, only around 18-25% depletion was seen. It is interesting to note that, even in the presence of promoters of lipid peroxidation, different pepper extracts protected the GSH depletion by 20%, when compared to that in their absence.

It has been reported that reduced GSH is a better index of assessing the lipid peroxidation than the quantification of TBARS<sup>40</sup>. An inverse relationship was established between TBARS and reduced GSH content. The protective effect of pepper extracts on lipid peroxidation with concomitant increase in GSH noted in the present study could be attributed to the inbuilt antioxidant system present in them. The results clearly pinpoints the antioxidant potential of different pepper species. *In vivo* studies should be carried out in the experimental animals in order to ascertain their antioxidant potential in biological system.

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