BENEFICIAL EFFECT OF *PHYLLANTHUS EMBLICA* FRUIT EXTRACT ON CIGARETTE SMOKE INDUCED IMPAIRED ANTIOXIDANT STATUS IN RATS

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Summary

Cigarette smoke contains oxidants which generate long lasting free radicals that cause oxidative stress in active and passive smokers leading to pathological conditions. Antioxidant nutrients are reported to prevent the oxidative damage induced by smoking. In the present study, we have evaluated the antioxidant role of *Phyllanthus emblica* (PE) fruit extract on cigarette smoke-induced oxidative stress in rats. Adult male albino rats were exposed to cigarette smoke and administered daily with PE fruit extract (Gr.A) or vitamin supplements (Gr.D). Another group of animals were exposed to only cigarette smoke (Gr.B) and control animals were subjected to sham session (Gr.C). Group A, B and D animals were kept in a smoke chamber for 30 min, twice a day for 4 weeks. Hemoglobin (Hb) % and malondialdehyde (MDA) content of RBC, lung and liver homogenate; superoxide dismutase (SOD), catalase (CAT), reduced glutathione (GSH) and glutathione –S-transferases (GST) of lung and liver homogenate of different treatment groups were estimated. Administration of PE fruit extract showed increase in the Hb level (+ 23.4 %) of Gr.A animals with concomitant decrease in MDA levels of RBC, lung and liver homogenate (48-54%, $p<0.001$) when compared to those of group B animals. The status of improvement of SOD, CAT, GSH and GST of lung and liver homogenate in PE fruit extract-administered group (Gr.A) ranged from 30 - 80% over the only smoke exposed group (Gr.B). PE fruit extract administered group showed better antioxidant profiles than that of the multivitamin supplemented group. The results of the present study suggest that *Phyllanthus emblica* fruit extract exerts its protective effect against cigarette-smoke induced oxidative damage through its potent antioxidant agents (gallo-ellagi tannoids).

Keywords: Cigarette smoke, Antioxidant status, *Phyllanthus emblica* fruit extract

Introduction

Cigarette smoking has been implicated as a significant risk factor in causing and in progression of several diseases, including atherosclerosis, chronic emphysema, chronic obstructive pulmonary disease and cancer[1]. There is overwhelming evidence from epidemiological studies worldwide showing deaths due to cardiovascular diseases linked to cigarette smoking. Several other studies also showed increased incidence of death from bronchitis and emphysema in smokers compared to that of non-smokers[2]. Although, the underlying mechanisms involved in the pathogenesis of diseases associated with smoking may be different, involvement of reactive oxygen species seems to have a definite role[3]. Tobacco smoke contains numerous compounds, many of which are oxidants and pro-oxidants, capable of producing free radicals and enhancing oxidative stress *in vivo*[4,5]. The increased oxidant burden is derived from the fact that each puff of tobacco smoke contains an estimated 10^{14} free radicals, most of these are tar semiquinones which can generate H_2O_2 and are long lasting[6,7]. Tar quinones can also react with oxygen and increase the generation of superoxide radicals in intracellular redox cycling reactions. H_2O_2 and superoxide radicals can act as prominent risk factors for increased lipid peroxidation in smokers[8]. Earlier studies have shown that smoking alters metabolism of trace elements[9] and thereby affects antioxidant enzyme activities that require trace elements as essential components.

There are many intrinsic radical scavenging systems in our body which involve enzymatic and non-enzymatic reactions. When there is excessive and chronic addition of free radicals [Reactive Oxygen Species (ROS) and/or Reactive Nitrogen Species (RNS)] from exogenous sources, the available antioxidant systems become unable to neutralize such abundant and pernicious radicals leading to oxidative damage of the tissues and manifestation of pathophysiological syndromes. Natural antioxidants present in foods of plant origin that scavenge reactive oxygen species may be of great value in preventing the onset and occurrence of oxidative stress and its related diseases[3].

Phyllanthus emblica L. (syn. *Emblica officinalis* Gaertn; Euphorbiaceae), commonly known as *Amla* in India, has played an important medicinal role for centuries in Ayurveda. The fruits of the plant are recommended as a *rasayana*[10], which promotes health and longevity by increasing defense against diseases, arresting the aging process and revitalizing the body in debilitated conditions[10]. *Phyllanthus emblica* is used for the treatment of liver disorders, indigestion, stomach ulcers, diabetes, inflammatory diseases, inhibition of tumor growth and in geriatric complaints. It also functions as potent antioxidant agent[11].

