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BOERAVINONE B AND CHEBULINIC ACID AMELIORATE ASPARTAME INDUCED TOXICITY IN HEPG2 CELLS BY REGULATING CELL DEATH PATHWAYS

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ABSTRACT: Aspartame is the most common artificial sweetener which is known to induce oxidative stress. Our goal was to determine whether Boeravinone B and Chebulinic acid protect HepG2 cells against Asp-induced oxidative stress in HepG2 on a multifarious level. In this investigation we have addressed ameliorative role of BB and CA in due course of Asp exposure by evaluating biochemical and molecular parameters. We found that Aspartame induced oxidative stress in HepG2 cells through elevation of ROS and RNS which was further attenuated by BB and CA. Associated levels of SOD, CAT, GPx, Protein carbonyls, Lipid peroxides and Caspase 3/7 in Asp treated cells were found to be risen but BB and CA supplementation restored these back to normal. It was revealed from fluorescence microscopy as well as flow cytometric analysis that BB and CA reversed the proportion of apoptotic, necrotic as well as autophagic cells which was found to be triggered during Asp exposure. mRNA expression of marker genes associated with Apoptosis, Necrosis and Autophagy was observed to be upregulated in stressed cells which was modulated positively by BB-CA phytotherapy. Thus BB and CA protected HepG2 cells from undergoing cell death pathways and ultimately improved a cellular health.

INTRODUCTION: Concerns about health and life quality have risen in recent decades, encouraging people to exercise, eat nutritious foods and limit their intake of sugar, salt and fat. Consequently, food products created with sweeteners rather than sugar have become more prevalent as customers' interest in lowering sugar intake has grown [1].Aspartame (E951) is one of the most commonly used synthetic sweeteners which is a dipeptide in nature. Research has revealed that Aspartame is rapidly hydrolyzed in the intestinal lumen resulting in three products viz 40% Aspartate, 50% Phenylalanine and10% Methanol [2].

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Most common symptom reported by customers after ingesting Aspartame (knowingly or unknowingly) is headaches [3]. Prenatally administered aspartame in male mice via feed was found to be the cause of bronchial as well as hepatocellular carcinoma [4]. Repeated oral administration of aspartame resulted in a large rise in production of tumor necrosis factor (TNF-α) and interleukin-1 (IL-1) of brain along with a remarkable drop in brain-derived neurotropic factor (BDNF) as well as serotonin levels [5].

Further, in organs such as liver and kidney, aspartame exhibited the tendency to modify the pool of general antioxidant enzymes. Long-term use of aspartame resulted in oxidative stress in blood cells [6]. Aspartame has revealed as the origin of oxidative stress in various immunological organs like lymph nodes, thymus, bone marrow and spleen of rats having deficiency of folate. In such sensitive organs, the generation of free radicals may

contribute to a lack of immunity which led the organs vulnerable for infections [7]. As oxidative stress impacts a wide range of cells, organelles and molecular pathways, gaining a fair understanding of such processes can lead to an emergence of new therapeutic possibilities like strengthening cellular antioxidant defenses to combat damaging cellular oxidative events. Indeed, previous evidences of antioxidant effect in cell and animal models of disease seemed promising. Boeravinone B is a rotenoid isolated from *Boerhaavia diffusa* having therapeutic effects in atherosclerosis, osteoarthritis, rheumatoid arthritis and systemic lupus erythematosus etc. [8]. On the other hand, Chebulinic acid is an ellagitannin present in the fruits of *Terminalia chebula*. It was reported to reduce the rate of neuronal cell death in severe brain traumas and other neurodegenerative disorders [9].

As liver is a prominent xenobiotic metabolic site, it is recognized to be the primary target organ for a wide range of chemicals and medications [10]. In present investigation we report the defensive roles of Boeravinone B and Chebulinic acid on Aspartame induced toxicity in Human Hepatocellular Carcinoma cell line along with their function in molecular perceptions related to apoptosis, necrosis, and autophagy. This work would give a theoretical and experimental foundation for the study of exogenous herbal supplementation as a way to reduce oxidative stress and prevent further damage.

MATERIALS AND METHODS:

Chemicals: Cell culture reagents including MEM, FBS, Antibiotics, PBS, Trypsin-EDTA, Aspartame, Vitamin C, RIPA buffer were purchased from HiMedia laboratory, India whereas Boeravinone B and Chebulinic acid were obtained from M/s Natural Remedies, Bangalore, India. GPX assay kit (Cat. No. CGP1), CAT assay kit (Cat. No. CAT100), SOD assay kit (Cat. No. 19160), Protein carbonyl content assay kit (MAK094), c-DNA synthesis kit, 2′7′-Dichlorofluorescin (Cat. No. D6883), Greiss reagent (Cat. No. G4410), DAPI (Cat. No. D9542), Rhodamine 123 (Cat. No. R8004) and Monodansylcadaverine (Cat. No. 30432) were procured from Sigma Aldrich, India. Annexin-V-FLUOS staining kit purchased from Roche, Germany, SYBR Green PCR kit and RNA isolation kit were bought from GeNei^{TM}, India while Caspase-Glo® 3/7 assay kit was purchased from Promega, India. Primers required for mRNA expression studies were obtained from Eurofins Genomics Pvt. Ltd., India. Remainder chemicals and reagents used for the experimentation were of highest purity available and analytical grade.

Cell Culture and Experimental Design: Human Hepatocellular Carcinoma cell line HepG2 was procured from National Institute of Cell Sciences (NCCS), Pune. The cells were cultured in MEM containing Earle's Balanced Salt Solution, non-essential amino acids, 2 mM L-glutamine, 1 mM sodium pyruvate, and 1500 mg/L sodium bicarbonate supplemented with 10% FBS and 1% Penicillin-Streptomycin solution at 37^{o}C in CO_2 incubator having 5% CO_2 in air atmosphere. Cells were trypsinized when they achieved 80-90% confluency and seeded according to sets of experiment. Throughout the study we kept 5 experimental sets and in triplicate as i. negative control having untreated HepG2 cells; ii. HepG2cells exposed to aspartame; iii. HepG2 cells exposed to aspartame supplemented with BB; iv. HepG2 cells exposed to aspartame supplemented with CA and v. HepG2 cells exposed to aspartame supplemented with vitamin C as a positive control in complete medium without Antibiotics incubated at 37^{o}C and 5% CO_2 in CO_2incubator for 24 hrs.

