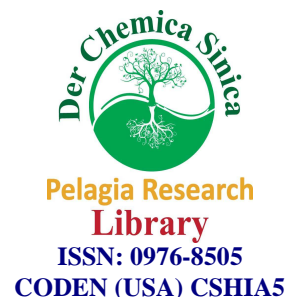




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Impact of ethanolic extract of *Boerhaavia diffusa* on lipid peroxidation and antioxidant status of novice smokers

Amir Khan^{*1}, Fouzia Ishaq² and Deepti Malhotra³

¹Department of Biotechnology & Biochemistry, Division of Life Science, Sardar Bhagwan Singh Post Graduate Institute of Biomedical Sciences & Research Balawala, Dehradun, UK, India

²Department of Zoology & Environmental Science, Gurukul Kangri University, Haridwar, Uttarakhand, India

³Department of Biotechnology, Shri Guru Ram Rai (P.G) College, Dehradun, UK, India

ABSTRACT

Smoking is responsible for the death of about 3.5 million subjects every year, 10,000 deaths per day. Smoking is a major risk factor for cancer, heart and lung diseases. The smoke is able to cause tissue oxidative damage at various levels. *Boerhaavia diffusa* (Spreadind Hogweed) is a medicinal plant which belongs to the family Nyctaginaceae is traditionally known as Punarnava in India. In this study we investigated the efficacy of antioxidative power of *B. diffusa* by analyzing all the parameters in plasma, VLDL-C, LDL-C, HDL-C, HDL2-C, HDL3-C, LDL-Protein, HDL-Protein, VLDL-Protein, MDA, CD and invitro oxidizability of LDL in absence and presence of *B. diffusa*.

Keywords: *Boerhaavia diffusa*, lipid peroxidation, total antioxidant power.

INTRODUCTION

WHO ranks smoking among the 10 greatest risks to health [1] and estimates about 1 billion men and 250 million women currently smoke cigarettes. At present, approximately 5 million people die each year from tobacco related illness and this figure is estimated to rise to about 10 million by 2025, most of the increase being in third world [2]. WHO indicates 47% of men, 12% women being smokers and smoking is responsible for the death of about 3.5 million subjects every year, 10,000 deaths per day. Smoking is a major risk factor for cancer, heart and lung diseases [3]. For every one person who dies of smoking attributable diseases, 20 more are suffering with atleast one serious smoking related illness [3]. Cigarette smoking is a mixture of over 4000 chemicals containing bioactive substances [4]. One puff of a cigarette exposes the smoker to more than 10^{15} free radicals and other oxidants and additional free radicals and oxidants are found in the tar of a cigarette [5]. Further damage may be caused by the endogenous formation of oxidants which affect the inflammatory immune response [6]. The burning of tobacco at temperature of 830-900° C leads to the production of about 5000 already identified toxic substances of heterogeneous mixture containing gaseous phase like CO, Nitrogen Oxides, Nitrosamine etc and solid phase which are products of pyrolysis like nicotine, phenols, aromatic polycyclic hydrocarbons in addition to free radicals [7]. The solid phase contains relatively stable free radicals while gaseous one contains small free radicals of oxygen, carbon and sulphur, high concentration of nitric acid and aldehyde moieties. The smoke is able to cause tissue oxidative damage at various levels [8] and contributes significantly to the appearance of endothelial dysfunction and to the alterations which induce arteriosclerosis [9]. Increase in lipid per oxidation products [10] particularly important for increase in LDL oxidation [11] accompanied by a decrease in HDL cholesterol level is reported [12]. Tobacco is associated in

