Potential antigenotoxicity assessment of *Ziziphus jujuba* fruit

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**ABSTRACT**

*Ziziphus jujuba* Mill. fruits are nutritionally rich and have a broad spectrum of health benefits. In this work we hypothesized that this natural product rich in polyphenols might protect humans against DNA damage and its consequences. This has led to our investigation to find out if the fruit extract showed an ability to decrease the frequency of DNA damage (antigenotoxicity) induced by two known genotoxins namely an alkylating agent methyl methane sulphonate (MMS) and a reactive oxygen species (ROS) inducer hydrogen peroxide (H₂O₂).

Human lymphocytes were incubated with the *Ziziphus* fruit ethanol extracts (ZFE) or betulinic acid (BA) followed by an exposure to either 50 μM of MMS or 250 μM of H₂O₂. Results suggest that ZFE (250, 500, 1000 μg/ml) and BA (10, 20, 40 μg/ml) were able to inhibit the DNA damaging effect caused by MMS and H₂O₂ indicative of their protection against the genotoxin. This could be attributed to the interactions of the phenolics, flavonoid and BA present in the fruits. Additional in vivo experiments were carried since BA is an important phytochemical detected in ample amounts in the fruit extract. Mice were primed with BA (2.5, 5.0 and 10 mg/kg body weight) for a period of 6 days. The animals were injected with MMS (10 mg/kg body weight) 24 h later and sacrificed. The genotoxic activity of MMS was inhibited in a dose–related manner by BA. BA reduced the frequency of MMS–induced DNA damage in liver, kidney and bone marrow cells of mice thereby exhibiting its antigenotoxic properties. It could also reduce total glutathione level, lipid peroxidation and hydrogen peroxide content in liver cells of mice through the up-regulation of antioxidant enzymes. Therefore taking into account the antioxidant and antigenotoxic properties, the consumption of the *Ziziphus* fruit should be more popularized worldwide.

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1. Introduction

Phytochemicals are ubiquitous in plant foods and are important components of the human diet. Among a myriad of other beneficial effects, they are known to have the potential to stimulate the immune system, prevent DNA damage and reduce oxidative damage to cells. Phenolic compounds (like flavonoids, phenolic acids and tannins) and terpenoids are the major contributors of the antioxidant and antigenotoxic properties of plants [1, 2, 3].

*Ziziphus* plants including *Ziziphus jujuba* Mill., is a small tree or shrub belonging to the family Rhamnaceae. The fruits are very popular in many regions of Asia for its high nutrition value [4] and are also used as food additive and flavouring agent [5]. The pharmacological activities are mainly attributed to the phytochemicals present in leaves, bark, fruit and seed of the plant [6, 7, 8]. Phytochemical investigations resulted in the isolation of bioflavonoids, triterpenoids, phenolic compounds, glycosides and saponins [9]. A total of 25 polyphenolic compounds were identified and classified as 10 flavan-3-ols, 13 flavonols, 1 flavanone, and 1 dihydrochalcone by Wojdylo et al. [10], in four Spanish jujube cultivars. Among the isolated triterpenoids, betulinic acid content was high in *Ziziphus* [11].

The various parts of the jujube plant have been reported to bring forth biological effects, such as the anticancer [12], anti-inflammatory, anti-obesity [13], anthelminthic [14], antioxidant, hepatoprotective and gastrointestinal protective activities [15]. To the best of our knowledge there has been no systematic investigation of the genotoxic and antigenotoxic effects of *Z. jujuba* fruits.

In conventional medicine, the use of entire plants or a concoction of plant products are preferred instead of extracted pure compounds. Evidence suggest that an equivalent dose of crude plant extract possess higher *in vitro* and *in vivo* activity than the isolated constituents [16]. Among the various bioactive compounds, phenolics compounds are one
of the most important bioactive compounds that play an important role in the antioxidant activity. In order to recover bioactive compounds from crude plant materials, optimization of extraction was done as the first important step.

Betulinic acid is a bioactive secondary metabolite that is present in an abundant amount in the leaves and barks of Betula, Ziziphus and other plants. It is known for its anticancer properties [17, 18, 19, 20] and known to reduce MMS-induced genotoxicity in V79 cells [21].

Many of the biologically active compounds are of limited therapeutic use because of their toxicological, carcinogenic and mutagenic properties. The analysis of genotoxicity both in vivo and in vitro is an essential aspect for their potential use as a new therapeutic agent.

As part of our ongoing research for screening antigenotoxic natural products, in this paper we report the cytotoxic, genotoxic and antioxidant activities of Ziziphus fruit ethanol extract. We hypothesize that this natural product may have anti-genotoxic properties. Therefore, we investigated its anti-genotoxic potential against methyl methane sulphonate (MMS) or its effect in reducing DNA damage by a ROS inducer H2O2 in two different test-systems frequently used in genotoxicity studies. In order to recover bioactive compounds from Z. jujuba fruit, optimization of extraction process was done by using 100, 75 and 50% ethanol. The three fractions of the fruit extract were named as ZFE-1, -2 and -3. Various concentrations of ZFE extracts and BA were studied in vitro on human lymphocytes and for in vivo study various concentrations of BA were gavaged to Swiss albino male mice. Such studies with physiologically different types of test-systems using sensitive tests for antioxidant potential and for genotoxicity (induction of DNA breaks) could both provide more informative assessment of the cytotoxic/genotoxic effect and give valuable information about the protective potential of this compound against genotoxins.