Determination of Optimum Concentrations of ASP, BB and CA through ROS Accumulation: Effective concentrations of ASP, BB and CA were determined by means of ROS accumulation in HepG2 cells using a fluorescent dye 2,7-dichlorofluorescein diacetate (DCFH-DA). For determination of an optimum concentration of ASP which induces a considerable stress in HepG2 through ROS accumulation, the cells were exposed to increasing concentrations of ASP ranges from 1µg/mL to 10µg/mL for 24 hrs. After incubation, cells from each concentration of ASP were treated with 10µM DCFH-DA solution for 30 min at 37^{o}C in dark condition followed by washing with PBS. Then cells were harvested by using a Trypsin-EDTA solution independently and immediately analyzed on fluorescence spectrophotometer 8300 (Agilent Technology, USA) at 488 nm of an excitation wavelength along with 530 nm of an emission wavelength for each sample. Similarly, optimum concentrations of BB and CA in stressed

HepG2 cells induced with ASP were determined which effectively lowered ROS accumulation near the normal levels at minimum concentration. For this analysis the cells were exposed to optimum concentration of ASP supplemented with BB and CA at concentration ranges from 1µg/mL to 5µg/mL independently for 24 hrs and ROS levels were measured using the same protocol mentioned above. Once the optimum concentrations of BB and CA obtained in ASP treated HepG2 cells, we also investigated their independent role in untreated HepG2 cells. For this study, we exposed untreated HepG2 cells to pre-optimized concentrations of BB and CA individually for 24 hrs followed by Intracellular ROS measurement. Further we estimated ROS accumulation in all five experimental sets together taking predetermined concentrations and using the same protocol mentioned above.

Nitrite Estimation by Griess Reagent: Nitrite content was determined by measuring extracellular nitric oxide production in cultured HepG2 cells using Griess reagent. For this analysis, cells amassed from T-25 flask were seeded into 24-well plate at cell seeding density of 5×10^4/well and exposed to a pre-optimized concentrations of ASP (6µg/mL) alone as well as ASP in combination with BB, CA and vit. C (3µg/mL, 5µg/mL and 5µg/mL respectively) along with untreated HepG2 cells as a negative control for 24 hrs. Later on culture supernatant was collected from all five sets and nitric oxide content was measured [11].

Evaluation of Antioxidant Enzymes' Status: For measurement of antioxidant enzymes' activities such as Glutathione peroxidase (GPx), Catalase (CAT) and Superoxide dismutase (SOD), HepG2 cells were seeded in 6-well plate at cell seeding density of 3×10^5/well. Then cells from all five sets of experimentation were harvested after 24hrs of incubation followed by centrifugation for 5 min at 500×g. Pellet obtained was homogenized in 100µL of RIPA buffer independently followed by centrifugation at 3000×g for 10 minutes at 4°C to separate the cell debris. Thus supernatant obtained was used as a source of SOD, CAT and GPx. Enzyme assays of corresponding enzymes were performed according to the protocol mentioned in product information bulletin of commercially available SOD, CAT and GPx assay kits with slight modifications and were run in triplicate.

Cytosolic protein content was quantified by Lowry's method [12] using bovine serum albumin as standard.

Estimation of Total Protein Carbonyls Content: Cells seeded in 24 well plate at seeding density of 1×10^5 cells/well and treated accordingly experimental sets for 24 hrs. After incubation, cells were harvested and homogenized in RIPA buffer individually followed by centrifugation for 10 min at 3000×g and 4°C. The supernatant thus obtained was used as source of cellular proteins. The total protein carbonyl content was estimated in all five sets by using the protocol according to product information bulletin of Protein Carbonyl Content Assay Kit with slight modifications in triplicate. Total protein content of each sample was ascertained with Lowry assay using Bovine Serum Albumin as a standard. The results were noted as nmol of protein carbonyls per mg of protein [12].

Measurement of Lipid Peroxidation Levels: A method involving measurement of levels of thiobarbituric acid reactive substances (TBARS) was used for assessment of lipid peroxidation. Cells garnered from all experimental sets and resuspended in ice-cold 0.15M KCl solution followed by homogenization and all the samples obtained were centrifuged at 3000×g for 10 min at 4°C. Thus supernatant resulted was used for estimation of lipid peroxide levels [13].

Assessment of Caspase 3/7 Activity: The activity of enzyme Caspase 3/7 was measured with Caspase-Glo® 3/7 assay kit which compute the activities of Caspase 3 and 7 in cultured cells based on luminescence. For this investigation, cells from all five sets of experimentation were harvested as mentioned above and homogenized individually in 100µL of RIPA buffer followed by centrifugation. Thus, the supernatants obtained were used as source of Caspase 3/7 and activities were measured according to product information bulletin of Caspase-Glo® 3/7 assay kit. All the samples were analyzed for luminescence on Fluorescence/ Luminescence Spectrophotometer 8300 (Agilent Technology, USA).

Fluorescence Microscopic Examination to Identify DNA Disintegration, Mitochondrial Membrane Integrity, Apoptosis and Necrosis: Fluorescence microscopic analysis for the determination of DNA disintegration, mitochondrial membrane potential, apoptosis and necrosis in HepG2 cells was carried out on Olympus BX43 fluorescence microscope. A method of DAPI (4′,6-diamidino-2-phenylindole) labeling was carried out to confirm ASP's ability to cause DNA damage along with the protective role of BB and CA in ASP exposed HepG2 cells. Following the treatment of 24 hrs, cells from five experimental sets were treated with paraformaldehyde (5%) for 1 hr for fixation and washed thrice with PBS. The fixed HepG2 cells were stained with 5µL of 5 mg/mL DAPI in dark at 37^{o} C for 20 min which were examined using DAPI filters (excitation λ- 358 nm and emission λ- 461 nm) and images were captured. The fluorescent cationic probe, Rhodamine 123 (50 mmol) was used to stain the cells at 37 °C for 20 minutes in order to determine integrity of mitochondria. The photographs were captured by observing stained cells under fluorescent microscope using FITC filter (excitation λ- 494 nm and emission λ- 518 nm). Using Annexin-V-FLUOS Staining Kit (Roche), we analyzed the apoptotic as well as necrotic cells following the treatments. In order to observe the apoptotic cells, FITC filter (excitation λ- 494 nm and emission λ- 518 nm) was used while necrotic cells were observed using TRITC filter (excitation λ- 544 nm and emission λ- 570 nm).

