Protective role of *Monotheca buxifolia* and *Bosea amherstiana* against H_2O_2 -induced DNA damage in human lymphocytes and its effect on oxidative enzymes

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Abstract: Experimental based evidence suggests that most of the medicinal plants possess a wide-ranging pharmacological and biological activity that may possibly protect tissues against O_2-induced damages. The objectives of the current study are: first, to investigate the effects of *Monotheca buxifolia* and *Bosea amherstiana* on H_2O_2 induced DNA damage in human lymphocytes and second, to determine its effect on oxidative enzymes. Cells were treated at concentration of 100µg/mL with both plants. Alkaline Single Cell Gel Electrophoresis/comet assay were used for DNA damage analysis. Activities of antioxidant enzymes TBARS, SOD, CAT and POD were assayed on treatment with the extracts. Both plants species possess the protective role against H_2O_2-induced lymphocytes DNA. Dichloromethane (DCM) fraction of *Monotheca buxifolia* (H DNA 94.79±0.29%) and methanolic fraction of *Bosea amherstiana* (H DNA 93.63±2.23%) possess high protection Significantly decrease occur in status of antioxidant enzymes. This study indicates that both plants have potential in preventing oxidative damages/stress related diseases and would be suitably used as supplements in combination with conventional drug for the treatment of cancer like diseases.

Keywords: DNA damage, antioxidant enzymes, comet assay, medicinal plants, lymphocytes.

INTRODUCTION

For millennia, medicinal plants have been used in folk medicine. In recent era the scientific approach has been developed. Nevertheless, adverse effect has may be caused by some of them or have potency of interactions with other medication (Zink *et al.,* 1998) Furthermore, herbs have minimum potential risk on health (Basaran *et al.,* 1996). Generally, the primary sources of antimutagens and natural toxins are green plants (Plewa *et al.,* 1993), and many plants consist of genotoxic and cytotoxic material resulting from their long term uses of such plants. In many countries, there is ironic tradition of using medicinal plants for curing of various diseases like infectious, injuries and inflammation etc (Barboza *et al.,* 2009, Zampini *et al.,* 2007, Carrizo *et al*., 2002, Zampini *et al.,* 2005). Considering the huge potential of plants as source for antimicrobial drug, several authors have investigated the antimicrobial activities of herbal plants (Nascimento *et al.,* 2000, Laciar *et al.,* 2009, Lucia *et al.,* 2012, Satorres *et al.,* 2013). *Bosea amherstiana* and *Monotheca buxifolia* are local species of found in hilly areas of Pakistan. Both plants have therapeutic uses as antiseptic, gastrointestinal disorders and wound healings. These plants are found in abundant in Dir Lower, Attock, Xhob Kala Chitta and Chitral (Nasir *et al.,* 1972).

The genotoxicity can be assessed by *Allium cepa* test, comet assay and others. Thus, the generation Of DNA damages are considered to be an important incident in carcinogenesis. Currently toxicological evaluation of medicinal plants is the need for the modern drugs. Due to traditional uses medicinal plants are considered to be safe. However some scientific research shows that some plants have mutagenic effects (Cardoso *et al.,* 2006, Déciga *et al.,* 2007, Mohd *et al.,* 2007) or carcinogenic and toxic possessions (De Sá *et al.,* 1999). For this reason, medicinal plants screening for their mutagenic effects screening is important. Plants that exhibit mutagenic effects should be considered as possibly hazardous and its further investigations should be recommended before its administration Plants with apparent anti mutagenic potency can also be used as a remedial agent and merit further in depth investigation of its pharmacological properties. Mutagenicity can also be used as an anticancer tools, as most anti cancer medicines are mutagenic in nature (e.g., the spindle disturbing substances taxol and vinblastine).

In this study, our purpose was to contribute to the safe use of medicinal plants by means of the evaluation of the possible genotoxic effects of *Monotheca buxifolia* and *Bosea amherstiana* extracts by single cell alkaline gel electrophoresis the comet assays and their effect on oxidative stress enzymes.

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MATERIALS AND METHODS

Plant materials

Aerial parts of *Monotheca buxifolia* and *Bosea amherstiana* were collected from, Dir, KPK, Pakistan, in March 2014 and September 2014 respectively. The sample was authenticated by Prof. Dr. Jehandar Shah, Taxonomist; Ex Vice Chancellor Shaheed Benazir Bhutto University Shiringal, Pakistan.

