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Resveratrol protects acrylamide / glycidamide induced genotoxicity in hepatic cell line

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Abstract
Resveratrol has been regarded to exhibit antigenotoxic effects. Resveratrol was isolated from healthy grape berries and wine. The *trans*-resveratrol was purified, characterized by HPLC and LC-MS. In this study the protective effect of *trans*-resveratrol against genotoxicity induced by acrylamide (AA), a toxicant formed in carbohydrate-rich foods subjected to temperatures, above 140º C and its metabolite glycidamide (GA) resulting from cytochrome P450 2E1 (CYP2E1) was investigated in HepG2 cells. Pretreatment of HepG2 cells with resveratrol (RES) showed ameliorating effects on AA and GA induced cytotoxicity, decreased the reactive oxygen species (ROS), and prevented DNA damage, micronucleus induction. Also resveratrol exhibited decreased in the activity, mRNA levels of *CYP2E1* and increased activity and gene expression of *GST*, thus preventing the accumulation of GA. Also, RES showed the upregulation of few of the DNA repair genes thus preventing mutation accumulation induced by AA and GA. In conclusion RES potential antioxidant property protects HepG2 cells from genotoxicity induced by AA and GA.

Keywords: acrylamide, glycidamide, genotoxicity, oxidative stress, resveratrol, DNA repair genes

Introduction
The global increase in exposure of humans to bakery products such as French fries, potato chips, crisp bread, bread crust and cereal formulations containing AA, a toxic compound intern exerts a cytotoxic, genotoxic, and neurotoxic effect on human health and has raised public health concerns [1, 2]. Also, the metabolic conversion of AA to GA *via* an epoxidation reaction that is facilitated by cytochrome P450 (CYP2E1) is critical for the genotoxicity of AA, as it was reported to be a more potent mutagen including its ability to bind DNA causing genetic damage [3, 4, 5]. Thus, Human exposure to AA through their diet needs to be given importance without negligence. Resveratrol (3, 5, 4'-trihydroxy-trans-stilbene) is a natural polyphenol, produced in grapes, blueberry, raspberry, and mulberry fruits in response to injury or due to the attack by pathogens such as bacteria or fungi [6]. It has been reported to have antioxidant, antimutagenic, anticarcinogenic and anti-inflammatory properties [7, 8]. Although resveratrol is effective in scavenging free radicals and an excellent antioxidant, it remains unclear whether this compound is effective in AA-induced genotoxicity. Hence, the present study was investigated to study the protective effect of resveratrol towards AA and GA-induced genotoxic effects. HepG2 cells were used in the present investigation since HepG2 have been used in toxicogenomics studies because of its competent in the biotransformation of xenobiotic compounds to active mutagens and carcinogens. These, in turn, activate the DNA damage response of the cell [9, 10].

Materials and Methods
Chemicals
Minimum Essential Media (MEM), Phosphate buffer saline (PBS), fetal bovine serum (FBS), trypsin, Dimethyl sulphoxide (DMSO), nicotinamide adenine dinucleotide phosphate (NADPH), was purchased from Himedia, India. Acrylamide, glycidamide, 2,7-dichlorofluorescein diacetate (DCFH-DA), 1-chloro-2,4-dinitrobenzene (CDNB), penicillin/streptomycin, methyl thiazol tetrazolium bromide (MTT), Hoechst 33258 stain solution, cytochalasin B, *t*-resveratrol, *p*-nitrocatechol, Bovine Serum Albumin (BSA) were purchased from Sigma-Aldrich Chemicals, India. RNA isolation kit was purchased from GE Life Sciences, India. cDNA synthesis kit from Thermo Fisher Scientific, India and Syber green (Fast start Essential DNA Green Master) from Roche Diagnostics, Germany. The other Chemicals used were of the highest analytical grade and were from standard chemical companies.

Primers

The primer sequence for the genes – *CYP2E1, EPHX1, GAPD, GSTA2, GSTA4, DDB1, RAD52, RPA1, XRCC1, XRCC5* were obtained from the literature [10, 11, 12]. The primers were synthesized by Sigma-Aldrich Chemical Company, Bangalore, India.

Resveratrol isolation and characterization

The red variety of healthy grape berries (*Vitis vinifera*) *was* procured from local market, Mysore, India. Resveratrol was isolated from the skin of grape berries as described by [13]. Breifly the skins were removed manually, dried in an oven at 60º C for 24 h, and were powdered. 20 g of the grape skin powder was homogenized for 30 s in a magnetic stirrer with 200 mL of ethanol: water in the ratio of 80:20 and maintained at 60°C for 30 min in dark, with gentle stirring and was kept overnight at 37°C in the dark. The extract was filtered through Whatman 1 Filter paper and the filtrate obtained was centrifuged at 10,000 g for 5 min. The supernatant obtained was concentrated by rotary evaporation (Buchi, Switzerland) at room temperature. The residue left was dissolved in 100% methanol.

Also resveratrol was isolated from wine prepared in the laboratory. The resveratrol was exracted from wine by ethylene acetate extraction. The solvent extraction fractions were pooled and the solvent was evaporated to dryness and the residue was dissolved in 100% methanol. The isolated compound from both skin of grape berries and wine was filtered through a 0.2 µm membrane filter and was purified by analytical HPLC, LC-10A (Shimadzu, Japan), using reverse phase C-18 column (300×6 mm, 5 µm hypersil). Separation was based on isocratic condition with methanol: water (1:1), as a mobile phase at 1mL/min flow rate. *Trans*-Resveratrol was detected using a UV-Vis detector at 320 nm at 40 ºC. The chromatographic peak of resveratrol isolated was identified in comparison with retention time of standard *trans*-resveratrol obtained from Sigma Chemicals. Resveratrol was quantified using calibration of external standard *t*-resveratrol [14]. The purified fractions of *t*-resveratrol were further purified by preparative HPLC using the same analytical condition. The Purified fractions collected were concentrated by rotary evaporation and the residue obtained was dissolved in 100% methanol. The isolated resveratrol was characterised by LC–MS (Q-TOF-Ultima Global) and the molecular mass was determined by negative electrospray ionization.