2. Materials and methods

2.1. Chemicals

Betulinic acid (BA, CAS no. 472-15-1), 1, 1-diphenyl-2-picrylhydrazyl free radical (DPPH, CAS no. 1707-75-1), 2,4,6-Tris (2-pyridyl)-s-triazine (TPTZ, CAS no. 3682-35-7), triton X-100 (CAS no. 9002-93-1) and methyl methanesulphonate (MMS, CAS no. 66-27-3) were purchased from Sigma–Aldrich (St. Louis, MO, USA). Ethanol and aluminium chloride anhydrous (AlCl3, CAS no. 7446-70-0) were purchased from Merck Specialties’ (Mumbai, India). Acetonitrile (HPLC grade) and dimesylsulphoxide (DMSO) was procured from Qualigens (Mumbai, India). Other reagents like ethylene diamine tetra acetic acid (EDTA) di-sodium salt, normal melting point agarose (NMPA) and low melting point agarose (LMPA), HiSep™ LSM 1084, Tris buffer, phosphate-buffered saline (PBS) (Ca2+, Mg2+ free), RPMI-1640 media, were procured from Hi Media, Mumbai, India. Ascorbic acid, gallic acid, Folin–Ciocalteu reagents were purchased from SRL (Mumbai, India). Sodium di-hydrogen phosphate (NaH2PO4), di-sodium hydrogen phosphate (Na2HPO4), hydrogen peroxide (H2O2), ferric chloride (FeCl3), sodium hydroxide (NaOH) and sodium carbonate (Na2CO3) were obtained locally and were of analytical grade.

2.2. Plant material and extraction procedure

Ziziphus jujuba fruits were collected from the campus of the University of Calcutta, Kolkata. The plant was authenticated by plant taxonomist and the voucher specimen (CUH accession number – 20034) was deposited at the Calcutta University Herbarium (CUH), Department of Botany, University of Calcutta, Kolkata. The fruits were de-seeded and air-dried. For optimization of extraction process of the bioactive compounds [22] we have used ethanol (100 %) and ethanol: water (1:4 and 1:1) extracts of Ziziphus jujuba fruit (ZFE). A total of 100 g sample was soaked in 1L each of 100, 75 and 50% ethanol and kept in dark at 40 ℃. After 7 days the extract was filtered (Whatman, No. 1) and evaporated to dryness. Finally semi-solid residue of the Ziziphus fruit pulp was obtained in the following amount: 15 g of ZFE-1 (extracted with 100% ethanol), 23.035 g of ZFE-2 (extracted with 75% ethanol) and 26.605 g of ZFE-3 (extracted with 50% ethanol). The extracts were preserved in – 20 ℃ until further use.

2.3. Phytochemical analysis

Total soluble phenolic compound (TPC) in the ethanol extract of Ziziphus fruit pulp (ZFE-1, ZFE-2 and ZFE-3) was determined with the Folin–Ciocalteu reagent [23]. Briefly, 0.5 ml of ZFE (ZFE-1, ZFE-2 and ZFE-3) was mixed with 5 ml of Folin–Ciocalteu reagent (in 1:10 dilution with distilled water) and 4 ml of Na2CO3 (1M) solution. The reaction mixture was incubated at 37 ℃ for 15 min and the absorbance was measured at 765 nm (Beckman Coulter, USA). Results were expressed as gallic acid equivalent per gram of extract (mg GAE/g) calculated from a standard calibration curve of gallic acid (R2 = 0.9236). Three independent experiments were conducted with three replicates.

The total flavonoid content (TFC) in the extract was measured according to the method of Ordonez et al. [24], with some modifications. In brief, 0.5 ml of 2% solution of AlCl3 in ethanol was mixed with 0.5 ml of ZFE (ZFE-1, ZFE-2 and ZFE-3). The reaction mixtures were incubated for 1 h at 37 ℃. The absorbance was measured at 420 nm (Beckman Coulter, USA) using ethanol as sample blank. Standard curve of quercetin (0–40 μg/ml) was used to determine the total flavonoid content and was expressed as quercetin equivalent per gram of extract (mg QE/g). Three independent experiments were conducted with three replicates.

Among the constituent photochemical present in ZFE, the presence of betulinic acid (BA) was done by high performance liquid chromatography using a HPLC system equipped with a DAD detector (Agilent, USA). The analytical column used was an Agilent Eclipse plus C18 column (150 mm × 4.6 mm, 3.5 μm). The mobile phase consisted of acetonitrile–water (86:14, v/v) at the flow rate of 0.5 ml/min and the detection wavelength by UV detector was set at λ = 210. All samples were filtered through nylon syringe filters (0.2 μm) and the volume of injection was 20 μl. All chromatographic analyses were carried out at ambient temperature. The chromatographic peak of BA in ZFE (ZFE-1, ZFE-2 and ZFE-3) and retention time was confirmed by standard BA. The amount was calculated with the help of standard curve and expressed as μg BA/mg of extract.

2.4. Determination of antioxidant activity

Free radical scavenging activities of ZFE (ZFE-1, ZFE-2 and ZFE-3) and BA were determined by the method of Shimada et al. [25], with some modifications. In brief, 1.0 ml freshly made DPPH solution (100 μM) in ethanol was added to the different concentrations of ZFE (125, 250, 500 and 10,000 μg/ml) and BA (10, 20, 40, 80 and 100 μg/ml). The samples were incubated at 37 ℃ in dark for 20 min and the absorbance was measured at 517 nm. In each experiment, DPPH in ethanol was used as blank. The antioxidant activity of the tested samples, expressed as percentage inhibition of DPPH, was calculated according to the formula:

\[ IC(\%) = 100 \times \left( 1 - \frac{A_{sample}}{A_{blank}} \right) \]

Where Ab = absorbance of blank sample and As = absorbance of a tested sample.

Percent inhibition was plotted against concentration and a linear regression was applied to obtain the IC50 value.