Flow Cytometric Analysis: Flow cytometry was implemented to quantify the percentages of cells with collapsed mitochondrial membrane integrity, apoptosis, necrosis and Autophagy as well. Briefly, HepG2 cells were cultured in 6-well plates at a cell seeding density of 2×10^{5}/well and were given all the treatments for 24 hours. Harvested cells were washed with and resuspended again in PBS which was taken for further analysis by applying a variety of fluorescent dyes using CytoFLEX flow cytometer (Beckman Coulter, India). Cells were stained with Rhodamine 123 at the concentration of 50 nM and allowed to stand for 20 min in dark. Subsequently, cells were examined for their fluorescence on flow cytometer by FL1-A filter. Apoptosis and necrosis were assessed by using Annexin-V-FLUOS Staining Kit (Roche). Cells were treated with earlier mentioned pre-diluted solutions (250 µL) of Annexin-V and Propidium iodide separately which are specific for apoptotic and necrotic cells respectively followed by incubation for 30 min in dark. Afterwards cells were analyzed independently for FITC and PE fluorescence using FL1-A and FL2-A filters. Similarly, MDC (50 µM) staining technique was used to calculate the percentage of autophagic cells which were monitored via FL1-A filter.

mRNA Isolation, cDNA Preparation, and Quantitative Real-Time PCR (qRT-PCR) Assay: Transcriptional changes were studied by evaluating the expression patterns of reporter genes associated with Apoptosis, Necrosis and Autophagy. In brief, HepG2 cells were seeded in 6-well plate at seeding density of 5×10^{5} cells per well followed by respective exposures. After 24 hrs of incubation, cells from all five sets were used for mRNA isolation using a manufacturer's protocol provided with GeneiPure™ Total RNA Isolation Kit. Total RNA isolated was quantified with the help of NanoDrop spectrophotometer (Eppendrop, USA). Out of the total RNA isolated, 1µg from each sample was mixed with Readyscript cDNA synthesis mix individually in 20µL total reaction volume and allowed for cDNA formation employing the protocol provided with ReadyScript™ cDNA Synthesis Mix kit, Sigma Aldrich, India. To perform quantitative Real time PCR analysis, reaction mixtures were prepared in total volume of 25µL consisting of 1µL of each primer (15 pM), SYBR Green, Nuclease free water and 100ng of respective cDNA templates in 96 well plate. The sequences of respective primers' pairs used in this investigation are enlisted in **Table 1**. The thermal cycling program was set as 95^{o}C for 5 min., 95^{o}C for 10 sec., 60^{o}C for 30 sec. and 40 amplification cycles were run. Alterations in mRNA expression patterns of specific genes were assessed using StepOnePlus, a system of Real Time PCR (Applied Biosystems, AB) and obtained data were analyzed by an automatic quantification mode provided with StepOnePlus Software version 1.0 (AB). The values of delta cycle threshold (ΔCT) for each gene were evaluated by deducting the CT values of GAPDH gene from the CT values of respective genes mentioned above and results obtained were then confirmed by the delta-delta Ct method ($2^{-\Delta\Delta Ct}$).

TABLE 1: LIST OF THE PRIMER PAIRS USED IN THIS INVESTIGATION FOR QRT-PCR ANALYSIS

Primer name	Primer Sequence	Total Bases
Bcl2 F	GTGCATTTCCACGTCAACAG	20
Bcl2 R	AACAGGCCACGTAAAGCAAC	20
P53 F	CAGTCTACCTCCCGCCATAA	20
P53 R	GCAAGCAAGGGTTCAAAGAC	20
Caspase 3 F	GGTTCATCCAGTCGCTTTGT	20
Caspase 3 R	AACCACCAACCAACCATTTC	20
BAK F	GGGGATTGGTGGGTCTATGT	20
BAK R	GCAGGGGTAGAGTTGAGCAG	20
AIF F	GTGGAAGATTGGCTGGAGAA	20
AIF R	GCCTCGGACTCTGTCTCACT	20
RIP1 F	GGAAAAACTGTGCCCGTAAA	20
RIP1 R	AGCCCATCCAGGGTTAGTTC	20
LC3 F	CCGCCTTTTTGGGTAGAAGT	20
LC3 R	AGTGAGGACTTTGGGTGTGG	20
Beclin1 F	CTGGATCAGGAGGAAGCTCA	20
Beclin1 R	TTGATTGTGCCAAACTGTCC	20
GAPDH F	CGAGATCCCTCCAAAATCAA	20
GAPDH R	GTTCACACCCATGACGAACA	20

Statistical Analysis: All experiments performed under this report were conducted in triplicate and in parallel and the data obtained are displayed as mean ± SD. Statistical interpretation of the revealed data was performed using Origin and Microsoft Excel. Experimental data obtained were examined and analyzed by the two-way ANOVA method and subsequently by the Tukey's multiple comparison test. A p value less than 0.05 ($p < 0.05$) was considered statistically significant. The coefficient of variance for all the experiments was quantified by a method reported previously [14].

RESULTS:

Optimum Concentrations of ASP, BB and CA through Evaluation of Intracellular ROS Accumulation: We found that 6µg/mL ASP exposure considerably increased ROS accumulation than that of the negative control as compared to rest of the concentrations. Further we observed that 3µg/ mL concentration of BB and 5µg/mL concentration of CA were effective in reduction of intracellular ROS accumulation in ASP treated HepG2 cells. Thus, the effective concentrations of ASP, BB and CA (6µg/mL, 3µg/mL and 5µg/mL respectively) obtained from this optimization were employed in all subsequent experiments. A reported concentration of vit. C was used in this investigation which was 5µg/mL. Furthermore, effect of pre-optimized concentrations of BB (3µg/mL) and CA (5µg/mL) individually in unrated HepG2 cells evinced that the levels of intracellular ROS accumulation in BB and CA treated cells were appeared below than negative control. It was observed from **Fig. 1A** that accumulation of ROS in ASP treated HepG2 cells was elevated by 83.47% in comparison with negative control. On the other hand, BB and CA persistently lowered it by 54.842% and 43.012% respectively which worked more superiorly than positive control showing 36.237% reduction in ROS accumulation compared with ASP treated cells.