The aim of the present study was to evaluate the effectiveness of prolonged administration of a standardized fruit pulp extract of *Phyllanthus emblica* in improving the antioxidant profile of rats, chronically exposed to cigarette smoke with altered health status. The effectiveness of PE fruit extract was compared with a well established marketed multivitamin-multimineral formulation using the same protocol.

Materials and Methods

Phyllanthus emblica (PE) *fruit extract — Phyllanthus emblica* fruit extract was prepared by heating the fresh fruit pulp in water, containing,1%NaCl (w/w), for 1 hour in a steam bath at 70±5ºC. The mixture is then filtered and immediately spray dried. An off-white free flowing powder, nearly 6% of the starting material, was obtained and designated as PE fruit extract and stored in a desiccator. The bioactive components of the PE fruit extract were characterized and estimated as before[12].

Multivitamin supplement- A commercially available multivitamin-multimineral capsule (Becadexamin®, Glaxo-SmithKline, batch no. BC 900) containing Vitamins A, D_3, E, B_1, B_2, Nicotinamide, B_6, Folic acid, B_{12}, C and minerals viz., Ca, Cu-sulphate, Mn- sulphate, Zn-sulphate, KI and light MgO was used as the comparator drug.

Animals and treatment- Male albino rats (Sprague Dawley strain) procured from Central Research Institute (Ayurveda), Govt. of India, Salt Lake City, Kolkata, were used in the study. The animals, weighing 180 to 200 gm, were divided into four groups, each containing 6 male rats and housed in colony cages maintained at an ambient temperature of 25°C ± 2°C with 12 hours light and dark cycle. The study was conducted in accordance with Good Laboratory Practice (GLP) Regulations of the WHO (WHO Document, 1998). The "Principles of laboratory animal care" (NIH Publication # 85-23, 1985) were also followed in this study. The 'Institutional Animal Ethics Committee' approved the present experimental protocol. All animals except group C were exposed to cigarette smoke (Cigarette without filter; 1.2mg nicotine and 12 mg tar/cigarette) in a ventilated chamber measuring 60 x 45 x 35 cm. Smoke was continuously generated by hanging 4 lit cigarettes at a time through a hook attached inside the chamber roof and rats were exposed to smoke for 30 min, twice a day, at an interval of 6-8 hours, 6 days a week, for 4 weeks. A tiny fan was attached inside the chamber to circulate the smoke uniformly. Control animals (Group C) were kept in a similar chamber for similar time- period everyday. For the rest of the time the animals were put in normal cages. PE extract or vitamin at a dose of 200 mg/kg was administered orally (the dose was calculated on the basis of an equivalent human dose) to Group A and Group D animals, respectively, before introducing to the first smoke exposure everyday. Group B and C animals received an equal volume of distilled water (per oral) every day. The study was continued for 28 days. One hour after the last smoke exposure, citrated non-clotted blood was collected by puncturing the orbital plexus and animals were sacrificed by exposure to an overdose of ether. The lungs and liver tissues were dissected out and were rinsed with cold phosphate buffer (PB, 100 mM, pH 7.4) and stored at -20 ºC.

Red blood cells were separated by centrifugation from whole blood and packed cell volume (PCV) was prepared, adjusted to 10 % v/v with PBS. The stored tissues were homogenized and the homogenate was centrifuged at 10,000 x g for 10 minutes at 4°C. The supernatant was stored at -20 ºC for further biochemical estimations.

The content of MDA, the marker of lipid peroxidation, of RBC, lung and liver homogenate was measured using thiobarbituric acid (TBA)[13]. Endogenous activities of antioxidants e.g. SOD[14], CAT[15], GSH[16], GST[17] of lung and liver homogenates were estimated.

Statistical analysis – Statistical analysis was carried out using SPSS software (Version7.5; Chicago, Illinois, USA). All data were expressed as mean ± SD. Groups of data were compared with repeated measure one way analysis of variance (ANOVA) followed by post hoc comparison between groups by Bonferroni test. Values were considered statistically significant at $p<0.05$.

Results

PE fruit extract-administered rats (Gr.A) showed significantly higher Hb% (+ 23.4%, $p<0.001$) than that of multivitamin supplemented animals (Gr.D: + 12.1%, $p=0.018$), each compared to that of the animals exposed to only cigarette smoke (Gr.B) (Tables 1 and 2). A significant decrease in Hb% (-24.9%, $p<0.001$) was observed in the animals exposed to cigarette smoke (Gr.B) compared to control (Gr.C).