Ferric Reducing Antioxidant Power (FRAP) assay was conducted according to the method of Benzie and Strain [26], with some modifications. The FRAP reagent was freshly prepared by mixing acetate buffer (10 mL, 300 mM, pH 3.6), 2,4,6-tripyridyl-s-triazine (TPTZ, 1 mL, 10 mM) and FeCl3-6H2O solution (1 mL, 20 mM) and then warming at 37 ℃ before use. 70 μl of ZFE-1, ZFE-2 and ZFE-3 (125, 250, 500 and 1000 μg/ml) or 10, 20, 40, 80 and 100 μg/ml of BA was allowed to react with 1
ml of the FRAP solution for 10 min at 37 °C in dark. Readings were then taken at 593 nm. Ascorbic acid was used as a standard and the results were expressed as ascorbic acid equivalent per g of extract (mg AAE/g of extract).

2.5. Cytotoxicity, genotoxicity and antigenotoxicity in vitro in human lymphocytes

Peripheral venous blood was obtained from three healthy donors (aged 20–25 years, non-smokers, non-alcohol consuming) not exposed to any drug therapy. Equal volume of freshly collected blood was mixed with equal volume of phosphate buffered saline (PBS pH 7.4) and layered on Histopaque and centrifuged at 800 g for 40 min [27]. The interface layer (buffy coat) enriched in lymphocytes was collected and washed twice with PBS (pH 7.4). The lymphocytes were re-suspended in RPMI – 1640 medium. The cell number and viability was confirmed by trypan blue dye exclusion test (>95%) according to the method of Tennant [28].

Our investigation was performed following all regulations and guidelines of Institutional Human Ethical Committee (IHAC), Central body of the University of Calcutta and informed consent was obtained from the human subjects, prior experiment preparation.

2.5.1. Cytotoxicity

The cytotoxicity of ZFE (ZFE-1, ZFE-2 and ZFE-3) and BA was estimated by MTT assay [29]. Lymphocytes (2 × 10^5 cells/ml) were incubated at 37 °C for 3 h in various concentrations of ZFE (125, 250, 500, 1000, 2500, 5000 and 10000 μg/ml) and BA (10, 20, 40, 80 and 100 μg/ml). Negative (RPMI-1640) and positive (100 μM dexamethasone) controls were maintained. After 3 h the media was removed and 10 μl of MTT solution (5 mg/ml of stock prepared in PBS) was added to 100 μl of cell suspension and allowed to incubate at 37 °C for 4 h. The dark blue coloured formazan crystals were dissolved in 100 μl DMSO and absorbance was recorded at 570 nm, with 630 nm as a reference wavelength 

Inhibition of cell proliferation was calculated as IC50 value.

2.5.2. Genotoxicity and antigenotoxicity evaluated by the comet assay

Human lymphocytes (2 × 10^5 cells/ml) were incubated for 3 h at 37 °C with various concentrations of ZFE (125, 250, 500, 1000, 2500, 5000 and 10000 μg/ml) or BA (10, 20, 40, 80 and 100 μg/ml) in RPMI-1640 media for 3 h) and processed for DNA damage analysis according to the method of Singh et al. [30], with modifications [31, 32, 33]. MMS (50 μM) and RPMI-1640 media treated cells were maintained as positive and negative controls respectively. Slides were coated with 1% normal human serum and incubated for 3 h at 37 °C. Slides were treated with neutralizing buffer (0.04 M Tris-HCl, pH 7.5). The slides were then neutralized by utilizing neutralizing buffer (0.04 M Tris–HCl, pH 7.5). The slides were stained with EtBr (20 μg/ml) and scored using a computerized system for image analysis (Komet 5.5, kinetic imaging; Andor Technology, Nottingham, UK) attached to a fluorescence microscope (Leica, Wetzlar, Germany, excitation filter 515–560 nm and barrier filter of 590 nm) equipped with a CCD camera. A total of 150 randomly chosen nuclei (50/slide) were analyzed from 3 slides per treatment set and expressed as percent of tail DNA. The parameter of tail DNA (%) was used to measure DNA damage in cells [34].

Antigenotoxicity was investigated on human lymphocytes measured as inhibition of DNA damage, by the addition of ZFE or BA that could decrease MMS and H_2O_2-induced DNA damage. The concentrations that were non-cytotoxic and non-genotoxic in MTT assay were chosen for antigenotoxicity experiments. The lymphocytes were pre-incubated for 3 h with different concentrations of ZFE (ZFE-1, ZFE-2 and ZFE-3) or BA and further exposed to MMS and H_2O_2.

Two sets of experiments were carried out. In one set lymphocytes were incubated for 3 h at 37 °C with ZFE-1,2,3 at concentrations 250, 500 and 1000 μg/ml or BA (10, 20 and 40 μg/ml) and embedded in agarose gel on a slide. This was followed by immersing the slides in a Coplin jar containing ice-cold H_2O_2 (250 μM) for 5 min [35]. In the second set lymphocytes incubated for 3 h at 37 °C with ZFE-1,2,3 at concentrations 250, 500 and 1000 μg/ml or BA (10, 20 and 40 μg/ml) were further incubated for 1 h with MMS (50 μM).

For both the sets after treatment slides were processed for lyses (1 h), unwinding (20 min) and electrophoresis (30 min) accordingly [34]. Scoring of slides was the same as for the genotoxicity assay.

The antigenotoxicity was measured as % Inhibition of DNA damage using the formula of Neffati et al. [36] and modified accordingly:

Antigenotoxicity (% Inhibition of DNA damage) = 100

\[
\frac{Tail \ DNA \ % \ in \ presence \ of \ ZFE \ or \ BA}{Tail \ DNA \ % \ in \ absence \ of \ ZFE \ or \ BA} \times 100
\]

The background value of tail DNA % in negative control was excluded from numerator and denominator.