the active smoker with the occurrence of ischemic cardiopathy, acute myocardial infarction [13], sudden coronary death, arterial hypertension [14], atherosclerosis [15] and in passive smoker with increased prevalence of CVD [16]. The term antioxidant refers to any molecule capable of stabilizing the deactivation of free radicals before they attack the cells. *In vitro*, LDL can be modified oxidatively in the presence of Transition metals such as iron and copper. LDL oxidized by the cell-free system is physiochemically and biologically indistinguishable from that by cellular system [17]. *In vitro* LDL oxidation is widely done through the measurement of CD(conjugated diene) and MDA(Malondialdehyde) formation [18]. *Boerhaavia diffusa* (Spreadind Hogweed) is a medicinal plant which belongs to the family Nyctaginaceae is traditionally known as Punarnava in India. The plant was named in the honour of its discoveror, Hermann Boerhaave in 18th century[19]. The roots, leaves, aerial parts or the whole plant of *B. diffusa* have been employed for the treatment of various disorders in the Ayurvedic herbal medicine. The first pharmacological studies have demonstrated that the roots of punarnava exhibit a wide range of properties: anti-inflammatory[20] [21], diuretic[22], blood pressure[22], laxative[23], anti-urethritis[24], antifibrinolytic[25], anticonvulsant[26], antibacterial[27], antihepatotoxic[28], diuretic[29], flower and seeds are used as a contraceptive[23], jaundice[30]. The plant is reported to be efficient for the treatment of tumors and cancer, to decrease the albumin urea, increase serum protein and lower serum cholesterol level [31]. Recent studies demonstrated that the leaves of *B.diffusa* reduce glucose level in blood. Ethanolic extract of *B. diffusa* showed immunosuppressive activity on human cells [32]. In this study we investigated the efficacy of antioxidative power of *B. diffusa* by analyzing all the parameters in plasma, VLDL-C, LDL-C, HDL-C, HDL2-C, HDL3-C, LDL-Protein, HDL-Protein, VLDL-Protein, MDA, CD and invitro oxidizability of LDL in absence and presence of *B. diffusa*.

MATERIALS AND METHODS

Chemicals: All chemicals used for this study were of analytical grade and obtained from HIMEDIA (India), Sisco (India), Ashirwad (India), Sigma-Aldrich (USA), Miles (USA) etc. All the Glasswares used were of borosil company and plastic wares from MS Tarson and Himedia. Micropipette from Eppendorf company, India. Instruments used during study were Electronic balance, pH meter, centrifuge, spectrophotometer, incubatory rotatory shaker, soxhlet apparatus, autoclave, deep freezer, refrigerator, magnetic stirrer, hot air oven, water bath etc.

Experimental Design: The research was carried out at the Department of Biotechnology and Pharmaceutical Chemistry, UCST, Dehradun. Normal control and Young smokers subjects were recruited from college campus. All the subjects where ethnically homogenous with similar nutritional habits free from alcohol consumption and were drinking maximum 3-2 cups of tea a day, had no vitamin intake 3 months before the initiation of the study. During recruitment the blood was drawn from the subjects in the morning after all night fasting, transferred into a heparinized glass tube. Plasma was collected by centrifugation and used for the analysis of Glucose, TG, TC, VDL-C, LDL-C, HDL-C and its sub fractions, HDL2-C and HDL3-C.

250g of powdered dried roots of *B. diffusa* were taken for extraction using 1lt ethanol, methanol and aqueous medium through soxhlet apparatus.

Collection of Blood and Plasma: At the end of the experiment treatment, overnight fasted subjects in each group were anaesthetized and blood drawn from cardiac puncture. The blood from each patient in a given group was collected in heparinized tubes, mixed gently by inversion 2-3 times and incubated at 4o C for 2-3 hrs. Plasma was separated from the blood by centrifugation at 25000 rpm for 30 min, aliquoted and either stored at 4°C or frozen at -20°C for use in other experiments.

Estimation: Determination of Nicotine [33], Carbon mono-oxide saturation [33], Plasma Triglycerides [34], Fractionation of plasma lipoproteins such as LDL [35], HDL and its subtractions HDL₂ and HDL₃ [36], Protein estimation [37], Plasma FRAP [38]. LDL oxidation in presence and absence of Vit. C [18] [39], Lipid peroxidation[40], ThioBarbituric Acid reactive substances (TBARS) in LDL [41].

Statistical evaluation: This was done by employing two-tailed Student t-test [⁴²]. P value less than 0.02 were considered significant.

RESULTS

Measurement of physiological parameter of non smokers and young smokers: The results for the physiological parameters of age, height, weight for male and females of both normal and young smokers shown in Table 1, do not have any significant difference. This depicts that they do not have any significant role in deciphering the smokers from the normal healthy persons.

