"*Bacopa monnieri* (Brahmi) induced Autophagy Inhibit Benzo[a]pyrene mediated cytotoxicity"

Thesis submitted to Department of life science for the partial fulfillment of the M.Sc. Degree in Life science

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CERTIFICATE

This is to certify that the thesis entitled **"*Bacopa monnieri* (Brahmi) induced Autophagy inhibit Benzo[a]pyrene mediated cytotoxicity"** which is being submitted by Ms.Aditi Nayak , Roll No- 412LS2044, for the award of the degree of Master of Science from National Institute of Technology, Rourkela, is a record of bonafide research work, carried out by her under my supervision. The results embodied in this thesis are new and have not been submitted to any other university or institution for the award of any degree or diploma.

SKBhutia
Sujit K Bhutia

DECLARATION

I do hereby declare that the Project report entitled **"*Bacopa monnieri* (Brahmi) induced Autophagy inhibit Benzo[a]pyrene mediated cytotoxicity"** submitted to the Department of Life Science, National Institute of Technology, Rourkela for the partial fulfillment of the Master Degree in Life Science is a faithful record of bonafide and original research work carried out by me under the guidance and supervision of **Dr. Sujit Kumar Bhutia**, Assistant Professor, Department of life Science, NIT, Rourkela.

Date: 10th May 2014
Place: NIT, Rourkela

Aditi Nayak

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LIST OF ABBREVIATIONS

PBS ---------- Phosphate Buffer Saline
et al ---------- And others
Conc ---------- Concentration
Hrs ---------- Hours
pH ---------- Hydrogen concentration
PM ---------- Particulate Matter
B[a]P ---------- Benzopyrene
PAHs ---------- Polycyclic Aromatic Hydrocarbons
ROS ---------- Reactive Oxygen Species
DEP ---------- Diesel exhausts particles
BM ---------- *Bacopa monnieri*
AIF ---------- Apoptosis Inducing Factor
DMSO---------- Dimethyl sulfoxide
FBS ---------- Fetal bovine serum
DMEM ---------- Dulbecco's modified Eagle Medium
% ---------- Percentage
Mg ---------- Milligram
µg ---------- Micro gram
nm ---------- Nano meter
CQ ---------- Chloroquine
µM ---------- Micro molar
mL ---------- Micro liter
Atg ---------- Autophagy gene
ER ---------- Endoplasmic reticulum
EPA ---------- Environmental Protection Agency
NAAQSs ---------- National Ambient Air Quality Standards
ETS ---------- Electron Transport Chain
mtDNA ---------- Mitochondrial DNA
mTOR ---------- Mammalian target of rapamycin
LC3 ---------- Light Chain 3
AhR ---------- Aryl-hydrocarbon receptor
ARNT ---------- Aryl hydrocarbon receptor nuclea translocator
BPDE---------- B[a]P-7,8-dihydroxy-9,10-epoxide
DISC ---------- Death inducing signal complex
GAPDH ---------- Glyceraldehyde-3-phosphate dehydrogenase
Mitophagy---------- Mitochondrial autophagy

ABSTRACT

Particulate matters like PAHs (Polycyclic Aromatic Hydrocarbons) are one of the most common and dangerous air pollutants which severely affect the organisms. Among all types of PAHs, Benzo[a]pyrene (B[a]P) has been found to be more toxic to the organism as well as the environment. Several evidences have suggested the cytotoxic effect of B[a]P. The sole objective of this study was to examine the cytoprotective role of the *Bacopa monnieri* (BM) against B[a]P induced cytotoxicity and to reveal the detailed mechanism under this protection. This study has showed the protective action of BM against the toxicity induced by B[a]P treated in HaCaT cell line through the cell survival assay. The morphology of B[a]P, BM and both B[a]P+BM treated cells were observed under bright field microscope and demonstrated that BM could thwart the induction of apoptosis by B[a]P. Caspase activity of B[a]P was also studied by caspase-Glo 3/7 assay reporting the high apoptotic capacity of B[a]P. More over to disclose the mechanism behind the protective action of BM, acridine orange staining was done showing the autophagy induction by BM. To become more assure about the autophagy induction by BM, an autophagy inhibitor known as Chloroquine (CQ) was treated in the cells which reflected that CQ had decreased the autophagy induction by BM in presence of B[a]P increasing cell death than BM . B[a]P treated cells only . Thus this study confirmed that BM can defend HaCaT cells from B[a]P through inducing autophagy which could have a curative application in the anticipation of B[a]P.