Cell Culture

The Human hepatocellular liver carcinoma cell line (HepG2) was purchased from National Centre for Cell Science (NCCS), Pune, India. The cells were cultured in Minimum Essential Medium (MEM) containing 10% fetal bovine serum, 1% penicillin/streptomycin (10 mg/mL) in a humidified atmosphere of 95 % air with 5% CO_2 at 37° C and were sub-cultured twice a week.

Cell viability assay

The effective concentrations of AA, GA and resveratrol on HepG2 cells were determined by MTT assay. About 1×10^5 cells/well seeded in 96 well plate were treated with AA, GA (0.0005-500 mg/L) and resveratrol (25-200 µM) dissolved in 0.2 % DMSO for 24 h at 37˚ C, 5% CO_2 and untreated cells were maintained as a control. Subsequently, the protective effect of resveratrol against AA and GA-induced cytotoxicity was tested. The cells were pre-exposed to resveratrol (100 µM) for 24 h followed by AA and GA exposure for an additional 24 h. Resveratrol treated cells were maintained as a control. The above-treated cells were treated with MTT at a concentration of 5 mg/mL for 4 h at 37˚ C followed by addition of the DMSO to dissolve the formazan crystal formed. The optical density at 570 nm was measured by Varioscan Flash microtiter plate reader (Thermo Scientific, Singapore). The cell viability was expressed as the percentage of viable cells after exposure to AA, GA and resveratrol as compared to the untreated control.

Measurement of ROS

The intracellular ROS produced was determined using the 2, 7-dichlorofluorescein diacetate (DCFH-DA) method [15]. Subconfluent HepG2 (2×10^5 cells/mL) cultures were exposed to AA and GA at a concentration of 5-500 mg/L for 24 h; the other set was pre-exposed to resveratrol (100 µM) for 24 h before treatment with AA and GA. After the AA and GA exposure for 24 h, PBS-washed cells were trypsinized and pelleted. The cells were resuspended in fresh media, treated with DCFH-DA (5 µM, for 40 min in the dark, at 37° C). The fluorescence intensity of the fluorescent dichlorofluorescein (DCF) formed was measured with an excitation wavelength of 485 nm and an emission wavelength of 550 nm, respectively.

Enzyme assays (GST and CYP2E1)

The effective concentration of AA (200 mg/L), GA (100 mg/L) and resveratrol (100 µM) (based on MTT assay) were used for further analysis. HepG2 cells were treated with respective concentrations of AA and GA to study the metabolic fate of AA, GA and its genotoxicity. Also, the study of protection offered by resveratrol on AA and GA-induced cells were carried by pretreatment of cells to resveratrol for 24 h, followed by AA and GA for another 24 h. The untreated cells were maintained as control. All the above treated and control cells were harvested, washed with PBS and pelleted and cells were lysed by lysis buffer and sonicated for homogenization. The total cellular protein concentration was estimated by Bradford assay using BSA standard [16]. All enzyme analyses were carried out with freshly prepared cell lysates of treated and untreated control cells. Total GST activity was measured according to Habig *et al.* [17]. The reaction was initiated by adding 2 µL each of 100 mM CDNB, 200 mM reduced glutathione (GSH) to 0.5 mg of protein cell lysate in phosphate buffer (0.1 M, pH 6.5), and incubated at 30º C for 5 min. The absorbance of CDNB conjugation with GSH was monitored at 340 nm using Varioscan Flash microtiter plate reader. The GST activity was expressed as nmol of CDNB-GSH conjugate formed/min/mg protein ($\varepsilon = 5.3\ mM^{-1}$).

CYP2E1-catalysed *p*-nitrophenol hydroxylation activity was assayed by measuring the amount of *p*-nitrocatechol formed as given by Chang *et al.* [18]. To an assay cocktail of 200 µL containing 5 mM *p*-nitrophenol, 0.5 mM NADPH, 1 mg of protein cell lysate was added and was made up to 200 µL with potassium phosphate buffer (50 mM, pH 7.4). The incubation was carried for 30 min at 37º C. The enzymatic reaction was stopped by adding 20% TCA, centrifuged and 2 M NaOH was added to the supernatant. The absorbance was read at a wavelength of 535 nm (Varioscan Flash ELISA reader, Thermo Scientific, Singapore). The amount of *p*-nitrocatechol metabolite formed was quantified using the *p*-nitrocatechol standard curve, and the activity was presented as nmol of *p*-nitrocatechol formed/min/mg protein.

Study of gene expression by RT-qPCR

Total RNA was isolated from HepG2 cells of AA (200 mg/L), GA (100 mg/L) treatment and resveratrol pretreatment (100 µM) using GE Life Sciences RNA isolation kit according to manufacturer's instructions. The RNA isolated was quantified in a spectrophotometer at 260/280 nm (Nanodrop, Eppendorf) and the reliability was tested using a 1% agarose gel. cDNA was synthesised, using Thermo Scientific First-strand cDNA synthesis kit as per manufacturer's instructions. cDNA synthesized was stored at −20° C for further use. The quantitative single-step RT-PCR assay was performed using gene-specific primers, and the sequences were adopted from the literature as mentioned above. Preliminary control experiments (data not shown) were performed to validate the RT-PCR conditions that permitted for linear amplification of PCR products. The PCR products were analysed by electrophoresis on 1.5 – 2% agarose gels containing ethidium bromide.