2.6. Genotoxicity and antigenotoxicity of BA in vivo on mice

In vivo genotoxicity studies were carried on BA because of its known antitumor properties and its occurrence in ZFE. Antigenotoxicity was aimed to find out if BA could reduce MMS or H_2O_2 – induced tail DNA % measured as % inhibition of DNA damage.

Male Swiss albino mice (8–10 weeks old and weighing 20–25 g) were acclimatized for 2 weeks prior to experimental tests. Animals were kept in polycarbonate cages (five animals per cage) bedded on rice husk and maintained at 25 ± 2 °C, 60 ± 5% relative humidity conditions and a 12 h light/dark cycle. Food (standard rodent pellet diet) and water was available ad libitum. Our investigation was performed following the Guidelines for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH Publication No. 85-23, revised 1996) and also approved by the Institutional Animal Ethics Committee (IEAC), University of Calcutta, registered under “Committee for the Purpose of Control and Supervision of Experiments on Laboratory Animals” (CPCSEA), Ministry of Environment and Forests, Government of India.

2.6.1. Experimental design

Male mice were divided into 8 groups.

Group 1: Ten mice were gavaged 0.9% saline solution (served as negative control) for 6 days.

Group 2: Ten mice were gavaged BA 2.5 mg/kg body weight for 6 days.

Group 3: Ten mice were gavaged BA 5.0 mg/kg body weight for 6 days.

Group 4: Ten mice were gavaged BA 10.0 mg/kg body weight for 6 days.

Group 5: Five mice of group 1 were injected (i.p.) with MMS (40 mg/kg bw) on day 6, 24 h prior to sacrifice on the 7th day.

Group 6: Five mice of group 2 were injected (i.p.) with MMS (40 mg/kg bw) on day 6, 24 h prior to sacrifice on the 7th day.

Group 7: Five mice of group 3 were injected (i.p.) with MMS (40 mg/kg bw) on day 6, 24 h prior to sacrifice on the 7th day.

Group 8: Five mice of group 4 were injected (i.p.) with MMS (4 mg/kg bw), 24 h prior to sacrifice on the 7th day.

After the treatment period the animals were sacrificed and bone...
marrow cells, liver and kidney tissues were obtained for further analysis.

2.6.2. Analysis of DNA damage by the comet assay

Alkaline comet assay was performed with the cells of bone marrow, kidney and liver, to evaluate the DNA damage following the method described earlier. The conditions for lysis (1 h), denaturation (20 min), electrophoresis (30 min) and neutralization were the same as for the comet assay on lymphocytes. Slides were stained with EtBr and visualized by fluorescence microscope (Leica, Wetzlar, Germany, excitation filter 515–560 nm and barrier filter of 590 nm) equipped with a CCD camera. Comet images were captured and analyzed using a computerized system for image analysis (Komet 5.5, kinetic imaging; Andor Technology, Nottingham, UK). 150 randomly chosen nuclei in total were analyzed from 3 slides per treatment and expressed as percent of tail DNA.

Antigenotoxicity was measured as the percentage inhibition of DNA damage in the BA-primed animals using the formula [36] mentioned earlier.

2.7. Analysis of biochemical stress markers

2.7.1. Lipid peroxidation assay

For lipid peroxidation, the level of malondialdehyde (MDA) was measured in liver homogenates according to the method of Buege and Aust [37]. MDA is the end product of lipid peroxidation which reacts with thiobarbituric acid (TBA) produce a pink colored complex that has a peak absorbance at 535 nm. Briefly, liver tissue was homogenized with chilled physiological saline and centrifuged at 8000 g for 5 min. The supernatant was mixed with 2 ml TCA-TBA-HCl and heated for 15 min in boiling water bath, cooled on ice and centrifuged at 10,000 g for 10 min. The supernatant was collected and absorbance was measured at 535 nm and expressed as μM MDA/g protein.

2.7.2. Quantification of H2O2

Oxidative stress in terms of H2O2 production was evaluated by the method of Jiang et al. [38], with minor modifications. Liver tissue was homogenized in 50 mM phosphate buffer (pH 6.5). The homogenate was subjected to peroxide-mediated oxidation of Fe3+ followed by reaction with Fe3+ and xylene orange and the absorbance was measured at 560 nm and expressed as nM/g protein.

2.8. Analysis of antioxidant enzyme activity

Catalase (CAT, EC 1.11.1.6), guaiacol peroxidase (GPOD, EC 1.11.1.7) and total glutathione level were measured in liver cells. Liver tissues were homogenized in ice cold homogenizing buffer (50 mM phosphate buffer) that has a peak absorbance at 535 nm. Briefly, liver tissue was homogenized with chilled physiological saline and centrifuged at 8000 g for 5 min. The supernatant was mixed with 2 ml TCA-TBA-HCl and heated for 15 min in boiling water bath, cooled on ice and centrifuged at 10,000 g for 10 min. The supernatant was collected and absorbance was measured at 240 nm and expressed as μM MDA/g protein.

2.9. Statistical analysis

All experiments were performed in triplicate, and data were expressed as Mean ± SEM (standard error of mean of 3 independent experiments). The data analysis was done using the Statistical Programme–SigmaStat 3.0 (SPSS Inc., Chicago, IL, USA). One-way analysis of variance (ANOVA) test was carried out at P ≤ 0.05.