Keywords: PAHs (Polycyclic Aromatic Hydrocarbons), B[a]P (Benzo[a]pyrene), BM (*Bacopa monnieri*), caspase-Glo 3/7 assay, CQ (Chloroquine), cell survival assay.

1. INTRODUCTION

Any unwanted or unsuitable change in the physiochemical and biological properties of the environment which has deleterious effects on each and every sphere of the environment is termed as Pollution. These undesirable changes are brought about by the pollutants and they are differentiated according to the type of pollution like air, water, soil or noise pollution etc. Among all pollutions air pollution is very much toxic to the environment and it is mainly caused by Carbon monoxide (CO), Sulphur dioxide (SO_2), Hydrocarbons and volatile organic carbons, Nitrogen oxides, Particulate matters, Photochemical smog, etc . These air pollutants are mainly released from anthropogenic sources like industries and traffic. Among almost two hundred hazardous air Pollutants-mainly corresponding to suspended particulate matter and gases-only six are monitored by the Environmental Protection Agency (EPA) which sets the National Ambient Air Quality Standards (NAAQSs) for air particles, carbon monoxide ozone, nitrogen oxides, sulfur oxides and lead [1]. In the present scenario the air pollutants like Particulate matters (PMs) and diesel exhaust particles (DEP) are the sole cause of many health hazards. Air pollution stimulates respiratory disorders, reproductive disorders and cancers. It is believed that air borne chemicals cause somatic mutations in normal cells, which then develop into malignant. Airborne pollutants affect health in changing degrees of severity, ranging from serious illness to premature death problems. It is believed that air borne chemicals cause somatic mutations in normal cells, which then develop into malignant. Airborne pollutants affect health in changing degrees of severity, ranging from serious illness to premature death problems. Air pollution by particles is characterized by the particle size, mass concentration, number concentration and chemical composition. PMs are usually defined as PM_{10}, $PM_{2.5}$ and $PM_{0.1}$ that correspond to airborne particles with an aerodynamic diameter equal or less than 10, 2.5 and 0.1 microns,

respectively [1]. PM_{10} and $PM_{2.5}$ are often classified as the "coarse" fraction; $PM_{2.5}$–$PM_{0.1}$ as the "fine" fraction of particles (FP) and $PM_{0.1}$ correspond to the "ultrafine" fraction of particles (UFP) [1]. The composition of PM_{10} varies, as they can absorb and transfer a multitude of pollutants. The main reason of PM toxicity may be due to the high metal content, PAHs and other organic component. PAH include benzo[a]pyrene, dioxin, dibenzofuran, anthracene and etc. In the past, EPA has assessed risks posed by mixtures of PAHs by assuming that all carcinogenic, but amongst them benzo[*a*]pyrene (B[*a*]P) and dioxin are most potent. It has been shown that they cause DNA damage and somatic mutations in normal cells culminating into malignancy. The industrial pollutants cause cytotoxicity mediated cell death and suppression of immune system which result in the mutated cells to proliferate leading to cancer. A rich amount of polycyclic aromatic hydrocarbons (PAHs) and their derivatives present in DEP have the potential to generate reactive oxygen species (ROS) [5, 6]. Superoxide is produced by the pollutant like 9, 10-phenanthraquinone (PQ), a PAH-quinone component of DEP [7]. The micromolar concentrations of quinone have deadly effect on human pulmonary epithelial A549 cells [8]. They also indicate aerobic and anaerobic perniciousness in yeast [9]. PM induced oxidative stress has been considered as an important molecular mechanism of mediated toxicity. At high levels of oxidative stress, disruption of the mitochondrial permeability transition pore and the electron transfer chain cause apoptotic, autophagy and necrotic cell death.

1.1) AIR POLLUTANT CYTOTOXICITY

Cell toxicity leading to cell death is the ultimate upshot of air pollutants. They mainly attack the power house of the cell i.e. mitochondria such as 2, 4-dinitrophenol (DNP) uncouples the ATP synthesis in Electron Transport Chain (ETS), Cyanide and CO block the transfer of electrons