In group B animals, lipid peroxidation (measured by MDA levels), of RBC (+ 219.6%, $p<0.001$), lung (+142.9%, $p<0.001$) and liver (+177.9%, $p<0.001$) homogenates were found to be significantly higher compared to those of the control (Gr.C). Significant decrease in MDA levels of RBC (-53.9%, $p<0.001$), lung (-48.3%, $p<0.001$) and liver (- 54.4%, $p<0.001$) homogenates were observed in PE fruit extract treated animals (Gr.A), which were also more pronounced than those of the multivitamin treated animals (Gr.D) (RBC - MDA: – 37.9%, $p<0.001$; lung-MDA: -32%, $p<0.01$ and liver-MDA: - 42.6%, $p<0.01$) (Tables 1 and 2).

Significant decrease in the levels of endogenous antioxidants, e.g. SOD (lung: -41.3%, $p<0.001$; liver: - 47.2%, $p<0.001$), CAT (lung: -41.5%, $p<0.001$; liver: - 46.0%, $p<0.001$), GSH (lung: -52.8%, $p<0.001$; liver: - 34.1%, $p=0.001$) with concomitant increase in GST (lung: +47.6%, $p=0.001$; liver: +64.0%, $p=0.001$) were observed in group B animals compared to those of the healthy control group (Gr.C) (Tables 1 and 2). Smoking induced reduction in the activities of antioxidant enzymes were significantly attenuated in animals treated with PE fruit extract (SOD-lung: +59.5%, $p<0.001$; liver: + 82.1%, $p<0.001$; CAT-lung: +33.3%, $p=0.132$; liver: + 55.5%, $p=0.007$; GSH-lung: +62.8%, $p<0.001$; liver: + 42.2%, $p=0.005$ and GST-lung: -29.0%, $p=0.003$; liver: -29.3%, $p=0.005$). A similar trend of improvement, but of lesser magnitude, was observed in multivitamin treated (Gr.D) animals (SOD-lung: +22.6%, $p=0.414$; liver: + 47.1%, $p=0.016$; CAT-lung: +25.5%, $p=0.401$; liver: + 44.1%, $p=0.044$; GSH-lung: +39.9%, $p=0.015$; liver: + 21.1%, $p=0.383$ and GST-lung: -25.8%, $p=0.004$; liver: - 21.9%, $p=0.128$) (Tables 1 and 2).

Table 1: Cigarette Smoke induced changes in Hb (%) and oxidative stress-related indices of albino rats of different treatment groups

Parameters	Group A	Group B	Group C	Group D
Hb (%):	15.38 ±0.75	12.46 ± 0.69	16.6 ± 0.83	13.98±0.82
MDA:				
RBC (nM/ml)	1.62±0.37	3.52±0.45	1.1±0.22	2.18 ± 0.39
Lung (nM/g tissue)	13.63 ± 4.02	26.35 ± 4.61	10.85 ± 1.26	17.92 ± 3.46
Liver (nM/g tissue)	15.9 ± 4.19	34.88 ± 7.53	12.55 ± 1.79	20.03 ± 2.63
SOD (U/g tissue /min)**:**				
Lung	276.0 ± 29.9	173.0 ± 36.0	294.5 ± 42.3	212.2 ± 31.8
Liver	386.3 ± 73.7	212 .2 ± 31.8	402.2 ± 52.9	312.2 ± 31.8
GSH (nM/g tissue)**:**				
Lung	2.98 ± 0.31	1.83 ± 0.24	3.88 ± 0.39	2.56 ± 0.48
Liver	4.38 ± 0.71	3.08 ± 0.32	4.67 ± 0.59	3.7 ± 0.60
GST (U/g tissue)**:**				
Lung	0.022 ± 0.004	0.031 ± 0.004	0.021 ± 0.004	0.023 ± 0.004
Liver	0.029 ± 0.007	0.041 ± 0.007	0.025 ± 0.005	0.032 ± 0.004
CAT (U/mg tissue /min)**:**				
Lung	1.20 ± 0.23	0.89 ± 0.2	1.5 3 ± 0.18	1.133 ± 0.23
Liver	3.53 ± 0.47	2.26 ± 0.47	4.2 ± 0.80	3.26 ± 0.50

Values are Mean ± SD; n=6 rats in each group. Group A: cigarette smoke + PE fruit extract (200 mg/kg), /day Group B: Only cigarette smoke, Group C: Control and Group D: cigarette smoke + vitamin (200 mg/kg).