TABLE 1: Measurement of physiological parameter of non-smokers and novice smokers

Parameters	Non-smokers	Young-Smokers
Number	9	11
Male	5	11
Female	4	-
Age (yrs)	19.77±0.186*	22±0.27*
Weight (kg)	52.55±0.15*	63.55±0.15*
Height (cm)	165.66±0.19*	167.63±0.17*
Number of cigarettes/day	-	7±0.301*
Smoking history (yrs)	-	3±0.30*
Smoking Index**	-	21±0.213*

*Values are mean±S.D. from all groups of subject **Number of cigarettes per day ×smoking history

Measurement of nicotine, carbon mono-oxide saturation and lipid profile of non-smokers and novice smokers: As shown in Table 2, the results show a modest and significant increase in blood nicotine and carbon mono-oxide. Plasma TC, VLDL-C, LDL-C, HDL-C, HDL₂-C, HDL₃-C and Non HDL-C levels in smokers as also observed to increase in smokers compared to non smokers as seen in Fig. 1. This maybe due to markedly increased production of oxidant and significantly diminished antioxidant defence. The results are in accordance to the reports of Science Daily. Similar results have been produced for increase in HDL-C and its subfractions HDL₂-C; HDL₃-C conc. [42] Increase conc of VLDL-C, LDL-C and TC has been also reported[43] .

Table 2.Measurement of Average value of nicotine, Sco%, TC and Lipid Parameters

Parameters	Non-smokers	Young-Smokers
Nicotine (µg/ml)	2.20±0.06*	5.32±0.07* (+145.82%)
Carbon monooxide saturation (Sco%)	1567.21±0.05*	1701.32±0.066* (+8.05%)
TC (mg/dl)	137.96±0.163*	215.002±0.01* (+55.84%)
VLDL-C(mg/dl)	17.126±0.021*	29.22±0.036* (+70.61%)
LDL-C(mg/dl)	88.60±0.13*	146.002±0.004* (+66.66%)
Non-HDL-C(mg/dl)	105.735±0.17*	160.61±0.15* (+51.89%)
HDL-C(mg/dl)	32.55±0.04*	54.38±0.08* (+68.75%)
HDL ₂ -C(mg/dl)	10.077±0.022*	19.14±0.002* (+89.93%)
HDL ₃ -C(mg/dl)	21.148±0.043*	34.24±0.042* (+61.90%)
MDA (Plasma)	0.4515±0.01*	0.8191 ±0.005* (+81.41%)

*Values are mean±S.D. from all groups of subject. TC (Total Cholesterol), VLDL-C (Very Low Density Lipoprotein, LDL (Low Density Lipoprotein), Non-HDL value= TC-HDL-C

MEASUREMENT OF LIPID PROTEINS: As shown in Fig. 2, VLDL-Protein significantly increased while LDL-Protein and HDL-Protein significantly decreased for young smokers compared to control value. Similar results have been reported [44] [45].