Gene expression studies were performed in the C1000 Touch thermal Cycler (Biorad CSH96) using Syber Green (Fast start Essential DNA Green Master) with cDNA at a concentration of 10 nmol and 200 nmol forward and reverse gene-specific primer. The PCR parameters were 95° C for 5 min, 95° C for 20 sec, 60° C for 40 sec (annealing temperature), 72° C for 20 sec, and 45 cycles. For RT-qPCR, *GAPD* was employed as the internal control, and the RNA level of each gene of interest was normalized to the level of *GAPD* for comparison. The delta-delta Ct (ΔΔCt) method was used to analyse the relative gene expression [19].

DNA damage Assay

DNA damage assay was carried by the isolation of genomic DNA. The cells harvested from AA (200 mg/L), GA (100 mg/L) and resveratrol pretreated (100 µM) AA/GA-induced cells were washed with PBS. Then were suspended in TNES lysis buffer (10 mM Tris, 400 mM NaCl, 100 mM EDTA, 0.6% SDS), pH 8.0 followed by addition of Proteinase K (20 mg/mL), homogenised and was incubated at 50º C for 24 h. After incubation 6 M NaCl was added and shaken vigorously for 20 sec and was centrifuged at 10,000 rpm for 5-10 min. To the supernatant an equal volume of cold 100% ethanol was added and was kept at -20º C overnight to precipitate DNA. After 24 h, it was centrifuged, the DNA pellet obtained was washed with 70% ethanol 2 to 3 times and air dried for 10-30 min. The DNA pellet was then resuspended in TE buffer (10 mM Tris–HCl pH8.0; 1 mM EDTA). The concentration and purity were determined by measuring absorbance at 260 nm and 280 nm using Nanodrop. The isolated genomic DNA of respective treatments was assessed for DNA damage by agarose gel electrophoresis carried in 1% agarose gel. The gel electrophoresed was stained with ethidium bromide (0.5 µg/ml), visualised and photographed (Cleaver Scientific Ltd Gel Doc system) for DNA damage.

Micronucleus (MN) assay

The MN assay was carried according to Varga *et al.* [20] with slight modification. Nearly 2 x 10^5 HepG2 cells grown on poly-L-lysine coated cover slip for 24 h, placed in 6 well plates were exposed to AA (200 mg/L), GA (100 mg/L) for 24 h. Pre-exposure with resveratrol (100 µM) for 24 h followed by AA/GA exposure was carried out as given above. After incubation, cold PBS-washed cells were resuspended in fresh medium containing cytochalasin B (6 µg/mL) for the next 6 h. The cells were trypsinized, washed with PBS and treated with 0.56% KCl solution for 15 min at 28° C. The cell fixation was carried using cold methanol: acetic acid (5:1) and was stained with fluorescent dye Hoechst 33258 (0.125 µg/mL in PBS). 1000 binucleated cells (BNC) were scored to evaluate the frequencies of MN formed in AA, GA exposed cells and resveratrol pre-exposed cells in comparison with control untreated cells. The MN image acquisition was taken under a confocal microscope (LSM-700; Carl-Zeiss, Germany) and the images were processed using the Zen software.

Statistical analysis

The results were expressed as a mean ± standard error from the replicates of the three independent experiments. The data were analysed using analysis of variance (ANOVA). Values less than 0.05 were considered as significant. All the data were analysed by the software program, Graph pad prism 5.0.

Results and Discussion

In the present study, *t*-resveratrol isolated was quantified by validated HPLC method from skin of grape berries and wine prepared from red grapes. Table 1 describes the concentration of *t*-resveratrol present in grape skin powder extract and wine extract. The identification of resveratrol was confirmed by LC–MS (ESI negative) with a molecular ion peak at m/z 227 (resveratrol has a molecular weight of 228).

Table 1: Amount of trans-resveratrol in grape berry skin and wine.

Sample	*trans*-resveratrol[a]
Grape berry skin (µg/g of dry skin)	459.97 ± 1.2
Wine (mg/L)	3.35 ± 0.8

[a] Mean value ± SD (n=3)

Effect of resveratrol on AA and GA-induced cytotoxicity in HepG2 cells

Cell viability of HepG2 cells was assayed by MTT by treatment of cells with different doses of resveratrol, AA and GA for 24 h. The resveratrol up to 100 µM did not present a significant difference in cytotoxicity compared to control HepG2 cells (Fig. 1A). Thus the protective effect of resveratrol on AA and GA-induced cytotoxicity was studied at 100 µM concentration of resveratrol. Cytotoxicity of AA and GA in HepG2 cells was observed to be concentration dependent. AA showed 50% reduction in cell viability at 200 mg/L and GA exhibited the same at 100 mg/L concentration. Hence GA was observed to be more cytotoxic compared to AA. Hence these concentrations were chosen for further experiments. However, cells pretreated with resveratrol (100 µM) for 24 h followed by AA and GA-treatments for another 24 h to assess the protective effect of resveratrol on AA and GA-induced cytotoxicity showed an increase in cell viability. Resveratrol restored viability of AA (200 mg/L) treated cells from 53.2% to 85.7% and GA (100 mg/L) treated cells from 52.9% to 76.8%. Thus, the protective effect of resveratrol on the cytotoxicity of AA and GA were observed. But at higher concentrations of AA and GA, the protection offered by resveratrol was hindered (Fig. 1B and 1C).