Table 2: Percentage improvement of Hb and oxidative stress-related indices of rats of treatment groups compared to rats exposed to cigarette smoke only (Group B) and vehicle control group (Group C) .

Parameters	Group A vs Group B		Group D vs Group B		Group B vs Group C		Group A vs Group C		Group D vs Group C		Group A vs Group D	
	%Δ	*p*	%Δ	*p*	%Δ	*p*	%Δ	*p*	%Δ	*p*	%Δ	*p*
Hb (%):	+ 23.4	<0.001	+ 12.1	0.018	- 24.9	<0.001	- 7.3	0.08	- 15.8	<0.001	+ 10.0	0.032
MDA:												
RBC (nM/ml)	- 53.9	<0.001	- 37.9	<0.001	+ 220.0	<0.001	+ 46.4	0.15	+ 98.2	<0.001	- 25.7	0.094
Lung (nM/g tissue)	-48.3	<0.001	- 32.0	0.003	+ 142.9	<0.001	+ 25.6	1.000	+ 65.1	0.016	- 23.9	0.305
Liver (nM/g tissue)	-54.4	<0.001	- 42.6	<0.001	+ 177.9	<0.001	+ 26.7	1.000	+ 59.6	0.063	- 20.6	0.809
SOD (U/g tissue /min)**:**												
Lung	+ 59.5	<0.001	+ 22.6	0.414	- 41.3	<0.001	- 6.3	1.000	- 27.9	0.004	+ 30.1	0.032
Liver	+ 82.0	<0.001	+ 47.1	0.016	- 47.2	<0.001	- 3.9	1.000	- 22.4	0.035	+ 23.8	0.117
GSH (nM/g tissue)**:**												
Lung	+ 62.8	<0.001	+ 39.9	0.015	- 52.8	<0.001	-23.2	0.002	- 33.8	<0.001	+ 15.9	0.382
Liver	+ 42.2	0.005	+ 21.1	0.383	- 34.1	0.001	- 6.2	1.000	- 20.1	0.064	+ 17.4	0.383
GST (U/g tissue)**:**												
Lung	- 29.0	0.003	- 25.8	0.004	+ 47.6	0.001	+ 4.8	1.000	+ 9.5	1.000	- 4.4	1.000
Liver	- 29.3	0.019	- 21.9	0.128	+ 64.0	0.001	+ 16.0	1.000	+ 28.0	0.284	- 9.4	1.000
CAT (U/mg tissue /min)**:**												
Lung	+ 33.3	0.132	+ 25.5	0.401	- 41.5	<0.001	- 21.6	0.078	- 26.1	0.109	+ 5.9	1.000
Liver	+ 55.5	0.007	+ 44.1	0.044	- 46.0	<0.001	- 15.9	0.365	- 22.1	0.069	+ 8.0	1.000

p values were obtained by ANOVA followed by *post hoc* comparison between groups by Bonferroni test. n=6 rats in each group.

Discussion

Oxidative damage is one of the deleterious effects of habitual cigarette smoking. Passive smokers are also exposed to the harmful effects of tobacco smoking[19]. Tobacco smoke contains polycyclic aromatic hydrocarbons, reactive oxygen species (ROS), cadmium and nicotine that could initiate or amplify oxidative damage. In the gas phase, short-lived oxidants such as $O_2^{-\cdot}$ and NO are found which immediately react to form highly reactive peroxynitrite ($ONOO^-$) molecule[20]. In the last couple of years, several attempts have been made to identify natural source of antioxidant, in the form of **Superfruit,** having exceptional nutrient richness, antioxidant quality and appealing taste, which can be effectively used to prevent oxidative stress induced altered health status including cigarette smoke. Several such fruits have already been identified in western world which includes *Morinda citrifolia, Euterpe oleracea, Hippophae rhamnoides, Punica granatum, Vaccinium macrocarpon* etc.

Phyllanthus emblica (PE) fruits have been used for thousands of years, in Ayurveda, for their preventive, curative and health restorative properties. PE fruits possess strong antioxidant properties due to the presence of high amounts of low and medium molecular weight hydrolysable (gallo-ellagi) tannoids (65-70%). These tannoids produce a sustained and cascading vitamin C-like effect without the pro-oxidant activity found in vitamin C[12]. The bioactives of PE fruit extract are excellent quenchers of free radicals and non-radicals, such as, hydroxyl, superoxide anion, peroxyl, peroxynitrile and singlet oxygen[11,12,21]. PE fruit extract inhibits lipid peroxidation and boosts body's antioxidant defense enzymes, superoxide dismutase, catalase and glutathione peroxidase[22].