Extracts preparation

Well established protocol was followed to get methanolic extracts from the powder plant materials (Saddiqe *et al.*, 2016, Khan *et al.*, 2011, Raziq *et al.*, 2011). Extracts were concentrated under vacuum at 40°C after filtration. Further fractions were obtained with different solvents on the basis of polarity i.e. *n*-hexane, dichloromethane, ethyl acetate and aqueous fractions. The methanolic as well as the subsequent solvent fractions was screened for comet assay and antioxidant enzymes.

Lymphocytes isolation from human peripheral blood

Blood samples from donors were collected from ten volunteers, which include four females and six males, healthy nonsmokers, ranging from 24 to 48 years old. Fresh blood (20-30mL) was obtained in heparinized tubes and lymphocytes were isolated with the help of separation kits accompanied with Ficol-Paque Plus lymphocytes isolations sterilized solution (Cinkilic *et al.*, 2013). Harvesting of cells were done within one day and cultured in atmosphere containing 5% CO_2 in air for 24 hours at 37°C.

Cell viability testing

Lymphocytes exposed to 100µg/mL concentration of each plant methanolic extracts and fractions for one hour at 37°C. Lymphocytes were exposed H_2O_2 (10 µM) in order to make it damage on ice for 5 min and centrifuged for 10 minutes and washed in the similar medium. All experimentations were conducted in triplicate. Viability of the cells was tested by using MTT assay (Cory *et al.*, 1991).

DNA damage estimations: The comet assay

Standard protocol for comet assay was performed as described by Ahmad *et al.* (2007), by methanolic, *n*-hexane, dichloromethane, ethyl acetate and aqueous extracts from *Monotheca buxifolia* and *Bosea amherstiana* being used for this study. The whole process was directed under low indirect incandescent light (60 watt). DNA status of human lymphocytes was estimated by using an alkaline electrophoresis with slight modification. Three layers were made in first layer labeled microscopic slides were dipped half in 1% agarose (normal melting). The slides were placed on the ice box to solidify and dried at room temperature. Well-ordered samples containing (10^5 cells/mL) lymphocytes were mixed in 70µl of 0.5% agarose (low melting) for first layer formation. Another layer of agarose (70µl) of the same concentrations was added to fill the residual pores. The gel was set at 4°C, and cells were lysed in lysis buffer for 24 hours in lysis buffer at 4°C. Cells were alkaline unwound by electrophoresis using the electroophoresis buffer at 25 V (0.714 V/cm) for 10 minutes adjusted to 300mA. Following electrophoresis, neutralization of slides was done in buffer solution and stained with Acridine orange. Scoring of the comet like images were done under epifluorescent microscope (400 X, Nikon AFX -1 Optiphot) Digital images were taken for succeeding analyses and scoring. Experiment was conducted in triplicates, and fifty images were selected randomly per slide to calculate average DNA damages. A computerized software (CASP) was used to calculate DNA damages (Ahmad *et al.*, 2007).

Estimation of oxidative enzymes

Catalase (CAT)

CAT enzyme was determined by the decreasing the absorbance due to consumption of H_2O_2 (Aebi *et al.*, 1984). Homogenated lymphocytes (50µl) were diluted in 2ml of 50mM phosphate buffer solution (pH 7.0). Two ml of dilute homogenates were added with 1ml of 50mM buffer (phosphate pH 7.0) containing 30Mm H_2O_2 to sample tube, likewise blank was prepared in distilled water as an alternative of sample. Mix directly and take the absorbance for 15 seconds and 30 seconds at 240nm. CAT activity was determined as change in the absorbance of 0.01 as unit/ min.

Peroxidase (POD)

POD enzymes in homogenate were calculated by spectrophotometric technique followed the procedure of Calberg and Manervik (1975). The solution consists of 0.1ml homogenate, 0.3ml of 40mM H_2O_2, 0.1ml of 20Mm guaiacole, and 2.5ml phosphate buffer solution 50mM (pH 5.0). Absorbance changes were calculated at 470nm.

Superoxide dismutase (SOD)

Super oxide dismutase enzyme activity was determined by the procedure established by Kakkar *et al*. (1984). Quantity of chromogen was measured at 560nm.

Estimation of Lipid per oxidation by (LPO/TBARS)

Malondial dehyde in homogenates were calculated by reaction with thio barbituric acid (TBA) followed the procedure developed by Iqbal *et al*. (1996). Briefly added, 0.29ml phosphate buffer solution (0.1M; pH 7.4), 0.1ml sample, 0.1ml ascorbic acid (100Mm) and incubated at 37^{o}C in water bath for 1 hour. Adding 0.5ml trichloro acetic acid (10%) to stop the reaction. Followed by adding 1ml of 0.67% tri chlorobarbituric acid, and placed for 20 minutes in water bath at 95^{o}C and prior to centrifugation the sample were put on ice bath. The TBARS amount in the samples was assessed by measuring the optical density of supernatant at 535nm using spectrophotometer. The

result was expressed as Nm TBARS/min/ml plasma at 37°C using molar extinctions coefficients of 1.56X 10^5 M^{-1} cm^{-1}.