Treatment of BB and CA Reduced the Rate of Extracellular Nitrite Production: After evaluation of ROS, we also investigated the production of Nitrites (NO^{-2}). Our findings suggested that the phytotherapy has lowered the rate of extracellular nitrite production during ASP exposure more efficiently than positive control. We observed that the amount of extracellular nitrites in ASP exposed experimental set was 90nM/mL and it was 73.077% higher in comparison with negative control (52nM/mL). Further these elevated levels were lowered by 54.44% that was up to 41nM/mL and 61.11% that was up to 35nM/mL when ASP treated HepG2 cells supplemented with BB and CA respectively **(Fig. 1B).**

BB and CA Ameliorated an Antioxidant Enzymes' Status in HepG2 Cells During Asp Exposure: Superoxide dismutase activity observed in only ASP exposed HepG2 cells was 84.726% inhibition/ 3×10^5 cells that was 90.413% greater than the activity observed in negative control

HepG2 cells which showed 44.496% inhibition/ 3×10^5 cells. Phytotherapy showed reduction in SOD activities as compared to the activity in ASP treated HepG2 cells by 51.353 % and 41.546% that was 41.217% inhibition/ 3×10^5 cells and 49.526% inhibition/ 3×10^5 cells observed in BB and CA augmentation respectively **(Fig. 1C)**. Further up to 5.963 Units/ 3×10^5 cells activity of Catalase and 1.994mmole/ min/ 3×10^5 cells activity of Glutathione Peroxidase observed in ASP exposed HepG2 cells which accounted for 71.056 % and 106.632 % higher in comparison with that of the activities of CAT and GPx observed in negative control (3.486Units/ 3×10^5 cells and 0.965mmole/ min/ 3×10^5 cells) respectively. A drop of 64.615% and 51.605 % in activities of CAT (2.11Units/ 3×10^5 cells) and GPx (0.965mmole/ min/ 3×10^5 cells) respectively in experimental set of BB supplementation was recorded. In addition to this 49.237% and 58.074 % reduction in activities of CAT (3.027Units/ 3×10^5 cells) and GPx (0.836mmole/ min/ 3×10^5 cells) was noted in ASP exposed HepG2 cells in combination with CA by comparing with the activities of corresponding enzymes observed in only ASP treated HepG2 cells **(Fig. 1D and 1D).**

Decrement in the Total Protein Carbonyls Content as a result of BB and CA Supplementation: We noticed that 0.532nmole of carbonyls/ mg of protein in ASP treated HepG2 cells which was 52.436% greater than negative control having 0.349nmole of carbonyls/ mg of protein. This enhanced content was subsequently reduced by 22.180% in BB supplementation which showed 0.414nmole of carbonyls/ mg of protein whereas supplementation of CA revealed 0.344nmole of carbonyls/ mg of protein which was 35.338% lesser than that of ASP exposed HepG2 cells **(Fig. 1F).**

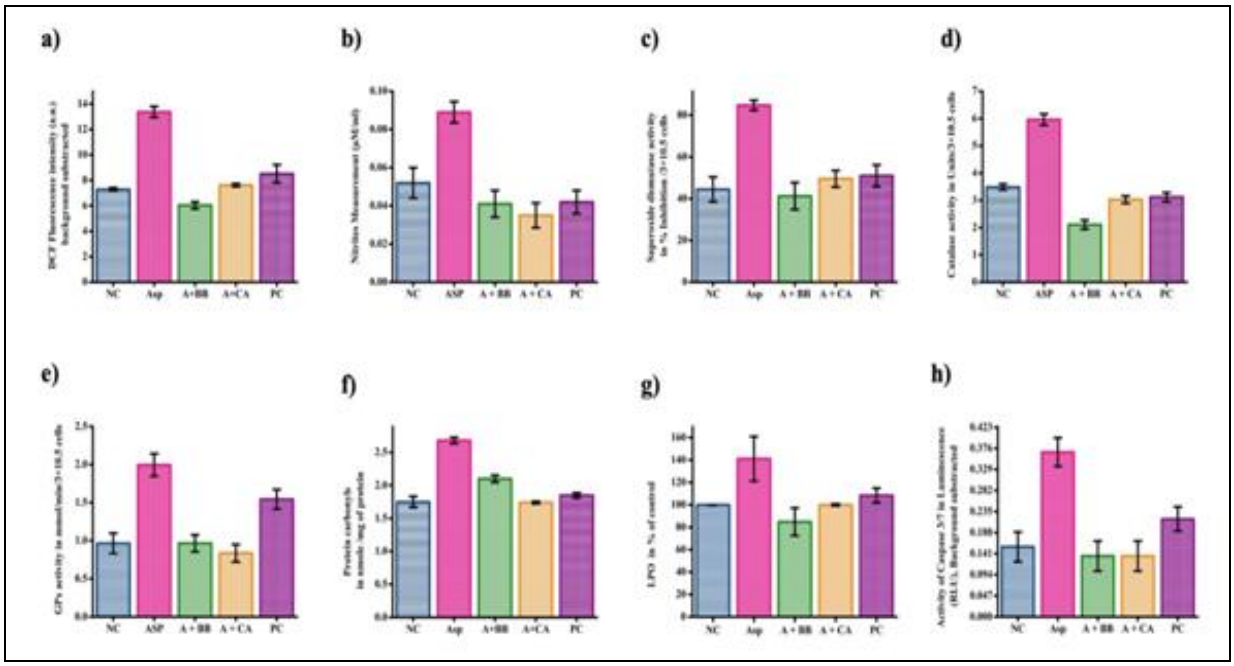

FIG. 1: EFFECT OF ASP AND SUBSEQUENT BB AND CA SUPPLEMENTATION ON OXIDATIVE STRESS AND ASSOCIATED PARAMETERS, A) INTRACELLULAR ROS PRODUCTION B) EXTRACELLULAR NITRITES C) SUPEROXIDE DISMUTASE ACTIVITY D) CATALASE ACTIVITY E) GLUTATHIONE PEROXIDASE ACTIVITY F) PROTEIN CARBONYLATION G) LIPID PEROXIDATION H) CASPASE 3/7 ACTIVITY. The data expressed as the average ± standard deviation of three separate experiments (n=3). Values of the treatment sets substantially differed from those of the ASP set (*$p\leq0.03$; $CV\leq0.05$).