3. Results

3.1. Phytochemical analysis of ZFE

The three fractions of the Ziziphus fruit-extract were named as ZFE-1 (extracted with 100% ethanol), ZFE-2 (extracted with 75% ethanol) and ZFE-3 (extracted with 50% ethanol). The total phenolics (TPC) and flavonoid (TFC) content of ZFE was evaluated using the standard curve of gallic acid (y = 0.088x + 0.132, R2 = 0.99) and quercetin (y = 0.037x + 0.0017, R2 = 0.99) (Table 1). TPC was highest in ZFE-2 (7.01 ± 0.22 mg GAE/g) and TFC was highest in ZFE-1 (1.38 ± 0.09 mg QE/g).

Fig. 1 and Table 1 represents HPLC-DAD chromatogram and quantification of betulinic acid (BA) in ZFE. The highest amount of BA was found in ZFE-1 (11.93 ± 0.14 μg/mg) followed by ZFE-2 and ZFE-3 respectively.

3.2. Antioxidant activity of ZFE and BA

Table 1 shows the results obtained by the two most widely used assays for radical-scavenging activities and antioxidant capacities: DPPH (2, 2-diphenyl-1-picryl-hydrazyl-hydrate) scavenging activity and FRAP (ferric reducing antioxidant power) activity.

Increasing of DPPH radical scavenging activity was observed in ZFE (125–500 μg/ml) and BA (10–100 μg/ml) (Fig. 2A, B). Commercially available BA was used for all experiments. The IC50 value for radical scavenging activity of ZFE was lowest in ZFE-1 (340.2 ± 2.77 μg/ml) and that of BA was 154.01 ± 6.35 μg/ml. The highest FRAP value was found in ZFE-2 (17.27 ± 0.11AAE mg/g extract) followed by ZFE-3 and ZFE-1. The lowest FRAP value has been observed in BA (Table 1, Fig. 2C, D). Antioxidant activity of ZFE showed a strong positive correlation with the total phenolics present. The coefficient of Regression (R2) values were significant between TPC and FRAP (R2 = 0.999), and TPC and DPPH (R2 = 0.926) [data not shown]. This suggests that the phenolics in ZFE were responsible for its antioxidant activity which was absent in pure BA.

Table 1

Analysis of phytochemicals and antioxidant activity of betulinic acid and Ziziphus fruit ethanol extracts.

| Ziziphus fruit extract | Total polyphenolic content (mg GAE/g)a | Total flavonoid content (mg QE/g)b | Betulinic acid (μg/AAE/g)c | FRAP value (mg AAE/g) | DPPH scavenging activity (IC50 value μg/ml) |
|-----------------------|---------------------------------------|----------------------------------|---------------------------|-----------------------|--------------------------------------------|
| ZFE-1                 | 6.54 ± 0.17                           | 1.38 ± 0.09                      | 11.93 ± 0.14              | 9.51 ± 0.13           | 340.52 ± 0.28                             |
| ZFE-2                 | 7.01 ± 0.22                           | 1.06 ± 0.14                      | 7.62 ± 0.09               | 17.27 ± 0.13          | 553.79 ± 0.22                             |
| ZFE-3                 | 6.86 ± 0.30                           | 1.18 ± 0.09                      | 1.67 ± 0.04               | 14.83 ± 0.11          | 413.45 ± 0.21                             |
| BA                    | –                                     | –                                | –                         | 3.19 ± 0.05           | 154.01 ± 0.04                             |

Ziziphus fruit extract (ZFE): ZFE-1 (100% ethanol), ZFE-2 (75% ethanol) and ZFE-3 (50% ethanol); BA-betulinic acid, GAE – gallic acid equivalent, QE – quercetin equivalent, FRAP – Ferric Reducing Antioxidant Power, AAE – ascorbic acid equivalent, DPPH – 1, 1-diphenyl-2-picrylhydrazyl free radical, IC50 – the half maximal inhibitory concentration.

a Values are expressed as mean ± SEM of three independent experiments performed in triplicates.

Ziziphus fruit extract (ZFE): ZFE-1 (100% ethanol), ZFE-2 (75% ethanol) and ZFE-3 (50% ethanol); BA-betulinic acid, GAE – gallic acid equivalent, QE – quercetin equivalent, FRAP – Ferric Reducing Antioxidant Power, AAE – ascorbic acid equivalent, DPPH – 1, 1-diphenyl-2-picrylhydrazyl free radical, IC50 – the half maximal inhibitory concentration.

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3.3. Cytotoxicity, genotoxicity and antigenotoxicity of ZFE and BA in human lymphocytes

To find the IC50 value the cytotoxicity of the ZFE was tested over a high range of concentrations from 125 to 10,000 μg/ml. The results of MTT assay demonstrated that ZFE (ZFE-1, ZFE-2 and ZFE-3) was cytotoxic to the human lymphocytes at concentrations 2500 μg/ml and above. Compared with the untreated control, the cells treated with 125, 250, 500 and 1000 μg/ml of ZFE did not show any cytotoxic effect (Fig. 3A). The lymphocytes exposed to BA did not show any cytotoxic effect at the concentrations tested.

Genotoxicity of ZFE and BA in human lymphocytes were assessed by the alkaline comet assay and expressed as tail DNA%. Compared to the negative control, tail DNA% of ZFE (ZFE-1, ZFE-2 and ZFE-3) was significant at concentrations 5000 μg/ml and above. ZFE was non-genotoxic to the lymphocytes at the concentrations-125, 250, 500, 1000 and 2500 μg/ml (Fig. 3C) and BA was non-genotoxic at all the concentrations (10, 20, 40 μg/ml) tested (Fig. 3D).

The anti-genotoxic potential of ZFE and BA was also detected using the alkaline comet assay utilizing tail DNA % as an endpoint. The non-cytotoxic, non-genotoxic concentrations of ZFE (250, 500, 1000 μg/ml) and BA (10, 20, 40 μg/ml) were selected for the antigenotoxicity assay.