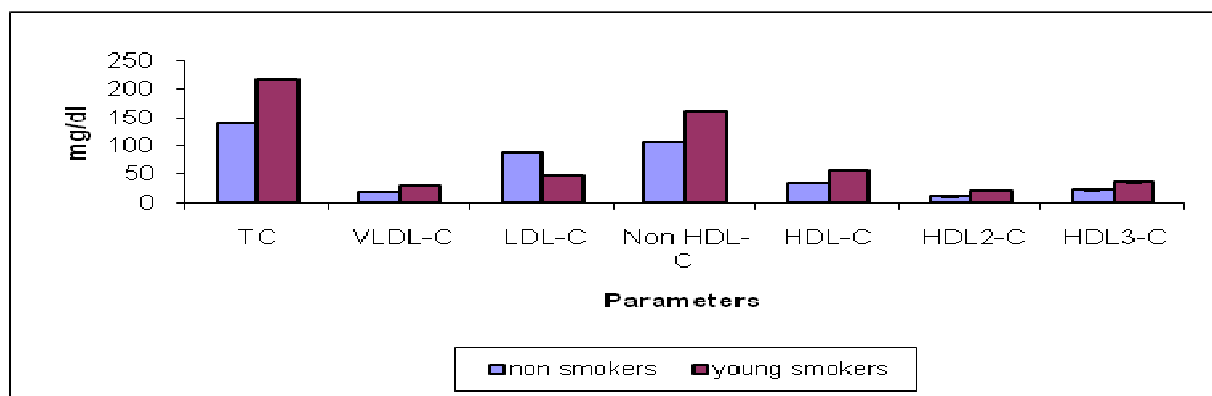


Fig. 1 Comparison of Lipid profile of Non smoker and Smoker

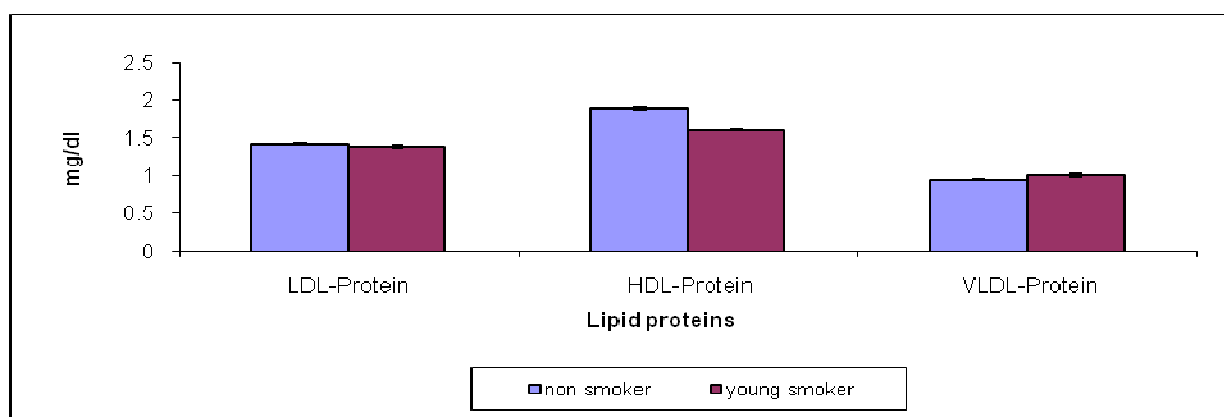


Fig. 2 Measurement of Lipid Proteins

ESTIMATION OF RATIOS OF LDL-C/HDL-C, LDL-C/TC, HDL₂-C/HDL₃-C and TC/HDL-C: Table 3 depicts the ratios of LDL-C/HDL-C and TC/HDL-C to decrease while LDL -C/TC and HDL₂-C/HDL₃-C to show increase.

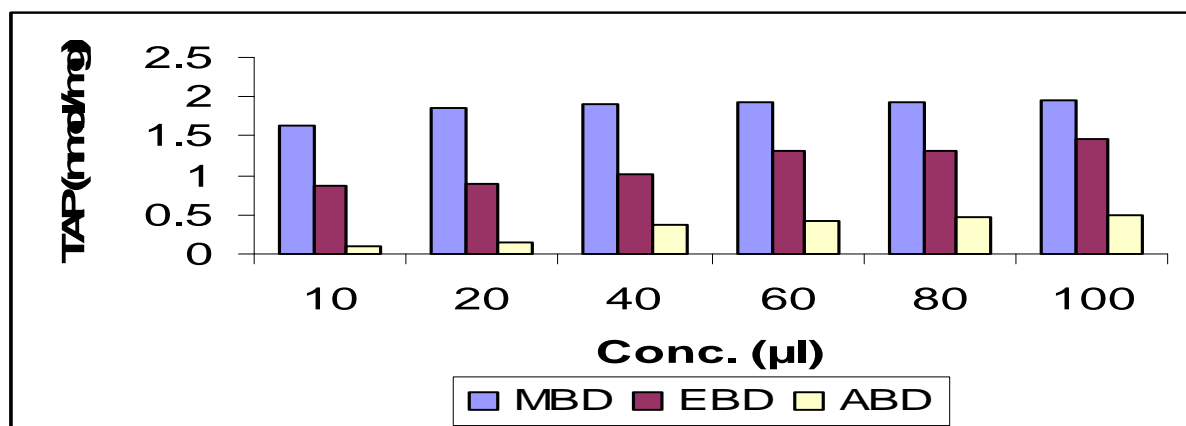
Table 3. Average value of ratios of different parameters

Parameters	Non-smokers	Young-Smokers
LDL-C/HDL-C	2.718±0.10*	2.6846 ±0.13* (-1.23%)
LDL-C/TC	0.634±0.008*	0.679±0.009* (+7.09%)
HDL ₂ -C/HDL ₃ -C	0.476±0.009*	0.558±0.01* (+17.22%)
TC/HDL-C	4.281±0.001*	3.953±0.14* (-7.66%)

*Values are mean±S.D. from all groups of subject

MEASUREMENT OF TOTAL ANTIOXIDANT POWER (TAP) IN DIFFERENT *B. diffusa* EXTRACTS:

Fig 3 depicts measurement of Total Antioxidant Power in three different solvent mediums for *B. diffusa* at different concentration. The maximal TAP as found in Methanolic extract of *B. diffusa* compared to ethanolic and aqueous extracts. The effectiveness of the methanolic extract of B.D. being high has been reported [46].