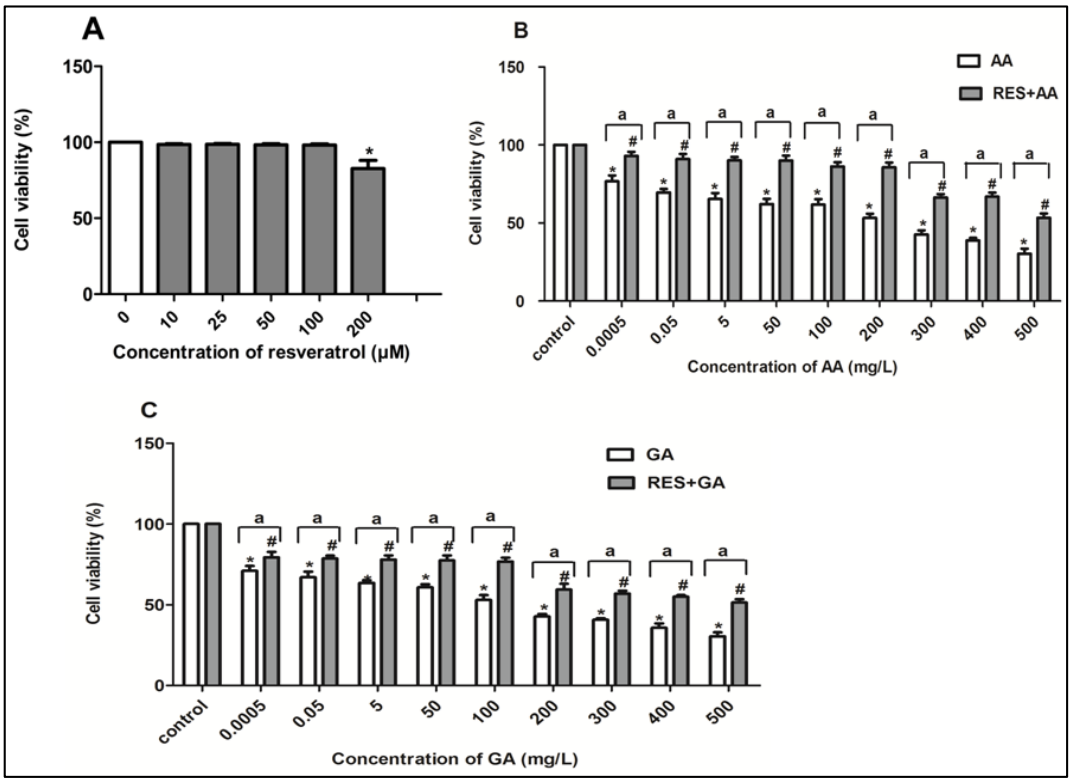

Fig 1: (A) Effect of different concentration of resveratrol on cell viability of HepG2 cells. Protective effect of resveratrol (100 µM) on (B) AA and (C) GA-induced cytotoxicity in HepG2 cells. All symbols represent a significant difference in cell viability. **P*< 0.05 (resveratrol, AA, GA-treated in comparison to control cells), #*P*< 0.05 (resveratrol pretreated, AA and GA-induced cells to the control cells. aaa*P*< 0.001 (resveratrol pretreated AA, GA-induced cells compared to AA, GA-induced cells

Effect of resveratrol on ROS formation induced by AA and GA in HepG2 cells

The ROS estimated in AA, GA treated cells at 5-500 mg/L concentrations showed a significant increase in ROS level and the increase was concentration dependent as in Fig. 2A and 2B. ROS formation was more with GA compared to AA. The oxidative stress resulting from the reactive oxygen species (ROS), plays a vital role in the genotoxicity of AA which intern damage DNA directly ensuing in mutations, tumour promotion, and cancer development [21, 22].These hostile effects can be prevented by antioxidants that can eliminate ROS [23]. Curcumin and mulberry digest (MBD) was found to have a protective effect on AA-induced oxidative stress [24, 25]. Our present study of pretreatment of HepG2 cells to resveratrol (100 µM) followed by exposure to AA, GA (500 mg/L), the ROS formation was reduced by 50% in comparison with AA-treated cells and 30.6% compared to GA-treated cells. These results suggested that the preventive effects of resveratrol might bring about ROS scavenging. The antioxidant property of resveratrol may be one of the hepatoprotective effects [26]. Therefore, resveratrol has been considered as protective to hepatic cells in relative to the genetic risk associated with the usage of AA and GA.