Lymphocyte cells were pre-incubated for 3 h with ZFE (250, 500 and 1000 μg/ml) or BA (10, 20, 40 μg/ml) and were challenged with MMS (50 μM) for 1 h. The results demonstrated that ZFE (Fig. 4A i) and BA (Fig. 4B i) were able to reduce the genotoxicity of MMS, measured as tail DNA%. The values of tail DNA % was ~62% for MMS (50 μM) alone and in combination with ZFE-1 was 39, 29, and 28% at the concentrations 250, 500 and 1000 μg/ml respectively (Fig. 4A i). A similar decreasing trend in the values of tail DNA % was scored for MMS in combination with ZFE-2 (45, 42 and 40 Tail DNA %) and ZFE-3 (55, 52 and 50 Tail DNA %) (Fig. 4A i). This decrease in the values of tail DNA % was calculated according to the formula of Neffati et al. [36] as anti-genotoxicity and expressed graphically as % inhibition of DNA damage in

![Fig. 1. HPLC-DAD chromatogram and quantification of betulinic acid (BA) in Ziziphus jujuba fruit extract (ZFE): ZFE-1 (100% ethanol), ZFE-2 (75% ethanol) and ZFE-3 (50% ethanol). (A) BA standard (B) ZFE-1 (C) ZFE-2 (D) ZFE-3. Chromatographic conditions: Agilent Eclipse plus C-18 column (150 mm × 4.6 mm, 3.5 μm), acetonitrile: water (86:14), λ = 210 nm, 0.5 ml/min flow rate.](image1)

![Fig. 2. Antioxidant activity of Ziziphus jujuba fruit extract (ZFE): ZFE-1 (100% ethanol), ZFE-2 (75% ethanol), ZFE-3 (50% ethanol) and betulinic acid (BA): DPPH free radical scavenging assay (A) ZFE-1, ZFE-2, ZFE-3 and (B) BA; FRAP assay (C) ZFE-1, ZFE-2, ZFE-3 and (D) BA. Results represent mean ± SEM of three independent experiments performed in triplicates.](image2)
As compared with MMS alone (50 μM), the % inhibition of DNA damage in ZFE pretreated lymphocyte cells were ~37 to 55% less in ZFE-1 (Fig. 4A ii), 27 to 32 % less in ZFE-2 (Fig. 4A iii), and 10 to 20 % less in ZFE-3 (Fig. 4A iv) and more than 60% less in BA (Fig. 4B ii). The percent inhibition of MMS-induced DNA damage was in the order ZFE1 > ZFE2 > ZFE3. Lymphocytes pretreated with BA showed a maximum inhibition of MMS-induced DNA damage with higher antigenotoxic activity. Therefore ZFE and BA exhibited antigenotoxic potential.

The DNA damage induced by H₂O₂ measured as tail DNA% was ~45.42 ± 4.39% (Fig. 5 A-i). Lymphocytes were pre-incubated for 3 h with ZFE (250, 500 and 1000 μg/ml) or BA (10, 20, 40 μg/ml) and were challenged with H₂O₂ (250 μM) for 5 min. ZFE-1 pre-treatment reduced the H₂O₂ – induced DNA damage from a value of 45.42 ± 4.39 (H₂O₂ Fig. 3. Cytotoxicity and genotoxicity of Ziziphus jujuba fruit extract (ZFE) and betulinic acid (BA) in human lymphocytes; ZFE-1, ZFE-2 and ZFE-3: Assessment of cell viability by MTT assay (A) ZFE-1, ZFE-2, ZFE-3 and (B) BA; Assessment of DNA damage by comet assay (C) ZFE-1, ZFE-2, ZFE-3 and, (D) BA. (* Significant with respect to control, p ≤ 0.05; Error bar represents ±SEM).

Fig. 4. A. (i) DNA damage measured as Tail DNA % in human lymphocytes exposed to MMS (50 μM) in combination with Ziziphus jujuba fruit extract (ZFE)-ZFE-1,ZFE-2and ZFE-3; Antigenotoxicity of ZFE against MMS (50 μM) measured as % inhibition of DNA damage by (ii) ZFE-1 (iii) ZFE-2 and (iv) ZFE-3; B. (i) DNA damage measured as Tail DNA % in human lymphocytes exposed to MMS (50 μM) in combination with BA (10, 20, 40 μg/ml); (ii) Antigenotoxicity of BA against MMS (50 μM) measured as % inhibition of DNA damage. (* Significant with respect to MMS, p ≤ 0.05; Error bar represents ±SEM).
alone) to 18.66 ± 2.05% tail DNA at 1000 μg/ml, ZFE-2 and ZFE-3 could reduce the value to 29.06 ± 3.55% and 36.23 ± 1.96% respectively (Fig. 5 A-i). This decrease in the values of tail DNA % of ZFE pre-treatment was concentration (250, 500 and 1000 μg/ml) dependent and antigenotoxicity expressed as % inhibition of DNA damage was ~44 to 60 % for the ZFE-1 (Fig. 5 A-ii), 21 to 36 % for ZFE-2 (Fig. 5 A-iii) and 11 to 20% for ZFE-3 (Fig. 5 A-iv).

Pre-treatment with BA reduced significantly the % tail DNA induced by H₂O₂ from a value of ~44.22 ± 4.39 (H₂O₂ 250 μM) to 17.86 ± 0.59 (10 μg/ml of BA), 15.82 ± 1.24 (20 μg/ml of BA) and 12.76 ± 2.35% (40 μg/ml of BA) (Fig. 5 B-i). Antigenotoxicity calculated as % inhibition of DNA damage showed a concentration dependent inhibition of H₂O₂-induced DNA damage by BA that was ~60 to 70 % (Fig. 5 B-ii).