from cytochrome oxidase complex to the terminal oxygen. Air pollutant induced cell death may occur due to programmed cell death (Type I apoptosis) or due to necrosis. Apoptosis is a form of regulated cell death represented by activation of caspases which is critical and injurious to cell [10]. Several studies have been done regarding the potential of air pollutants to spark off the process of apoptosis. Very few of them have been published and most of them riveting on the competition between ROS (Reactive Oxygen Species) production and the opposition by the antioxidants ultimately leading to oxidative stress [1]. It has been found that the air pollutants like PMs cause cell death by both ROS dependent and ROS independent pathway (Fig.1). The experiments carried out in respiratory cells using high doses of PMs have demonstrated their striking effects featuring oxidative stress on them [11, 12]. Oxidative stress produced by them induce intrinsic pathway of apoptosis [13]. Oxidative stress also causes mitochondrial lipid peroxidation during apoptosis [1]. Enzymes such as cytochrome P450 oxidase 1A1 present in the hepatic cell produce H_2O_2 while converting B[a]P (benzo [a] pyrene) into BPDE (anti-7,8-dihydrodiol-9,10-epoxybenzo [a] pyrene) [14,15]. This reaction actuates DNA damage and cancer [16]. Mitochondrial pathway involving electron transfer chain and the NADPH oxidase activity are induced to produce ROS activated by the metals like cadmium or hexavalent chromium [17, 18]. Polluting metals impose serious impact on human health while interacting with necessary elements in the metabolic pathways [19]. This ROS is also involved in Mitochondrial DNA (mtDNA) damage due to propinquity to the production area and the deficiency of protective histones protein [1]. Recently various new mechanisms of pollutants induced apoptosis have came into limelight such as 1-Nitropyrene and B[a]P causing apoptosis carried out through lipid accumulation [20], modification in the structure of plasma membrane

particularly alteration in Na^+/H^+ ion channels, prohibition of intercellular communication system through the gap junction [21], and modification in the constitution of lipid rafts [22].