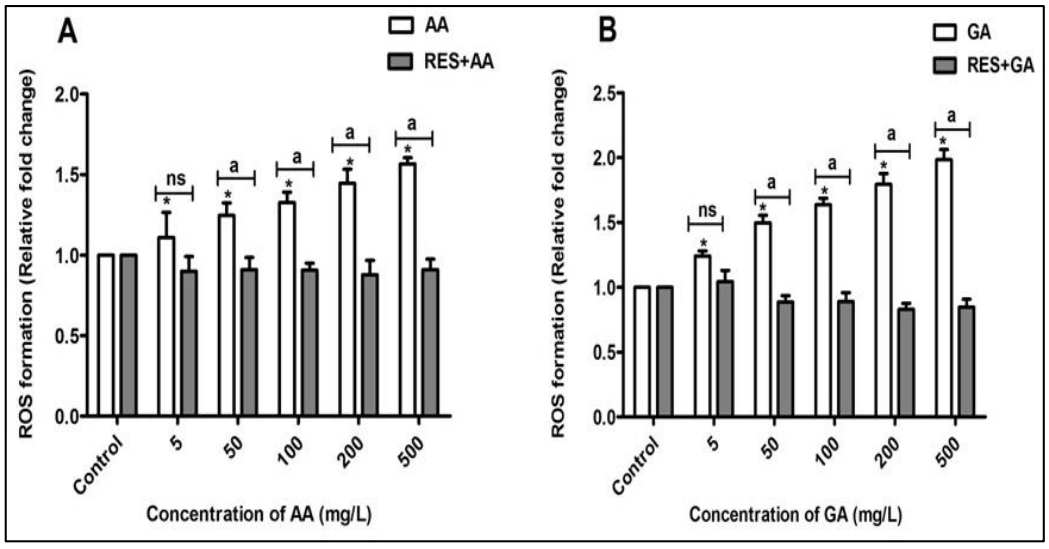

Fig 2: Protective effect of resveratrol (100 µM) on AA and GA-induced ROS formation in HepG2 cells. Significant difference in ROS formed between (A) **P*< 0.05 (AA-induced to the control untreated cells, a*P*< 0.05 (resveratrol pretreated AA-induced to the AA-induced cells (B) **P*< 0.05 (GA-induced to the control untreated cells, a*P*< 0.05 (resveratrol pretreated GA-induced to the GA-induced cells. ns-not significant.

Effect of resveratrol on CYP2E1 and GST activities induced by AA and GA

Earlier studies have demonstrated that the enzyme involved in the metabolism of AA to GA is CYP2E1 which is later metabolized by either conjugation with glutathione (GSH) by Glutathione S- transferase (GST) or hydrolyzed by epoxide hydrolase [27]. Enzymatic assays and RT-qPCR was used to find out the activity and mRNA expression of few detoxification enzymes of AA and GA metabolism which includes phase I - CYP450 (*CYP2E1*), phase II – GST (GST2, GST4), microsomal epoxide hydrolase (*EPHX1*) in HepG2 cells treated with AA (200 mg/L) and GA (100 mg/L). Also, the impact of these genes was studied in resveratrol pretreated AA, GA-induced cells. The total GST activity of AA, GA treated cells was significantly increased relative to untreated control cells with 0.81 and 1.9-fold respectively. Also, the resveratrol pretreated AA, GA-induced HepG2 cells presented a significant increase in GST activity of 1.82, 2.08 folds with respect to control cells, however when compared to AA-treatment, the GST activity was increased by 0.55 fold, but no significant change was seen in resveratrol pretreated to GA-induced cells (Fig. 3A). CYP2E1, estimated by CYP2E1-associated *p*-nitrophenol hydroxylation activity caused statistically significant 1.98 and 1.56 fold increase in AA and GA treated cells relative to untreated control cells. But the CYP2E1 activity in cells pretreated with resveratrol followed by AA, GA treatment, resulted in a significant decrease in activity by 1.76 and 0.54 folds respectively when compared to AA, GA treated cells (Fig. 3B).

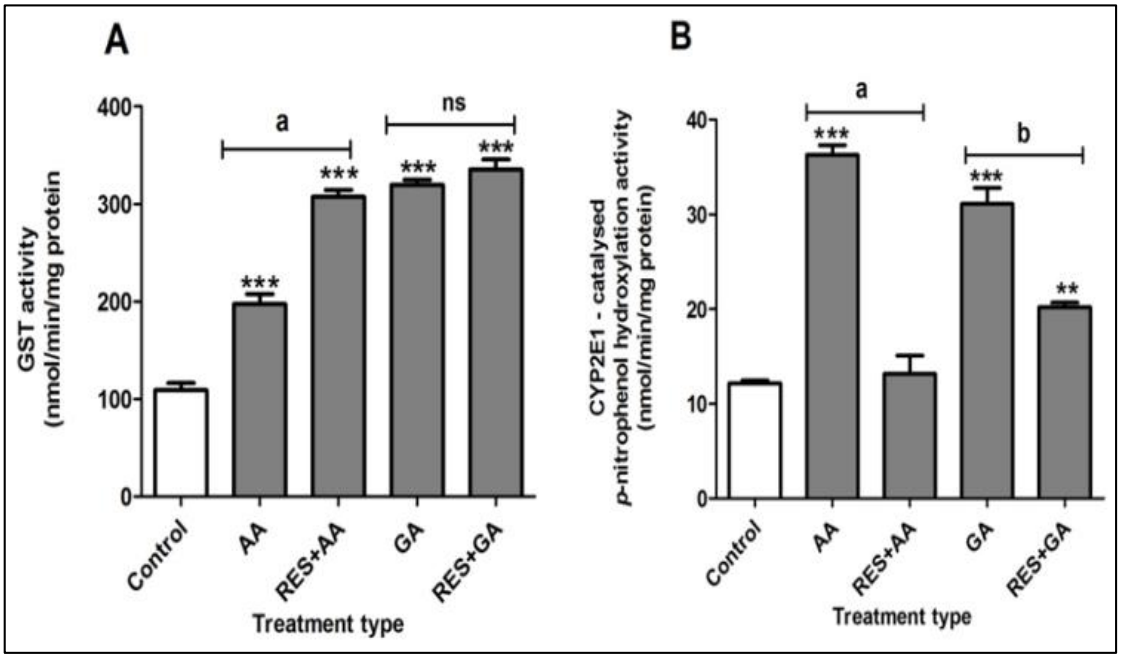

Fig 3: (A) GST, (B) CYP2E1 activity of AA (200 mg/L), GA (100 mg/L) treated and resveratrol (100 µM) pretreated HepG2 cells. ***P*< 0.01, ****P* 0.001 denotes significant difference between AA, GA-induced cells, resveratrol pretreated AA, GA-induced cells to the control untreated cells. [a]*P*< 0.001 (AA-induced cells to the resveratrol pretreated AA-induced cells), [bb]*P*< 0.01 (GA-induced cells to the resveratrol pretreated GA-induced cells. ns (not significant).