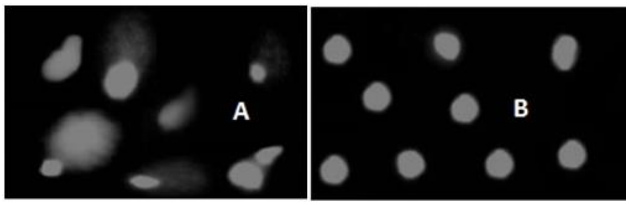

Fig. 1: Fluorescent photomicrograph (40x) (A) Lymphocytes induced with H_2O_2, Control (B) H_2O_2 induced lymphocytes protection by *Monotheca buxifolia* and *Bosea ahmerstiana*

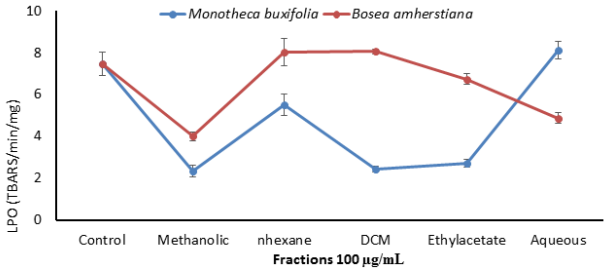

Fig. 2: Lipid Per Oxidation (LPO/TBARS) activity in human lymphocytes exposed to *Monotheca buxifolia* and *Bosea amherstiana.* Data are mean ± S.E. (n=3). Significantly different at p<0.05.

STATISTICAL ANALYSIS

The obtained data from the experiments were expressed as mean ±SE. One-way analysis of variance were used followed by LSD test using Statistix Version 8.1. Values P <0.05 were measured statistically significant.

RESULTS

Effects of Monotheca buxifolia and Bosea amherstiana on H_2O_2-induced DNA damage to lymphocytes

The lymphocytes were exposed to *Monotheca buxifolia* and *Bosea amherstiana* at concentration of 100µg/ mL for 1 hour at 37°C. H_2O_2 (10µM) was used to damage the DNA for 5min on ice. To determine the DNA damages and protective effects of plants extracts, comet assay was performed. The effects of pretreatments of the both plants tested on 10µM H_2O_2induced DNA oxidative damages in human lymphocyte are presented in table 1 and 2. Percent tail DNA shows that both plant had a significantly better level of protections against H_2O_2 exposure. The maximum protective effects of lymphocytes pre-treatment were noted with dichloromethane fraction of *Monotheca buxifolia* which is 5.21 ± 0.030 % Tail DNA and Olive Tail moment is 0.61 ± 0.03 (table 1) compared to the rest of treated samples of *Monotheca buxifolia*. In *Bosea amherstiana* the methanolic fraction has more protective effect against H_2O_2 induced lymphocytes which has 6.36 ± 2.23 Tail DNA % and their Olive Tail Moments is 0.84 ± 0.40. The control lymphocytes exposed to H_2O_2 without any prior treatment show high damage 32.37 ± 1.28 Tail DNA % and Olive Tail Moment 2.97 ± 0.29 (Table 1 & 2). Hence, the DNA damages induced by H_2O_2 was expressively more as compared to treated extracts (Fig. 1).

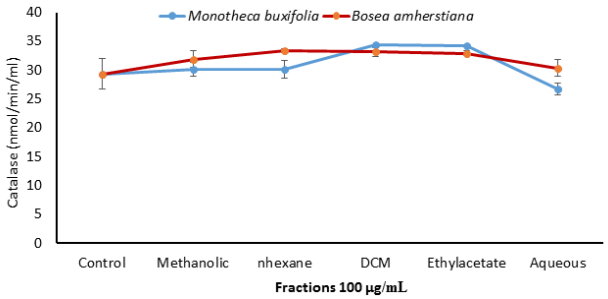

Fig. 3: Catalase (CAT) activity in human lymphocytes exposed to *Monotheca buxifolia* and *Bosea amherstiana.* Data are mean ± S.E. (n=3). significantly different at p<0.05.