Fig 3. Measurement of TAP of *Boerhaavia diffusa* Methanol, Ethanol and Aqueous extracts

MBD (Methanol extract of *B. diffusa*), EBD (Ethanol extract of *B. diffusa*), ABD (Aqueous extract of *B. diffusa*).

MEASUREMENT OF TAP IN LIPID PROFILE: Fig. 4 shows decrease in the total antioxidant power of LDL-C, HDL-C, HDL₂-C, HDL₃-C and VLDL-C for smokers compared to non smokers.

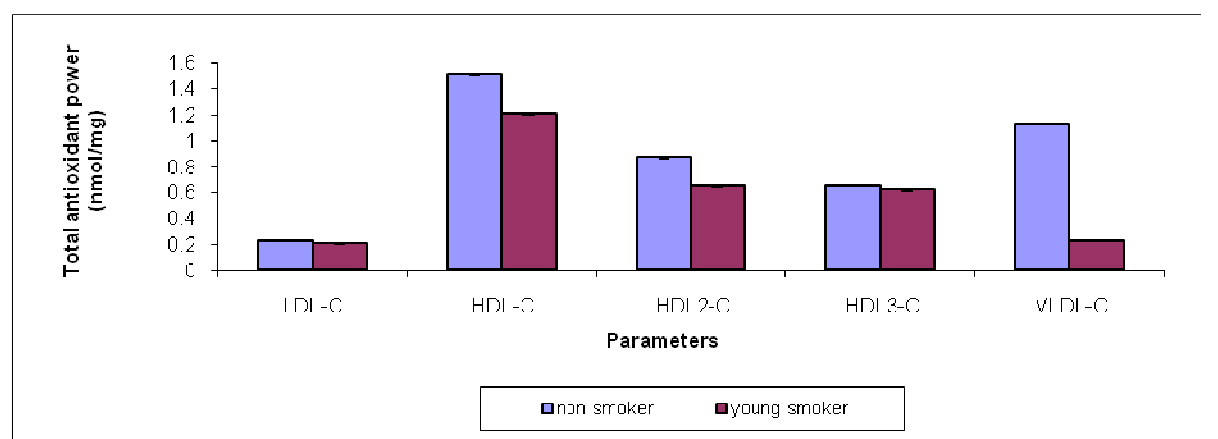


Fig. 4 TAP measurement in lipids

MEASUREMENT OF TAP IN PLASMA IN THE ABSENCE AND PRESENCE OF BD:

Results depicted in Fig 5 is the measure of total antioxidant power in plasma without and with extract of *B. diffusa* at two concentrations (10µl, 20µl) which shows significant reduced TAP in smokers than non smokers. As expected plasma treated with BD in both smokers and non smokers TAP significantly increased. The excessive increase in free radicals in smokers reduced plasma FRAP level from normal control value. The *invitro* treatment of non-smokers and young smokers with BD increases in each group. Oxidative stress increases in smokers due to a higher production of ROS (Reactive Oxygen Species) or deficiency in the antioxidant defense system, thus leading to various pathological conditions. An antioxidant compound (ours being *B. diffusa*) might contribute to TAP of such damage. Our results show a significant decrease in TAP status in the plasma of young smokers and significant increase after treatment with *B. diffusa*.

MEASUREMENT OF LDL OXIDATION: Table 4 shows the *exvivo* basal values of CD (Conjugated Diene) and MDA (Malondialdehyde) of LDL Oxidation in young smokers to significantly increase in comparison to nonsmokers value. After the Cu²⁺ mediated LDL Oxidation in each group were significantly increased. As expected *in vitro* LDL Oxidation was carried out in the presence of BD which decreased the maximal amount of CD and MDA of LDL oxidation after 4 hours incubation (fig. 6, 7). These results indicate a strong antioxidative property of BD which could be a good source of natural antioxidant effect of *B. diffusa* has been proved [47].