Effect of resveratrol on gene expression of Phase I and Phase II detoxification genes

The gene expression of few of the AA, GA metabolism was studied. The mRNA levels were calculated as the fold of expression (FOE), FOE >1 is considered as upregulation and FOE < 1 as downregulation of gene expression. The *CYP2E1* gene, involved in the degradation of AA to toxic metabolite GA was upregulated in AA-induced and downregulated in GA-treated HepG2 cells. On the other hand, the resveratrol pretreated AA, GA-induced cells resulted in downregulation of *CYP2E1* gene expression. There was no significant upregulation of *EPHX1* gene in AA and GA treated cells, a gene of GA metabolism. Both *GST2, GST4* of AA-treatment were upregulated by 6.49, 1.32 folds and resveratrol pretreated AA exposed cells showed upregulation by 4.64 and 2.45 folds when compared to control cells. However, only *GST4* gene was upregulated in both GA-treated and resveratrol pre-exposed GA-treated cells with an increase in the fold of expression of 3.1 folds compared to GA-treated cells (Fig. 4A and 4B). Thus pre-treatment of HepG2 cells with resveratrol showed a decrease in activity and gene expression of CYP2E1 and increase in GST activity and mRNA levels thus indicates the protective effect of resveratrol on genotoxicity.

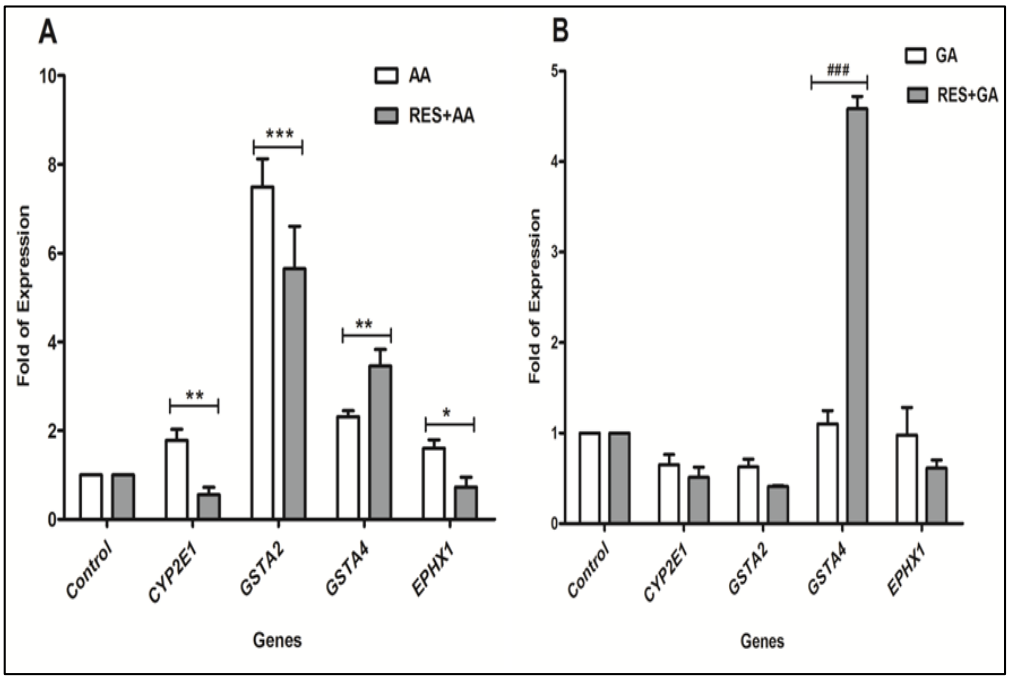

Fig 4: Regulation of gene expression of detoxification enzymes of resveratrol (100 µM) pretreated, (A) AA (200 mg/L) and (B) GA (100 mg/L) exposed HepG2 cells. ***P*< 0.05, ****P* < 0.01 represents significant difference between AA-induced cells to the resveratrol pretreated AA-induced cells. ###*P*< 0.001 indicates a significant difference between resveratrol pretreated GA-induced cells to the GA-induced cells. ns (not significant).

Protective effect of resveratrol on AA/GA induced DNA damage and MN formation

The bioactivation of AA to GA *via* CYP2E1 may have an impact on genotoxicity bringing about DNA damage [28]. The DNA isolation and agarose gel electrophoresis were performed to study the protective effect of resveratrol on DNA damage induced by AA and GA. The DNA damage was quantified by measuring the DNA band intensity by ImageJ 1.43 image analysis software [29]. The electrophoresis of genomic DNA of control HepG2 cells were intact with a clear band as seen in lane 1 of Fig. 5A. But the AA and GA-treated cells showed an increase in DNA degradation as observed by a diminished DNA band (lane 2 and 4 of Fig. 5A). The quantitative estimation of DNA damage induced by AA and GA reveals a decreased band intensity analysed by ImageJ. But cells pretreated with resveratrol at 100 µM concentration was shown to protect DNA from DNA degradation with intact DNA bands as observed in lane 3 and 5 of Fig. 5A. The visual observation of DNA band on agarose gel correlated with the quantitative estimation of DNA damage with an increase in DNA band intensity relative to the AA, GA induced cells (Fig. 5B). The antioxidants such as hydroxytyrosol (HT), extracted from olive oil, allicin from garlic were reported to produce a substantial protective influence against AA-induced oxidative DNA damage studied both *in vitro* and *in vivo* in HepG2 cells [24, 30]. The present work has shown that resveratrol pretreated HepG2 cells showed an increase in the intensity of DNA and hence protected cells from DNA degradation induced by AA and GA.