Anti-Lipid Peroxidation Potential of BB and CA: It was resulted from **(Fig. 1G)** that only ASP treated HepG2 cells showed 37.815% elevation in the lipid peroxides' levels by comparing with that of the negative control (assuming to have 100% LPO). Combination of BB during ASP exposure in HepG2 cells showed decrease in lipid peroxides by 37.195% whereas in case of CA supplementation, 27.439% decrement was observed. Results demonstrated that BB and CA phytotherapy was performed superiorly than both of the control sets during entire course of ASP.

Diminution in the Activity of Caspase 3/7 during BB and CA Supplementation: The data obtained from this study revealed that 0.368R.L.U. Activity of Caspase 3/7 was recorded in ASP treated HepG2 cells which was 135.897% greater than that of the negative control (0.156R.L.U). Additionally BB and CA supplementation showed an identical decline in the activity of Caspase 3/7 that was 0.136R.L.U in both the experimental sets which accounted for 63.043% lower than the activity observed in HepG2 cells exposed to ASP only.

Supplementation of BB and CA lowered DNA Disintegration, Disturbed Mitochondrial Membrane Integrity, Apoptosis and Necrosis: DNA damage in HepG2 cells was demonstrated by DAPI staining, which revealed that exposure to ASP accelerated DNA fragmentation. Subsequently, complementation of BB and CA has restored this rate up to normal levels **(Fig. 2A)**.

Further we investigated the mitochondrial membrane integrity in all experimental groups using Rhodamine 123 and found that ASP exposed cells were observed as less bright in fluorescence as compared to Control. While after augmentation of BB and CA resulted in brighter fluorescence of Rh123 alike control ones **(Fig. 2B)**. Additionally, staining with Annexin V and PI revealed increased number of apoptotic and necrotic cells in ASP treated sets. On the other hand, supplementation of BB and CA has brought down these numbers near normal **(Fig. 2C and D).**

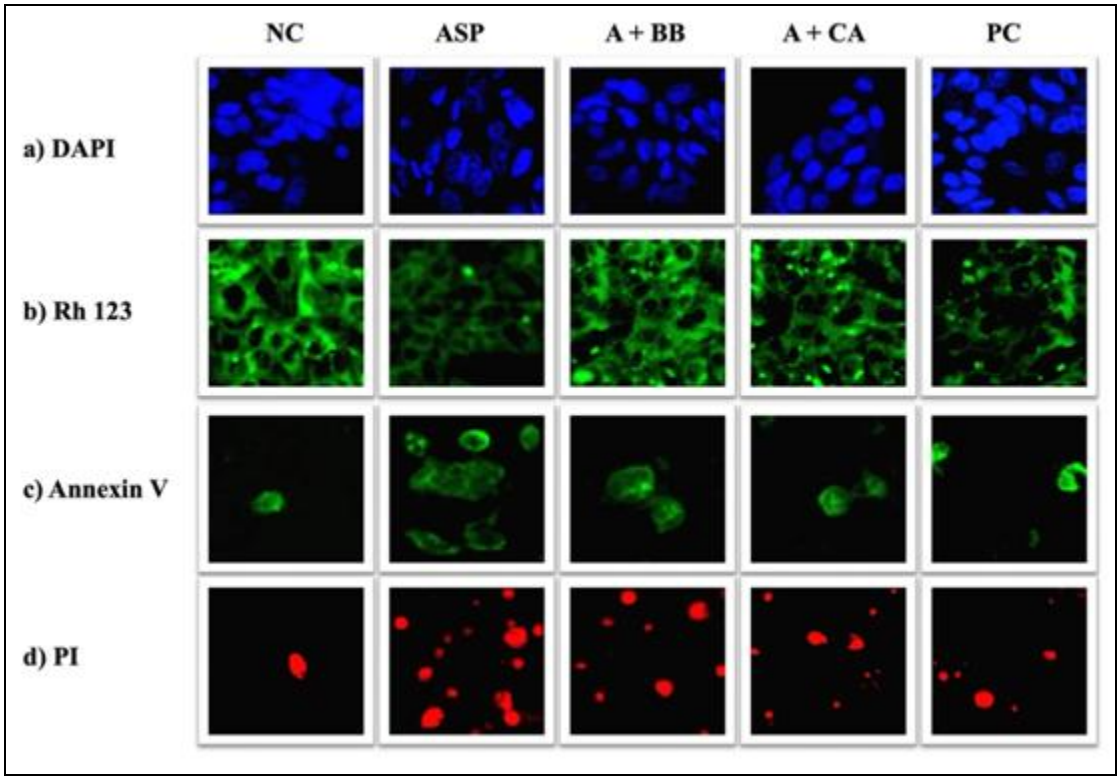

FIG. 2: EFFECT OF ASP ALONE AND ASP IN COMBINATION WITH BB AND CA ON DNA DAMAGE, MITOCHONDRIAL MEMBRANE POTENTIAL, APOPTOSIS AND NECROSIS IN FIVE EXPERIMENTAL SETS I) NEGATIVE CONTROL II) ASP III) A+BB IV) A+CA V) POSITIVE CONTROL USING FLUORESCENCE MICROSCOPY AT MAGNIFICATION OF 20X. A) DNA DAMAGE USING DAPI B) MITOCHONDRIAL MEMBRANE POTENTIAL USING RHODAMINE 123 C) APOPTOSIS BY ANNEXIN V AND D) NECROSIS USING PI