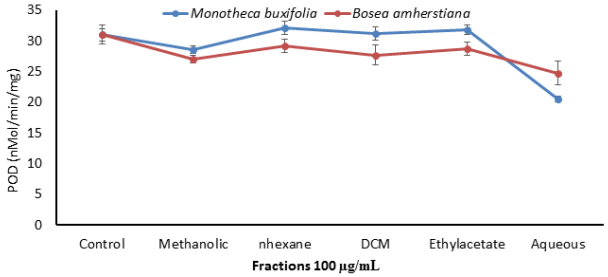

Fig. 4: Peroxidase (POD) activity in human lymphocytes exposed to *Monotheca buxifolia* and *Bosea amherstiana.* Data are mean ± S.E. (n=3). Significantly different at p<0.05.

Oxidative enzymes

Antioxidants enzymes activities of *Monotheca buxifolia* (MB) and *Bosea amherstiana* (BA) extracts treated and untreated cells were estimated. MB has effective role in decreasing Lipid Per Oxidase (TBARs) enzymes. Methanol, Dichloromethane and ethyl acetate fractions of MB are more effective (2.3, 2.4, 2.7 respectively) while aqueous fraction of MB slightly increases the LPO to 8.1. Methanolic and aqueous fraction of BA decrease LPO up to 3.99 while *n*-hexane and dichloromethane fraction increase the level of LPO (fig. 2). Both MB and BA has slight effect on catalase enzymes. Dichloromethane fraction of *Bosea amherstiana* increase CAT enzymes little bit while aqueous fraction of *Monotheca buxifolia* lower down the concentration of CAT enzymes compared to control (fig. 3). Dichloromethane fraction of MB slightly increase POD enzymes value to 31.2 and aqueous extract of MB lowered down POD enzymes 20.51. Overall both plants significantly effective in decreasing

Table 1: Head Length, Tail Length, Comet Length, Head DNA, Tail DNA, Tail Moment and Olive Tail Moment of H_2O_2 induced lymphocytes treated with *Monotheca buxifolia*

Fractions	Head L (µm)	Tail L(µm)	Comet L	H DNA (%)	T DNA (%)	TM	OTM
Methanolic	21.67 ± 0.89	3.67± 0.44	25.33 ± 1.11	86.80 ± 1.90	13.20 ± 1.90	0.49 ± 0.12	1.52± 0.29
*n*hexane	25 ± 6.7	4 ± 0.67	29 ± 7.33	92.85 ± 3.64	7.15 ± 3.64	0.30 ± 0.14	1.06± 0.67
DCM	23 ± 0	3 ± 0	26 ± 0	94.79 ± 0.29	5.21 ± 0.30	0.16 ± 0.01	0.61± 0.03
Ethylacetate	17 ± 2.67	3.33± 0.44	20.33 ± 0	92.01 ± 1.52	7.99 ± 1.52	0.27 ± 0.09	0.67± 0.26
Aqueous	22.33 ± 6.2	4 ± 1.33	26.33 ± 6.89	92.24 ± 1.92	7.76 ± 1.93	0.31 ± 0.12	0.87± 0.23
Control	13.67 ± 0.89	5 ± 1.33	18.67 ± 2.22	67.63 ± 1.28	32.37 ± 1.28	1.67 ± 0.51	2.97± 0.29

Table 2: Head Length, Tail Length, Comet Length, Head DNA, Tail DNA, Tail Moment and Olive Tail Moment of H_2O_2 induced lymphocytes treated with *Bosea amherstiana*

Fractions	Head L (µm)	Tail L(µm)	Comet L	H DNA (%)	T DNA (%)	TM	OTM
Methanolic	25.67 ± 5.78	3.33 ± 0.44	29 ± 6	93.63 ± 2.23	6.36 ± 2.23	0.21 ± 0.08	0.84 ± 0.40
*n*hexane	19 ± 5.33	5.33 ± 1.56	24.33± 6.44	87.99 ± 1.47	12.01± 1.47	0.66 ± 0.24	1.03 ± 0.73
DCM	18.33 ± 1.78	4 ± 1.33	22.33± 1.56	88.71 ± 2.18	11.29± 2.18	0.48 ± 0.26	1.01 ± 0.29
Ethylacetate	25.67 ± 3.11	4.33 ± 1.11	30 ± 4	90.52 ± 4.19	9.48 ± 4.19	0.46 ± 0.32	1.35 ± 0.75
Aqueous	17 ± 2.67	3 ± 0	20 ± 2.67	91.39 ± 2.20	8.61 ± 2.20	0.28 ± 0.07	0.68 ± 0.12
Control	13.67 ± 0.89	5 ± 1.33	18.67± 2.22	67.63 ± 1.28	32.37± 1.28	1.67 ± 0.51	2.97 ± 0.29

Data are represented as Mean ±SE (n = 3), significantly different at $p<0.05$.