Fig. 5. A. (i) DNA damage measured as Tail DNA % in human lymphocytes exposed to H₂O₂ (250 μM) in combination with Ziziphus jujuba fruit extract (ZFE)-ZFE-1,ZFE-2 and ZFE-3; Antigenotoxicity of ZFE against H₂O₂ (250 μM) measured as % inhibition of DNA damage by (i) ZFE-1 (ii) ZFE-2 and (iv) ZFE-3; B. (i) DNA damage measured as Tail DNA % in human lymphocytes exposed to H₂O₂ (250 μM) in combination with BA (10, 20, 40 μg/ml); (ii) Antigenotoxicity of BA against MMS (50 μM) measured as % inhibition of DNA damage. (* Significant with respect to H₂O₂, p ≤ 0.05; Error bar represents ±SEM).

Fig. 6. DNA damage measured as Tail DNA % induced by betulinic acid (BA) in vivo in mice in – (A) liver (B) kidney and (C) bone marrow cells; DNA damage measured as Tail DNA % induced by BA in combination with MMS in vivo in mice in – (D) liver (E) kidney and (F) bone marrow cells; Antigenotoxicity of BA against MMS (40 mg/kg b.w.) measured as % inhibition of DNA damage in (G) liver (H) kidney and (I) bone marrow cells. (* Significant with respect to MMS, p ≤ 0.05; Error bar represents ±SEM).
3.4. Genotoxicity and antigenotoxicity of BA in vivo on mice

Absence of genotoxicity was observed in mice primed with BA at concentrations 2.5, 5 and 10 mg/kg b.w. The comet parameter % tail DNA in liver, kidney and bone marrow (Fig. 6A, B, C) cells were not significantly high with the lowest concentration giving the closest values to the negative controls. MMS (40 mg/kg b.w.) induced a significant increase in DNA damage (tail DNA %) in all the organs mentioned above.

To study antigenotoxicity, animals were primed with BA (2.5, 5 and 10 mg/kg mice b.w.) for 6 days and administered with a single dose of MMS (40 mg/kg b.w.) 24 h prior to sacrifice. The genotoxicity of MMS, measured as tail DNA % in liver, kidney and bone marrow cells were reduced significantly by BA at all the concentrations when compared to the group treated with MMS alone (Fig. 6D, E, F). Antigenotoxicity calculated as % inhibition of DNA damage showed a concentration dependent inhibition of MMS-induced DNA damage by BA that was ~28 to 28 % in liver (Fig. 6G, ~48 to 22 % in kidney (Fig. 6H) and ~37 to 22 % in bone marrow cells (Fig. 6 I).

3.5. Changes in biochemical stress markers in mice liver

The MDA and H2O2 content in liver tissues of mice primed with BA (2.5., 5 and 10 mg/kg mice b.w.) did not show any difference when compared to the control mice (Fig. 7A and C). A significant increase in MMS-induced level of MDA and hydrogen peroxide (H2O2) content was observed in liver cells of mice administered with MMS alone (Fig. 7 B and D). BA in combination with MMS treatment in BA primed animals resulted in lower MDA and H2O2 contents in liver cells which were significant when compared with MMS alone.

3.6. Relative changes in antioxidant defense responses in mice liver

The CAT and GPOD enzyme activity and total glutathione level in liver tissues are presented in Fig. 8. The CAT and GPOD enzymes in mice liver cells were significantly higher in mice treated with different concentrations of BA (Fig. 8 A and C) and lower in mice treated with MMS (Fig. 8 B). BA when administered with MMS could significantly increase CAT and GPOD activity in liver cells of mice than in mice administered MMS alone (Fig. 8B, C, D) Hepatic glutathione level that was decreased significantly by MMS treatment was ameliorated by BA pre-treatment (Fig. 8E and F).

4. Discussion

In order to be used in human practice, natural plant products must be biosafe, non cytotoxic and non genotoxic. Taking into account the available published data about the biological activity of Ziziphus, we hypothesized that this natural product would have anti-genotoxic properties preventing DNA damage induced by a genotoxin such as methyl methane sulphonate (MMS) or reducing DNA damage by a ROS inducer H2O2.

Therefore our initial experiments were focused on the cytotoxicity and genotoxicity of the fruit extract.

We investigated the genotoxic activities of various concentrations of Ziziphus fruit ethanol extracts (ZFE-1, 2 and 3) and betulinic acid (a known anticancer agent present in ZFE) using human lymphocyte cells in vitro and Swiss albino male mice in vivo. Our results showed that ZFE and BA were non cytotoxic and non genotoxic to the human lymphocytes.

Genotoxicity of ZFE and BA in human lymphocytes assessed by the alkaline comet assay and expressed as % tail DNA were not significantly different when compared to the negative control at concentrations below 5000 μg/ml of ZFE. Treatment with BA under in vivo conditions at concentrations 10 mg/kg b.w and below, did not cause significant increase in DNA damage to the liver, kidney and bone marrow cells of mice with the lowest concentration giving the closest values to the negative controls. The results obtained by us are in agreement with those reported by others. Ziziphus fruit extract was reported to be non toxic to PC12 cells [43], HepG2 cells [44] and normal human lymphocytes [45]. On the contrary cytotoxic activity of Ziziphus fruit extract was reported on tumor cells [45], breast cancer cells [46] and Triticum vegetal cells [47]. Similar to our findings, Frolova et al. [48], demonstrated the absence of mutagenic and genotoxic activity of BA in the Ames test and SOS chromotest. A lack of genotoxicity of BA, a triterpenoid, has also been reported in normal human lymphocyte and fibroblast cells [19, 49, 50].