BB and CA Help Perpetuate Mitochondrial Membrane Potential and Rescued ASP Exposed HepG2 Cells from Undergoing Cell Death Pathways: In current investigation, flow cytometric analysis was employed to evaluate mitochondrial integrity using Rhodamine 123. 77% Cells from negative control shown intact mitochondrial membrane potential whereas during exposure of ASP, it dropped down up to 60.42%. After intervention with BB and CA it was recovered again to 76.16% and 74.82% respectively **(Fig. 3A)**. Further flow cytometric

evaluation with Annexin V revealed that 13.30% cells from ASP exposed set have undergone apoptosis in comparison with negative control (4.52%) while augmentation with BB and CA reduced this percentage up to 5.28 and 5.86 respectively **(Fig. 3B)**. Estimation of necrotic cells by Propidium iodide divulged that 9.26% among ASP exposed cells gone through necrotic pathway compared with 5.52% cells from negative control which was eventually recuperated to normal levels by BB and CA supplementation and observed to be 6.74 and 6.32% respectively **(Fig. 3C)**. Studies with MDC brought to light that 19.23% cells from ASP treated group entered autophagic cell death pathway in comparison with negative control having 5.20% which were brought down up to 10.50% by BB and 9% by CA augmentation during course of ASP exposure **(Fig. 3D).**

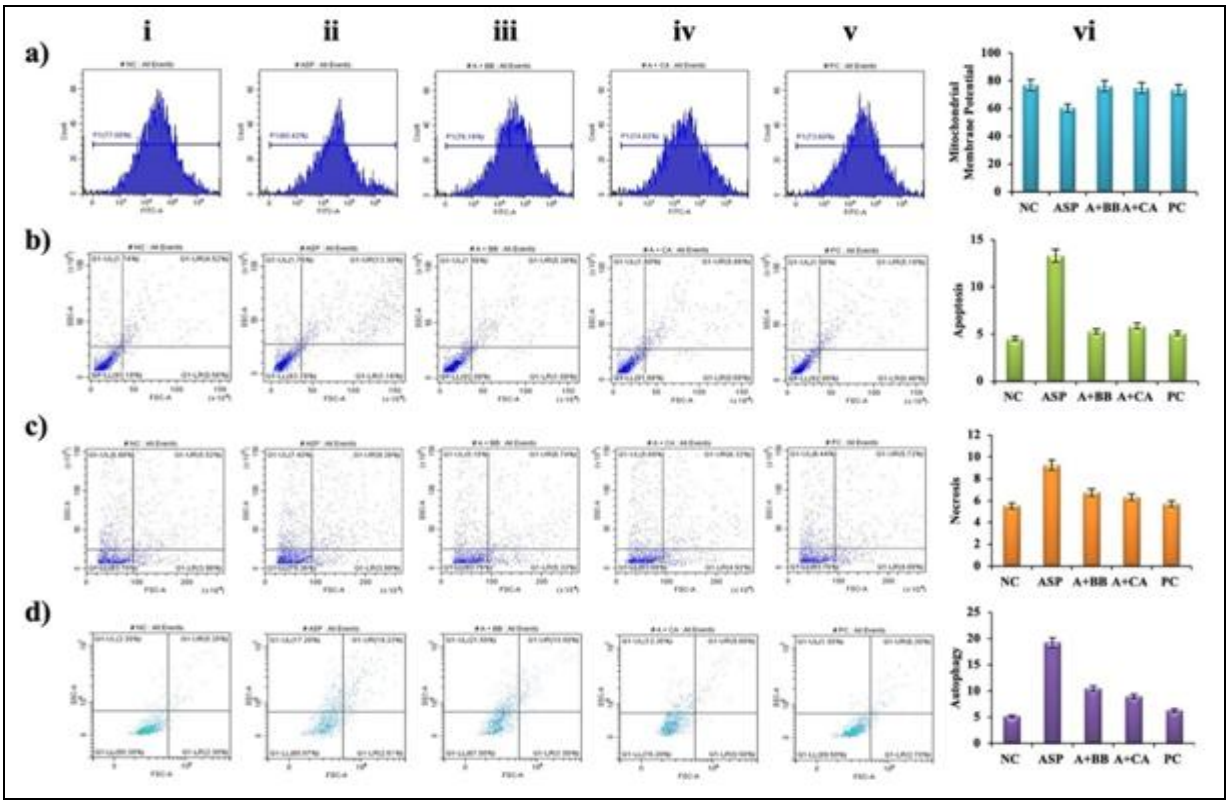

FIG. 3: FLOW CYTOMETRY FOR QUANTITATION OF A) MITOCHONDRIAL MEMBRANE POTENTIAL, B) APOPTOSIS, C) NECROSIS AND D) AUTOPHAGY ACTIVATION IN FIVE EXPERIMENTAL SETS I) NEGATIVE CONTROL II) ASP III) A+BB IV) A+CA V) POSITIVE CONTROL AS A RESULT OF ASP EXPOSURE AND PROTECTIVE MECHANISMS OF BB AND CA DURING ASP TREATMENT. VI) A BAR GRAPH REPRESENTING CORRESPONDING DATA ANALYSIS OF ALL EXPERIMENTAL SETS. The data expressed as the average ± standard deviation of three separate experiments. (n=3). Values of the treatment sets substantially differed from those of the ASP set (*p≤0.03; CV≤0.05).

Transcriptional Changes in the Markers of Apoptosis, Necrosis and Autophagy: An alteration in mRNA expression levels of genes associated with Apoptosis viz. *P53*, *Bcl2*, *Bak*, *AIF* and *Cas3*, an expression of *RIP1* associated with Necrosis along with expression of genes associated with Autophagy viz. *Beclin1* and *LC3* was scrutinized by using *GAPDH* as an internal control.