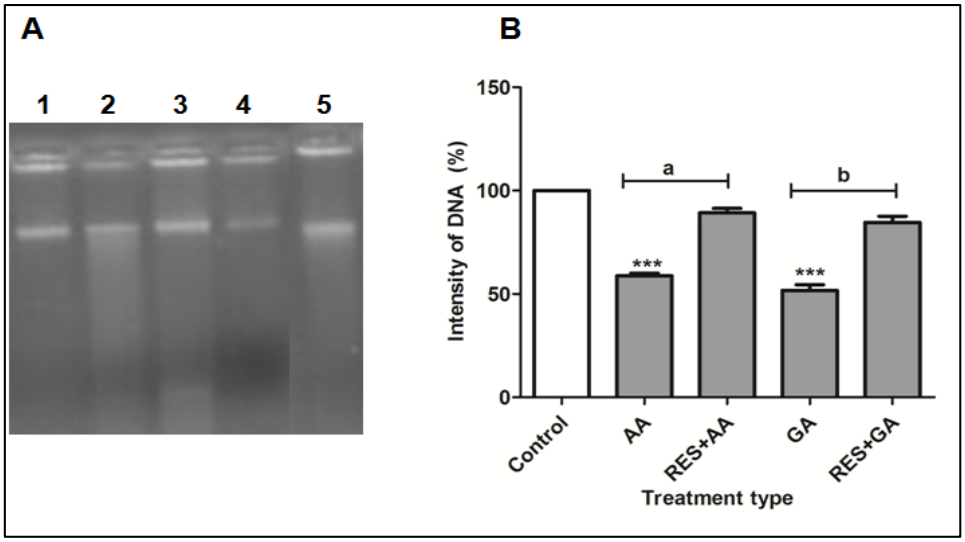

Fig 5: Protection of genomic DNA against AA and GA-induced DNA damage by resveratrol. (A) DNA isolated from control (lane 1), AA (lane 2), GA (lane 4) and resveratrol pretreated AA/GA-induced (lane 3 and 5) HepG2 cells resolved electrophoretically on 1 % agarose gel. (B) Quantitative estimation of DNA band intensity by ImageJ analysis. ****P*< 0.001 (significant difference between AA, GA induced cells to the untreated control cells), [aaa]*P* < 0.001, [bbb]*P* < 0.001 represents significant difference between resveratrol pretreated cells to AA/GA- induced cells.

The frequency of micronuclei (MN) has been used to measure the environmental or endogenous stresses that result in the genome damage leading to the cause of cancer. MN, a chromosome breakage or impairment of the mitotic spindle is reported as sensitive biomarkers for cytogenetic damage and is closely related to the DNA damage repair and genome instability [31, 32]. The MN was analysed by confocal microscopy. The Hoechst stained HepG2 cells analysed by confocal microscopy showed the presence of micronucleus in AA and GA treated cells an indication of chromosomal DNA damage (Fig. 6A). Also the cells were scored for micronucleus in 1000 binucleated cells. The frequency of MN increased significantly in AA up to 9.25 folds and 12.42 folds in GA-treated cells when compared to the untreated control cells. The frequency of MN showed a significant reduction ($P < 0.01$) of 3 to 4 folds in cells pre-treated with resveratrol (100 µM), 24 h before AA and GA treatment. These results suggested that resveratrol at 100 µM was able to reduce the frequency of MN induced by AA and GA in HepG2 cells (Fig. 6B).

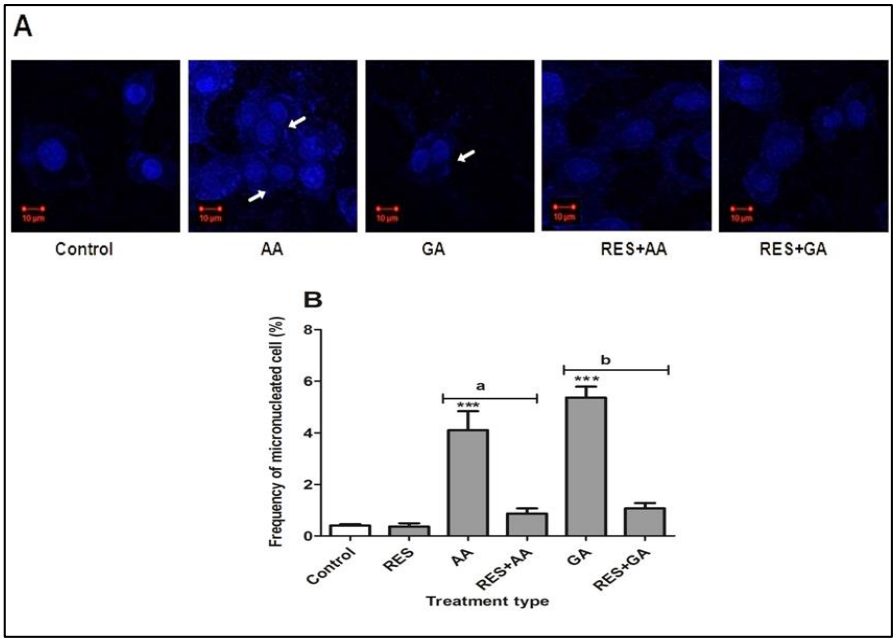

Fig 6: Protective effect of resveratrol (100 µM) on micronucleus formation of AA (200 mg/L) and GA (100 mg/L) induced HepG2 cells. (A) Cells treated with AA, GA, resveratrol pretreated AA, GA-induced and control cells stained with Hoechst 33258, analyzed by confocal microscopy for MN formation (scale bar-10 µm) (arrows designates MN). (B) Cells of respective treatments and control were scored for MN in 1000 binucleated cells. Values are ± SEM from the experiment carried in triplicates. Significant difference of frequency of MN between (***$P < 0.001$) AA, GA-induced and control cells, ($^{aaa}P < 0.001$, $^{bbb}P < 0.001$) resveratrol pretreated AA, GA-induced cells compared to AA, GA-induced cells).