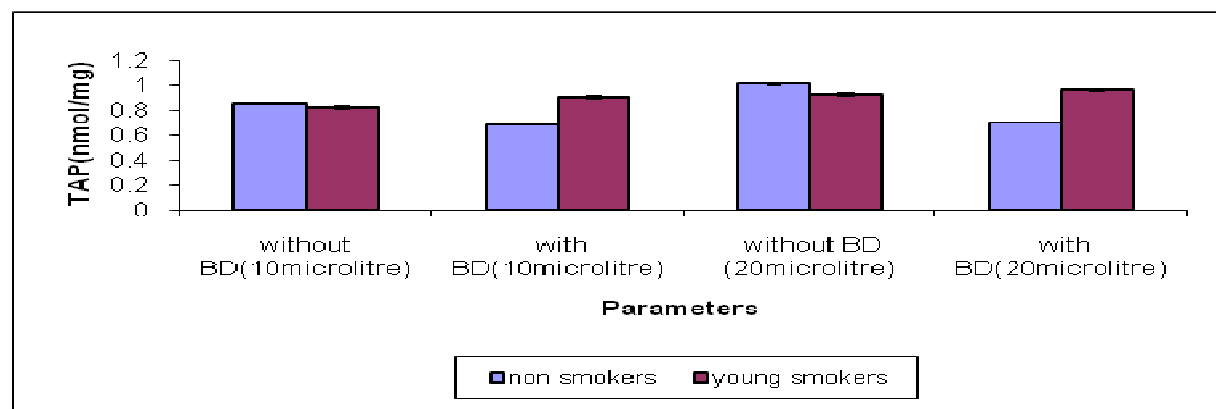


Fig. 5 Measurement of TAP in plasma

Table 4. LDL oxidation with or without *B. diffusa*

Conc(μl)	Incubation time (32°C) in hrs	CD formation (nmole/mg protein)		MDA formation (nmole/mg protein)	
		Non smokers	Young smokers	Non smokers	Young smokers
10	0	180.10±0.037*	239.36±0.03* (+32.77%)	0.3815±0.003*	0.481±0.003* (+26.15%)
10	CuSO ₄ (4 hrs)	270.12±0.04*	311.12±0.03* (+30.11%)	0.8012±0.003*	0.6221±0.003* (+29.15%)
10	CuSO ₄ + BD (4 hrs)	209.14±0.04*	270.10±0.6* (-13.18)	0.1755±0.001*	0.4129±0.0005* (-33.62%)