BB and CA Restored the Expression of Genes Involved in Apoptosis: It was revealed from **(Fig. 4A)** that the mRNA expression of *P53* was upregulated in ASP exposed HepG2 cells by 0.49-fold in comparison with negative control having relative quantitation 1.00. Implementation of phytotherapy displayed that an elevated mRNA expression of *P53* was significantly lowered than in control cells which were noted to be 0.93 and 0.91-folds in BB and CA augmented experimental sets. Besides this, down regulation in mRNA expression levels of *Bcl2* gene in the event of ASP exposure was noted which appeared to be 0.46-fold than that of the negative control (1.00). Further elevation was evident up to 0.9-fold along with 0.85-fold during sequential BB and CA supplementation in

HepG2 **(Fig. 4B)**. Investigation on mRNA expression of *Bak* illustrated in **(Fig. 4C)** revealed that up regulation of gene expression in ASP exposed HepG2 cells up to1.54-folds which was dropped down to the extent of 1.05 and 0.88 throughout BB and CA combination with ASP treatment respectively. According to the data acquired from **(Fig. 4D)**, the relative expression of *AIF* also was modulated up in comparison with negative control by 0.39-fold. In case of BB and CA combination during ASP treatment, expression levels were decreased up to 0.93 and 0.96-folds respectively which were below than control. Upregulation to 1.66-folds was observed in expression of *Cas3* during course of ASP exposure in HepG2 cells by comparing with that of the negative control. On the other hand, supplementation of BB reduced this level up to 1.43 and supplementation of CA reduced up to 1.40-folds in subjection of ASP **(Fig. 4E).**

Positive Regulation of Marker Gene Associated with Necrosis as a Result of Phytotherapy Implementation: The data perceived from **(Fig. 4F)** that mRNA expression of *RIP1* was raised by 2.13-fold in HepG2 cells during the course of ASP treatment compared to negative control. Further augmentation of BB showed the reduction in relative quantitation of the same up to 2.18 while 1.21 was recorded in CA supplementation during ASP exposure.

BB and CA Exerted a Protective Role against Autophagy: The same expression pattern was followed in mRNA expression of *Beclin1* gene which was summarized in **(Fig. 4G)**. ASP treatment upregulated the gene expression of *Beclin1* to 1.2-fold compared with negative control. On the other hand, application of BB and CA phytotherapy in ASP treated HepG2 cells minimized these values up to 0.75 and 0.87-fold respectively which was appeared below than negative control. It was recapitulated from fig. 4hthat during course of ASP exposure in HepG2, mRNA expression of *LC3* gene was modulated up which was observed to be 1.69-fold in comparison with that of the negative control. Subsequently augmentation of BB and CA during ASP exposure dropped down this enhanced relative quantitation up to the extent of 1.32 and 1.00 respectively.

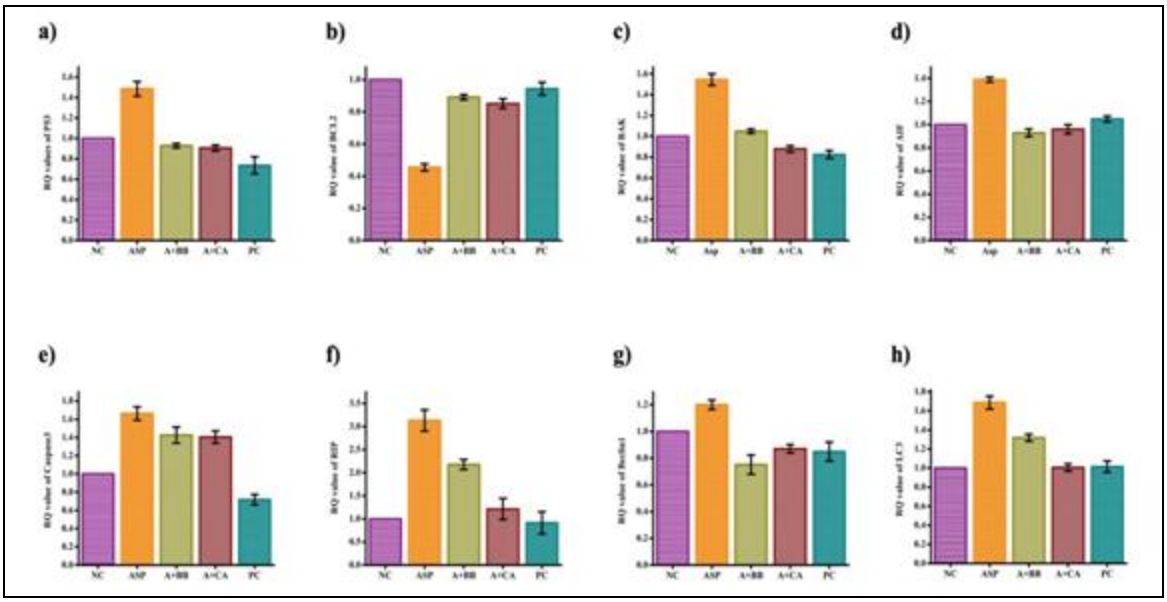

FIG. 4: AMENDMENT IN EXPRESSION PATTERNS OF GENES ASSOCIATED WITH APOPTOSIS, NECROSIS AND AUTOPHAGY AS A PHYTOTHERAPY IMPLEMENTATION A) *P53* B) *BCL2* C) *BAK* D) *AIF* E) *CAS3* F) *RIP1* G) *BECLIN1* H) *LC3*. THE GENE EXPRESSION DATA EXPRESSED AS THE AVERAGE ± STANDARD DEVIATION OF THREE SEPARATE EXPERIMENTS (N=3). Values of the treatment sets substantially differed from those of the Asp set (*p≤0.03; CV≤0.05).

DISCUSSION: For the pharmaceutical, cosmetics, chemical and food sectors, an insufficiency of an effective and precise method to assess the safety of chemicals as well as additives is a significant obstacle. In fact, the safety of food additives, as a class of natural or manufactured substances, has been widely questioned. Recent studies have focused on toxicities related with food additives

including Aspartame and further complications arise due to this. It was recently revealed that aspartame administration, either alone or in combination with a modest systemic inflammatory response increases oxidative stress along with inflammation in the brain [15].