In continuation to our findings that ZFE and BA was non cytotoxic and non-genotoxic to the human lymphocytes, we were interested to investigate whether ZFE and BA had antigenotoxic properties. The antigenotoxicity was measured as the reduction or inhibition of DNA damage induced by the two known genotoxins-methyl methane sulphonate (MMS) and H2O2. The concentrations of ZFE and BA that were found non-genotoxic were utilized for such antigenotoxicity studies. The results indicated that ZFE and BA demonstrated antigenotoxic potential and were able to inhibit the DNA damaging effect of MMS and H2O2 in human lymphocytes. Recently, Etebari et al. [44] reported that Ziziphus jujuba could prevent genotoxicity induced by MMS in HepG2 cells. This reduction of DNA damage was attributed to the interactions of the phenolics, flavonoid, BA and the antioxidants present in Ziziphus.

Our in vitro study was further validated in vivo in animal model to find out whether BA could prevent genotoxic (DNA) damage caused by MMS. Such studies are scarce and BA is known as an important bioactive chemical present in the Ziziphus fruit. We found that betulinic acid (BA) applied at nontoxic concentrations (2.5–10 mg/kg b.w.) showed antigenotoxic potential against the alkylating agent MMS (40 mg/kg b.w.). BA reduced the frequency of MMS-induced DNA damage in liver, kidney and bone marrow cells of mice thereby exhibiting its antigenotoxic property. Acsö et al. [21], also reported that treatment with BA and MMS resulted in lower micronucleus frequencies than those observed for V79 cultures treated with MMS alone.

It is well known that the antioxidant enzymes present in plants are responsible for their antigenotoxic activity. A majority of the phytochemicals with antioxidant activity also show anti-mutagenic and anti-genotoxic potential [51, 52, 53]. Therefore, the investigation on the antioxidant properties of ZFE was of prime importance. Potent antioxidant activities of Z. jujuba were reported for extracts from its seeds, fruits and leaves [54, 55]. Zhang et al. [54] carried a systematic study on the antioxidant capacities of various tissues of jujube plant and found that the highest antioxidant capacity was in the peel of the fruits. The radical scavenging activities of ZFE were confirmed in both DPPH and FRAP assays demonstrating the antioxidant property of the fruit. This is in accordance with the previous reports [54, 56]. DPPH and FRAP values of BA were low when compared to ZFE which is in accordance with the previous report by Nurul et al. [57]. The low antioxidant activity of BA might be due to the absence of phenolic group in its structure. Antioxidant activity of ZFE showed a strong positive correlation with the total phenolics present and is in accordance to that reported in five Chinese jujube cultivars [5, 54, 58]. This suggests that the phenolics in ZFE were responsible for its antioxidant activity. It was also observed that the amount of BA in ZFE was higher with the increasing amount of ethanol in the extraction solvent (ZFE-1 > ZFE-2 > ZFE-3). As a result the different concentrations of ZFE-1 were more potent in decreasing the DNA damaging effects of H2O2 and MMS followed by ZFE-2 and ZFE-3. In vivo study on mice confirmed that MMS caused oxidative damage in mice liver by inhibiting an enzymatic system (CAT, GPOD), reducing total glutathione level and enhancing lipid peroxidation (MDA) and H2O2 content. BA when administered with MMS could significantly increase CAT and GPOD activity in liver cells of mice than in mice administered MMS alone. Our study established that BA might act by up regulating the antioxidant status of the cells further making them more resistant to oxidative DNA damage. These results are consistent with other studies where BA was found to inhibit oxidative damage in mice splenocytes and thymocytes exposed to dexamethasone [59, 60]. The antioxidant, immunomodulative and hepato-protective properties were connected to
Fig. 7. Biochemical stress markers in liver cells of mice primed with betulinic acid (BA) and treated with MMS: MDA (A) BA, (B) BA followed by MMS treatment; H$_2$O$_2$ in (C) BA, (D) BA followed by MMS treatment. (* Significant with respect to MMS, $p \leq 0.05$; Error bar represents ±SEM).

Fig. 8. Antioxidant enzymes in liver cells of mice primed with betulinic acid (BA) and treated with MMS: Catalase activity – (A) BA, (B) BA followed by MMS treatment; Guaiacol peroxidase activity – (C) BA, (D) BA followed by MMS treatment; Total glutathione content – (E) BA, (F) BA followed by MMS treatment. (* Significant with respect to MMS, $p \leq 0.05$; Error bar represents ±SEM).
its ability to reduce oxidative stress [61, 62].

5. Conclusions

The *Ziziphus* fruit ethanol extract was found to be rich in polyphenols with antioxidant activity. The fruit extract and betulinic acid showed an ability to decrease the frequency of DNA damage induced by two known genotoxins namely an alkylating agent methyl methane sulphonate (MMS) and a ROS inducer hydrogen peroxide (H2O2) in human lymphocytes in vitro and in vivo in Swiss albino male mice. This anti-genotoxic property was manifested by the up-regulation of antioxidant enzyme activities. However, the biological effects of other identified and unidentified compounds in these fruits should be also investigated. Considering the antioxidant and antigenotoxic properties, *Ziziphus* fruit can be promoted as potential genoprotective compound and the consumption of the *Ziziphus* fruit should be more popularized worldwide.

Declarations

**Author contribution statement**

Priya Goswami, Ritesh Banerjee: Performed the experiments; Analyzed and interpreted the data; Contributed reagents, materials, analysis tools or data; Wrote the paper.

Anita Mukherjee: Conceived and designed the experiments; Contributed reagents, materials, analysis tools or data; Wrote the paper.

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**Competing interest statement**

The authors declare no conflict of interest.

**Additional information**

No additional information is available for this paper.

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