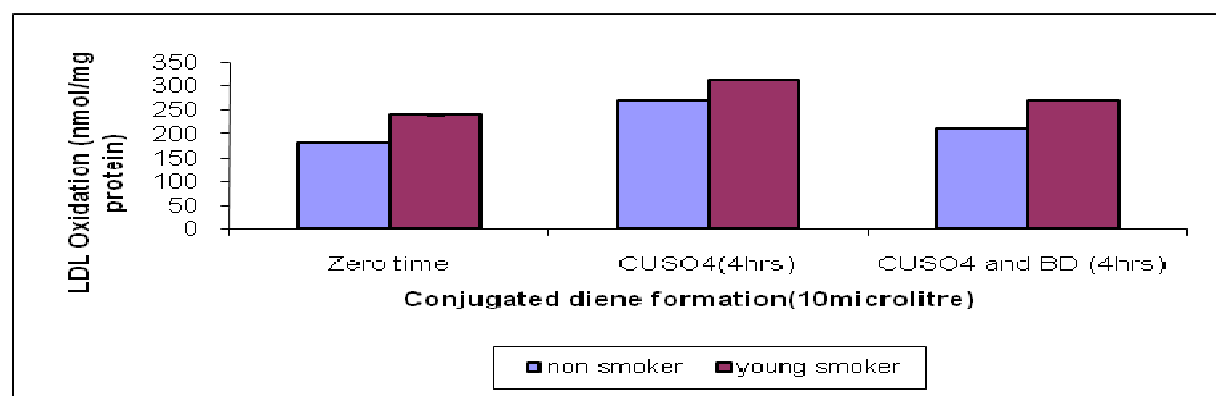


Fig. 6 LDL oxidation for CD formation

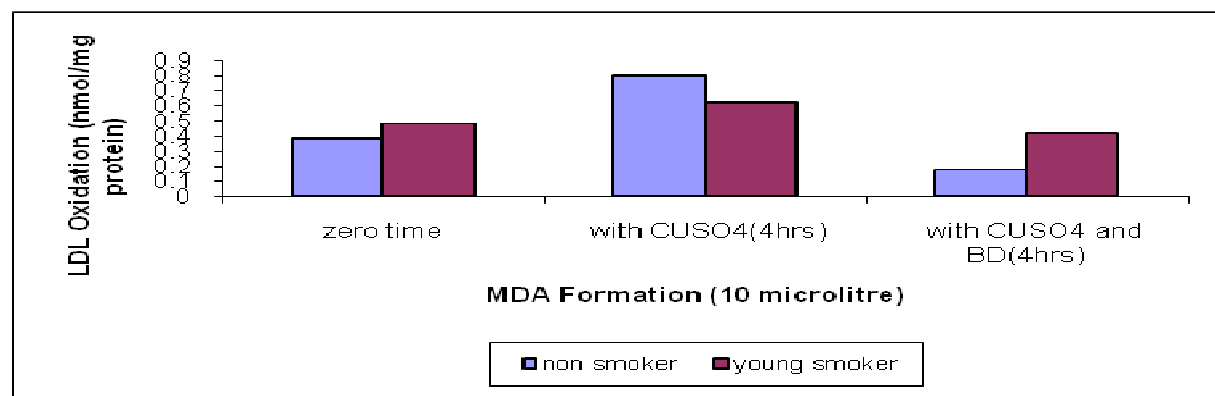


Fig. 7 LDL oxidation for MDA formation

DISCUSSION

Cigarette smoking is firmly established as a primary risk factor for atherosclerotic cardiovascular disease. Increased oxidative stress is one of the principal mechanisms by which it may exert its pathological influence. The cigarette smoke induced extensive proatherogenic changes that occurred in young smokers, were reflected on a variety of parameters, such as, plasma and lipoprotein lipids including cholesterol and plasma lipid peroxidation products, plasma total antioxidants. Treatment of smoke exposed rats with *B.diffusa* significantly reduced the overall oxidative burden and effectively ameliorated the above altered parameters, thus, indicating a strong hypolipidemic/anti-atherogenic and antioxidant effect of ethanolic root extract of this plant. The increase in plasma TG levels is apparently due to an increase in VLDL-C which can be the result of either increased VLDL production or decreased VLDL clearance. It is possible that massive free radical load in smoke control rats may stimulate VLDL production by increasing adipose tissue lipolysis, increasing hepatic de novo fatty acid synthesis, and decreasing hepatic fatty acid oxidation, all of which provide fatty acid substrate for esterification into TG and assembly into VLDL particles in the liver. Therefore, *B.diffusa* root extract may exert their cholesterol lowering effect in cigarette smoke exposed rats in a similar manner as previously reported for hyperlipidemic animals [48, 49] and humans [50, 51], which in turn is reduced by a decline in its protein mass [48, 52]. Antioxidant property of *B.diffusa* ethanolic extract possibly is due to the presence of phenolic compounds –Flavonoids, Flavones and Flavonols which have been reported to be a source of antioxidative characteristics naturally [53].

The high scavenging property of ethanolic extract of *B.diffusa* plant may be due to hydroxyl groups existing in the phenolic compounds chemical structure that can provide the necessary component as a radical scavenger. Free radicals are often generated as byproducts of biological reactions or from exogenous factors. The involvements of free radicals in the pathogenesis of a large number of diseases are well documented [54-62]. A potent scavenger of free radicals may serve as a possible preventative intervention for the diseases. Ethanolic extract of *B.diffusa* plant in this research exhibited antioxidant. The antioxidant potential may be attributed to the presence of polyphenolic compounds.

CONCLUSION

B.diffusa mediated multiple therapeutic benefits described in the present study, daily intake of BD as dietary supplement by novice/young/old moderate or heavy smokers as well as chronic smokers including passive smokers including passive smokers maybe useful in the presentation and treatment of tobacco include hyperlipidemia and atherosclerosis. In addition, daily intake of BD will be efficacious and cost effective and a good source of natural antioxidant.

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