Although, several detrimental consequences of aspartame have been proven in studies, the position of aspartame is controversial and still it is available in market for unlimited use. Considering these findings, we strongly believe that a thorough examination of currently used food additives in the light of health safety is necessary. Therefore, our attention has shifted towards the intervention of exogenous antioxidants of plant origin to restrain this impairment caused due to Aspartame in cultured human hepatocytes through oxidative stress which aim to betterment of life. Chebulinic acid (CA) and boeravinone B (BB) were discovered to help stressed *S. cerevisiae* cells rejuvenation by reducing the biochemical parameters associated with programmed cell death (PCD) as well as necrosis [16]. Subsequent studies with CA and BB on stressed *S. cerevisiae* demonstrated that by altering the mRNA expression of genes implicated in several cell death pathways, CA and BB phytotherapy shield cells from the cytotoxicity caused by MG [17].

Our data suggest that supplementation of BB and CA in HepG2 cells during ASP exposure is advantageous in scavenging produced oxidants and preventing additional cellular losses. Increased RNS production, in addition to ROS, causes severe abnormalities in cellular redox homeostasis, culminating in post-transcriptional damage to cellular and mitochondrial proteins, which ultimately responsible for disturbing cellular metabolism and leads chronic disorders [18]. This increase in nitric oxide following aspartame ingestion, on the other hand, should not be underestimated as chronic exposure to free radicals, even at low concentrations might damage biologically vital molecules [19]. The reduction of extracellular nitrite and restoration of cellular homeostasis in stressed hepatocytes following BB and CA treatments demonstrates the therapeutic importance of natural antioxidants, which might be linked to higher organisms. The findings suggested that antioxidant potential of BB and CA is linked to scavenge oxidants by themselves with the donation of hydrogen or electrons. According to earlier report, grape seeds' tocotrienol-rich fraction resulted in boosting in activities of SOD, CAT, and GPx, so as to minimize oxidative stress induced with tert-butyl hydroperoxide in HepG2 cells [20].

Protein carbonylation, also used as an indicator of oxidative damage that has been connected to programmed cell death (PCD) and diseases linked with it. BB and CA treatments reduced protein carbonyls in stressed cells, suggesting that they could be used medicinally to combat against protein carbonylation and various metabolic disorders associated with it during the process of PCD in higher organisms. According to recent reports, lipid peroxides are formed when structural damage to lipids occurs. Secondary messengers of oxidative stress, lipid peroxides and their derivatives can lead to cell death and several diseases linked with aging, which can be addressed with herbal antioxidants [21]. Based on data from our present study, it is confirmed that BB and CA have efficiently lowered the lipid peroxidation levels during ASP treatment and rescued HepG2 cells from possible PCD. However, present study showed a decrease in caspase3/7 activity as a result of BB and CA augmentation, indicating lowered apoptosis.

Apoptosis is a physiological cell-death mechanism that is frequently coupled with cells shrinkage, chromosomal DNA breakdown, disturbed mitochondrial membrane potential etc. This is the reason why cells were tested using DAPI and data displayed that BB-CA phytotherapy minimized the rate of DNA disintegration in due course of ASP exposure and protected cells from undergoing apoptosis. Mitochondria are crucial and injury to them can increase their membrane permeability and diminish their membrane potential. However, because exogenous antioxidants have a protective function in preserving mitochondrial integrity, it was feasible to prevent cells from going through cell death pathways. Similar results obtained with Annexin V staining which confirmed that ASP induces apoptosis in HepG2 which was significantly attenuated by BB and CA. An earlier research demonstrated that Olaquindox caused apoptosis in HepG2 cells, demonstrating the connection between ROS and programmed cell

death and showed that Olaquindox triggered apoptosis through mitochondrial pathways dependent on caspases9 and 3, which involved *P53* and *Bcl-2* family proteins [22]. Our findings are in agreement that increased *P53* levels followed by DNA damage further enhance the expression of *Bak* which is pro-apoptotic while decreased the expression of *Bcl-2* protein which is anti-apoptotic. This mismatch in the *Bak*/*Bcl-2* ratio could lead to dissipation in mitochondrial membrane potential. Finally, defective mitochondria could release cytochrome-c as well as *AIF* into the cytosol from which Cytochrome-C activates *Cas3* through procaspase 9 activation on the other hand *AIF* regulates apoptosis independent of caspases. The current investigation confirms that ASP induce mitochondrial apoptosis pathway in hepatocytes, but BB and CA phytotherapy rescues them from apoptosis and proved anti-apoptotic.

In mammals, the kinase *RIP1*has been demonstrated to play a vital role in necrosis in response to *TNF, TRAIL*, or *FasL* activation which interacts with a second kinase, *RIP3*, and the cell enters a necrotic state [23]. The data recapitulated from our investigation that ASP exposure allowed HepG2 cells to undergo Necrosis through up regulation of *RIP1*while, stressed hepatocytes were protected from necrosis by BB and CA augmentation. Additionally, it was confirmed by propidium iodide staining which have shown resemblance with our molecular studies with necrosis.

The autophagy route is linked to a number of proteins, with the *Beclin-1* and microtubule associated protein 1 light chain 3 (*LC3*) being particularly important [24]. Our findings are consistent with recent investigation in which the markers of autophagy *Beclin1* and *LC3* were highly active in the rat group that received 400 mg/kg of Olaquindox, showing that the pathway of autophagy was actively participated in apoptosis induced by Olaquindox [25].

Results with *Beclin1* and *LC3*along with flow cytometric analysis using MDC proved that ASP initiates autophagic cell death also while the protective potential of phyto-components against Autophagy was clearly obvious in stressed hepatocytes.

CONCLUSION: In summary, the present investigation revealed that Aspartame is responsible for triggering cell death pathways through induction of oxidative stress in cultured human hepatocytes but intervention of BB and CA phytotherapy rescued them from this holocaust. Moreover, excessive ROS induced by ASP was a key commencing factor for the programmed cell death which was confirmed by Caspase 3/7 analysis and its mechanism might be linked with the mitochondrial pathway. Genes associated with necrosis along with autophagy also found to be activated during treatment of ASP but augmentation of Boeravinone B and Chebulinic acid in due course of ASP exposure proved beneficial by ameliorating aforementioned devastating events and rescued cells from undergoing cell death pathways.

Authors' Contribution: Conceived and designed the experiments: PKP. Performed the experiments: SSK. Data analysis: SSK, HKB & PKP. The original manuscript was written by: SSK. Examined and amended the manuscript: SSK, HKB & PKP.

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