POD enzymes (fig. 4). The control value of SOD enzymes is

20.7 MB has no effect in decreasing or increasing SOD while BA relatively contribute in lowering down the values of SOD (fig. 5). Overall both contribute in lowering down the oxidative enzymes level during stress condition. The results of both plants are highly significant.

DISCUSSION

Estimation of single strands breaks DNA damages from exposure to various extracts of *Monotheca buxifolia* and *Bosea amherstiana*. Both plants were significantly protecting lymphocytes DNA against H_2O_2-induced at concentration of 100µg/mL. This might be due to the dihydroxy structure being essential for DNA protection H_2O_2 (Duthie *et al.*, 1997). Furthermore, many types of natural antioxidants, including polyphenolic and flavonolic compounds, prevent adhesive molecules expression and the adhesions of monocyte to endothelial cell, and cell inflammation are also suppressed, proliferation, transformations, invasions angiogenesis and survival (Li *et al.*, 2012). Free radicals are involved in human disease and induce cellular damages such as cancer, atherosclerosis and inflammatory disorder, and poly phenols tends to reduce mutagenicity and oxygen free radicals (Gargouri *et al.*, 2011).

The antioxidants protections depend on stimulating lymphocytes *in vitro* treated with H_2O_2. In this case, strands breaking are effective measures of incident damages, and the repairing of cells is not a factor since the cells are reserved cold during the entire treatment. The supplementation effects are again striking, with a noteworthy decrease in the damages received at the higher H_2O_2 tested dosage. Similar effects have been studied by Green *et al.*, 1994 by treating human lymphocytes *in vitro* with the ionizing radiations. The response, in terms of DNA strand breakage, was expressively reduced in blood sampling taken a shortly after a high dosage of vitamin C, compared with samples from the same subjects prior to ingestion of the antioxidant. In normal condition, the action of reactive specie is conflicting by a coordinated and balanced system of antioxidant enzymatic defenses, such as POD, SOD and CAT. Similar studies has been carried out which support our results by (Desaint *et al.*, 2004; Morel and Barouki 1999; Venkatesan *et al.*, 2007).

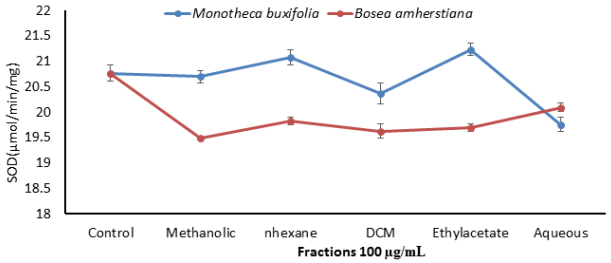

Fig. 5: Superoxide Dismutase (SOD) activity in human lymphocytes exposed to *Monotheca buxifolia* and *Bosea amherstiana*. Data are mean ± S.E. (n = 3). significantly different at $p<0.05$.

The increasing of lipid peroxidation or alteration of non enzymatics and enzymatic anti oxidation system

contribute the oxidative stress. Various information shown that different non-enzymatic and the enzymatic system in mammalian cells have the competency of free radical's reduction. Lipid per oxidation is a bio chemical marker for the free radical mediated injury. The enzymatic component related with defense against ROS includes catalase, SOD, peroxidase and enzyme of ascorbate glutathione cycle. SOD and catalases have been recognized as enzymatic protector against per oxidation reaction (Monk *et al.,* 1989). SOD is an important component of anti-oxidative defense mechanism in plants and it dismutates two super oxide's radical to water and O_2. *Monotheca buxifolia* has significantly decrease lipid per oxidation. Catalase is generally existing oxido reductase that decomposes H_2O_2 to water and molecular oxygen and it is one of the key enzyme involved in elimination of toxic peroxide. Both plants have slight or no effects on catalase (CAT) and POD enzymes while *Bosea amherstiana* significantly decrease the level SOD enzymes.

CONCLUSIONS

In conclusion, alkaline Single Cell Gel Electrophoresis is rapid, simple and economical way for estimating DNA damage/repair. *Monotheca buxifolia* and *Bosea amherstiana* protects DNA from damages. This may due to antioxidant bioactive compound present in medicinal plants which prevent DNA from oxidative damages. This study indicates that both plants have potential in preventing oxidative damages/stress related diseases and would be suitably used as supplements in combination with conventional drug for the treatment of certain diseases.

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