Effect of resveratrol on gene expression of DNA repair genes of AA/GA-induced HepG2 cells.

The DNA damage induced by AA and GA made us to study the genes of DNA repair pathways involved in repair of the DNA abnormality which might be due to the DNA adducts, single- and double strand breaks, abasic sites. The DNA repair pathways involved in the removal of DNA damage include Base Excision Repair (BER), Nucleotide Excision Repair (NER), mismatch repair (MMR) and double-strand break (DSB) repair pathways [33]. The genetic damage has been reported to be neutralized by these DNA repair processes and consequently maintains genome integrity [34]. However the DNA repair genes affected by the inherited mutations are strongly related to a high incidence of cancer risks [35]. The mRNA expression of few genes of DNA repair pathway such as BER - *XRCC1*, NER - Replication protein A (*RPA1*) and DSB repair - DDB1, *RAD52* and *XRCC5* genes were studied. The gene expression studies of AA and GA-induced cells presented the downregulation of all the above DNA repair genes which might be due to the mutation or epigenetic inactivation of DNA repair genes. The epigenetic changes imply the modification of DNA cytosine (methylated by DNA cytosine methyltransferases), post-translational modification of histone and deposition of specific histone variants besides particular gene sequences. The epigenetic modifications of genes, in general, are reversible [36, 37].

Studies have shown that resveratrol has been involved in maintaining the genome stability by accelerating the DNA repair, thus protecting from DNA damage induced by exogenous, endogenous chemicals and alkylating agents [38]. The present investigation of resveratrol pretreatment to AA-exposed cells showed the upregulation of *XRCC1*, *RPA1*, *DDB1*, *RAD52* and *XRCC5* genes. The double-strand break repair *RAD52* showed the maximum fold of expression which was followed by *XRCC1*, *XRCC5*, *DDB1* and *RPA1* genes (Fig. 7A). Similarly, the cells pretreated to resveratrol followed by GA exposure showed the upregulation of *XRCC1*, *RPA1*, *DDB1*, *RAD52*, and *XRCC5* repair genes which were downregulated in GA-exposed cells. But the BER pathway gene, i.e. *XRCC1* (X-ray repair cross-complementing group) showed a maximum fold increase of expression followed by *RPA1*, *RAD52*, *DDB1* and *XRCC5* genes (Fig. 7B).

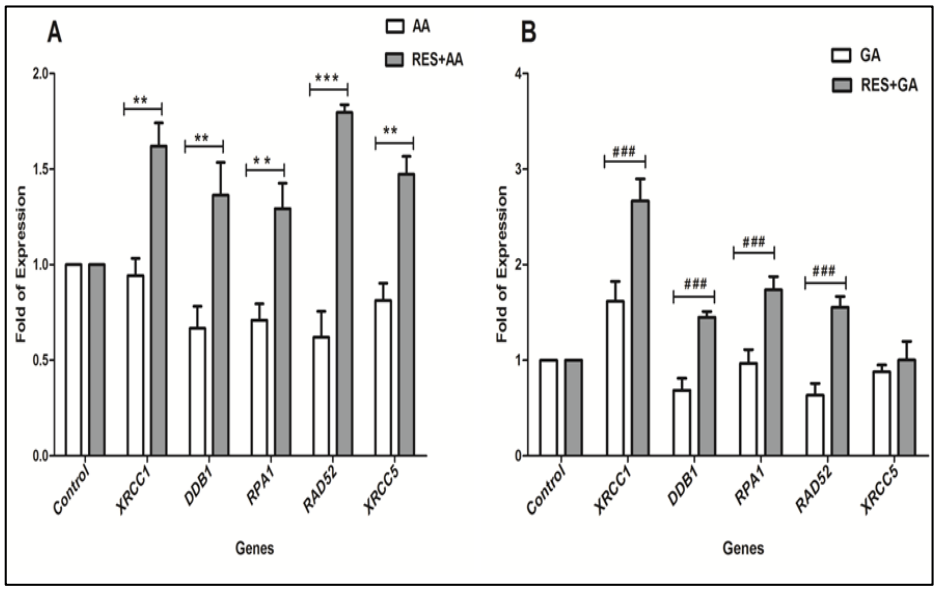

Fig 7: Effect of resveratrol (100 µM) pretreatment on gene expression of DNA repair genes of AA (200 mg/L) and GA-treated (100 mg/L) HepG2 cells. Significant difference of (A) resveratrol pretreated AA-induced cells to AA treated cells (**$P < 0.01$, ***$P < 0.001$) (B) resveratrol pretreated GA-induced cells to GA-treated HepG2 cells ($^{###}P < 0.001$).

Thus this data imply that resveratrol plays a vital role on protection from epigenetic inactivation of DNA repair genes and thus promotes the DNA repair pathway and thus maintains the genome integrity.

Conclusion

In conclusion, this study confirms that pre-treatment of hepatic cells to resveratrol could reduce AA, and GA-induced cytotoxicity and genotoxicity because of its direct ROS scavenging activity and use of resveratrol may be a credible way to prevent AA and GA-mediated genotoxicity. Based on the observed effects on CYP and GST isozymes, we have demonstrated that AA and GA can affect its metabolism by influencing the expression levels of *CYP* and *GST*. Thus potentiate the risk of toxicity, mutagenesis, carcinogenesis and malignant transformation resulting in DNA damage, chromosomal damage, and inactivation of DNA repair genes. This study confirms that pre-exposure to resveratrol could reduce the genotoxicity of AA, GA and thus maintains the genome stability. However, the *in vivo* effects of resveratrol on protection against AA and GA-induced toxicity require further study.

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