In the present study, we compared the protective effect of PE fruit extract with that of a marketed multi-vitamin supplement against smoking- induced oxidative damage using a suitable animal experimental model.

A highly significant deterioration antioxidant status of rats after sub-chronic exposure to cigarette smoke (Group B) was observed when compared to control animals. These changes were manifested by marked increase in MDA levels of RBC and GST activity in lungs and liver homogenate and concomitant decrease in Hb%, SOD, CAT, GSH level.

MDA levels have been used as a convenient index of the lipid peroxidation related oxidative damage of tissues. Several studies in animal models have indicated that chronic exposure to cigarette smoke increased the MDA levels of RBC as well as other tissues, mainly due to oxidative stress, compared to that of control animal[23]. In the present study, animals administered with PE extract significantly reduced the MDA levels, both in RBC and other tissues, compared to those of only smoke exposed animals, presumably by reducing oxidative burden by its strong cascading antioxidant action. Multivitamin supplementation also improved the antioxidant status of smoke-stressed rats but to a lesser extent than that exhibited by PE fruit extract.

Superoxide dismutase (SOD) and catalase (CAT) are present in various compartments of animal and human body. Some investigators reported compensatory increase in SOD and/or CAT activity in animals as well as in humans after cigarette smoking[24]. Many studies have, however, reported that this increment is transient and antioxidant enzymes may be down regulated during excess and/or chronic oxidant exposure[25]. MDA, a byproduct of lipid peroxidation, may also decrease enzyme activity by oxidizing the active site or by forming protein cross-links[26]. In the present study, we observed significant decrease in SOD and CAT activities in lungs and liver homogenate of cigarette smoke exposed animals compared to those of healthy control animals. PE fruit extract administration significantly increased both lungs and liver SOD levels nearly to the normal values. Supplementation of multivitamin could partly reverse this decrease in SOD level. Similar trend of increment in CAT activity was observed in the present study after the PE fruit extract administration.

GSH is an important protective antioxidant in the lungs and its levels are decreased following inflammation and oxidative stress of lungs increasing the potential for damage to the underlying epithelial cells both *in vivo* and *in vitro*. In alveolar type II cells, it was observed that incubation with GSH, increased intracellular GSH levels and thereby protected the cell against oxidative stress[27]. GSH converts lipid and hydrogen peroxides to nontoxic hydroxy fatty acids and water, respectively. The level of GSH in tissues decreases to neutralize excessive lipid peroxidation due to reactive oxygen species, generated during exposure to chronic cigarette smoke. Our findings suggested that supplementation with PE fruit extract decreased the oxidative stress produced by cigarette smoking and thereby reduced their damaging effects on vital organs, particularly lungs, which was reflected by increased levels of GSH in PE fruit extract treated group. Apart from radical scavenging activity of the phenolic antioxidants, the major bioactives of PE fruit extract can also modulate expression of the rate limiting enzyme for the synthesis of GSH[28]. Therefore, in addition to the radical scavenging abilities of PE extract, its bioactives may provide additional protection from oxidant-induced injury by increasing the systemic levels of GSH and related enzymes.

Glutathione-S transferases (GSTs) occur in a variety of organisms and are present in almost every tissue. These enzymes are principally involved in the detoxification processes by catalyzing the reactions in which GSH is conjugated with electrophiles to form a thioether[29]. Oxidative stress induced toxicity initially (acute stage) increases GSTs activity and thereby off-sets the pathogenic role of reactive species. Supplementation of PE fruit extract during smoking exposure reversed this enzyme level significantly and the effect was even better than that of the multivitamin supplementation.

The beneficial effects of dietary intake of potent antioxidants, e.g. PE fruit extract on smoking induced injuries can be manifold. It can scavenge reactive ROS and RNS by its cascading antioxidant effect and thereby minimize their deleterious effects on proteins, lipids and nucleic acids in cells. Additionally, such type of polyphenols (as in PE fruit extract) may augment the capacity of endogenous antioxidant defenses and modulate the cellular redox satus[30]. Multivitamin-multimineral supplementations, in contrast, can improve the redox status to a limited extent; also, their long term administration may not be free from health hazards[31].

The above findings would seem to suggest that standardized PE fruit extract[11, 12] may provide surveillance against oxidative stress in habitual human smokers. Work in this direction is currently in progress.

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