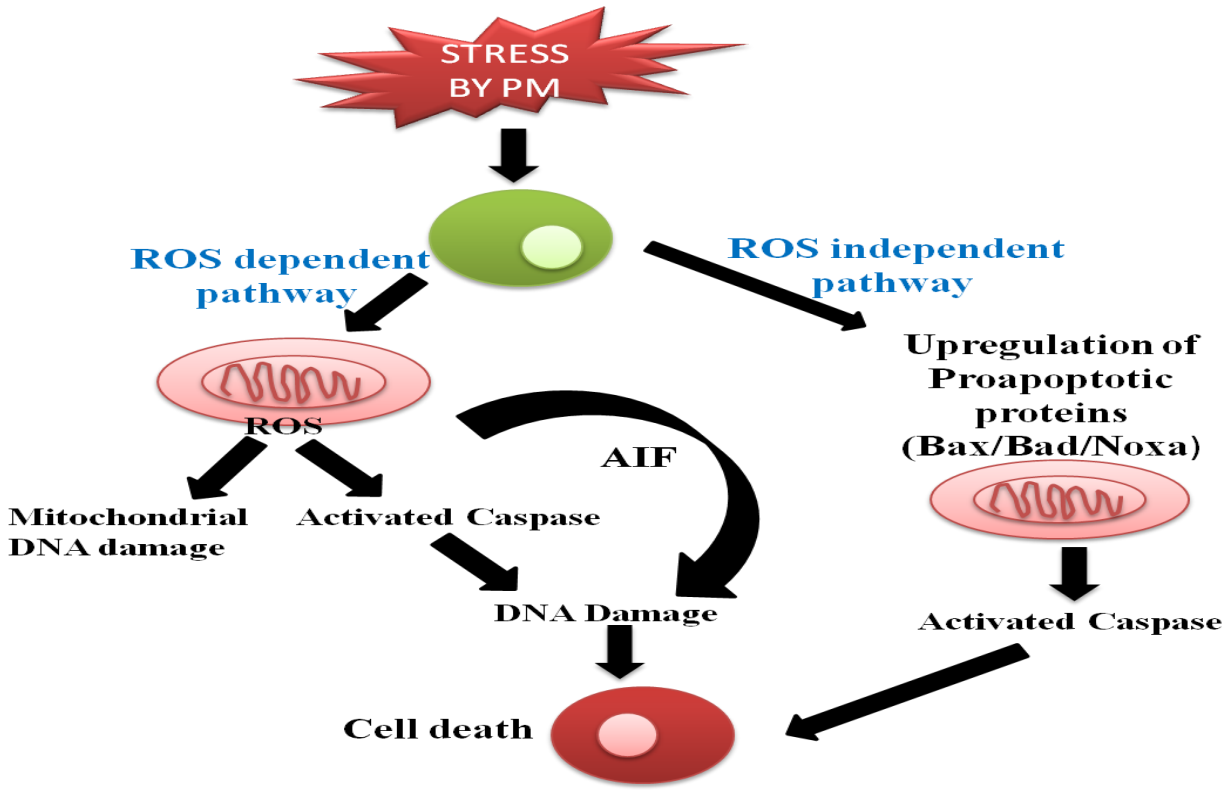

Fig.1. Particulate matter (PMs) induce apoptotic cell death both by ROS dependent and ROS independent pathway. PMs are the potent producer of ROS which cause oxidative stress. This finally cause mitochondrial DNA damage, caspase activation and triggering of apoptosis inducing factor (AIF) which stimulate apoptosis. In the ROS independent pathway PMs upregulate the expression of proapoptotic proteins (Bax/Bad/Noxa) which ultimately activate caspase leading to apoptosis.

1.2) AUTOPHAGY: A CYTOPROTECTIVE MECHANISM

Autophagy is a fundamental homeostatic process in both normal and stressful situation of all mammalian cell types which has a significant role in cell survival mechanism [23]. Two most

important physiological functions is governed by autophagy for e.g. recycling indispensable cell components and expelling undesirable cytoplasmic substances, such as impaired cell organelles like mitochondria and endoplasmic reticulum (ER) i.e. true autophagy or extracellular foreign particles i.e. xenophagy [23].

Autophagy is a fundamental catabolic cellular process first reported by Clark about six decades ago in mammalian cell [24]. Afterward it was clearly described by de Duve and his colleagues [25, 26, 27]. About 35 autophagy genes (Atg) genes have been identified from genetic analysis of yeast till now [28, 29]. As autophagy is evolutionarily conserved the homologous of yeast's autophagy genes must be present in mammals [29, 30]. The complete process of autophagy is divided into three important steps i.e. nucleation, elongation and maturation (Fig.2). In the first step cellular and metabolic stress causes inactivation of mammalian target of rapamycin (mTOR) which is a potent inhibitor of autophagy [31, 32]. This leads to hypophosphorylation of Atg13. This phosphorylated form then binds to Atg1 facilitated by Atg 17 [30]. Then Atg 1 inscribes Atg9 resulting in the lipid extraction from cell organelles like Golgi bodies, endoplasmic reticulum (ER), and the nucleus [30]. The stressed cells initiate the formation of phagophores by forming a lunate shaped double layer membrane. The elongation step is followed by the nucleation where class III of phosphoinositide (PI) 3-kinases i.e. vesicular protein sorting 34 (Vps34) facilitates the elongation of phagophores in association with Beclin-1 (mammalian homologue of yeast Atg6) [33]. This reaction gives rise to the formation of phosphatidylinositol-3-phosphate (PI3P) which has a vital role in the synthesis of autophagosome [33]. Activating molecule in Beclin1-regulated autophagy protein- 1 (Ambra-1), ultraviolet radiation resistance-associated gene (UVRAG), and Bax interacting factor-1 (Bif-1) are involved in enhancing the interaction between Vps34 and Beclin-1 [30]. On the other side this interaction is inhibited by

Bcl-2, Bcl-xL, and Run domain Beclin-1 interacting cysteine- rich containing protein (Rubicon) [34]. Two ubiquitin-like conjugation systems contribute to the enlargement, molding and sealing off the autophagosome membrane [35]. At first binding of Atg12 with Atg7 (E1 ubiquitin-like activating enzyme) in the presence of ATP takes place, after which Atg12 connects to Atg5 through Atg10 (E2-like ubiquitin carrier); then Atg5-Atg12 conjugate system interacts with Atg16 forming a trimer. This large complex of Atg5-Atg12-Atg16 assists in membrane expansion and in shaping its edges. The second ubiquitin-like conjugation system involves the cleavage of microtubule-associated light chain 3 (LC3) (mammalian homologue of Atg8) by Atg4 (a cysteine proteinase, also known as autophagin) giving rise to LC3 I. This LC3 I is activated by binding with E1-like Atg7 in an ATP dependent reaction. After wards activated LC3I combines with E2-like carrier Atg3 which stops the LC3 lipidation. Then LC3I is conjugated to phosphatidylethanolamine (PE) forming LC3II (or LC3I-PE) [36]. This step of conversion of LC3 from LC3-I (free form) to LC3-II (conjugated form) is considered as a decisive step in the formation of autophagosome [36]. Green fluorescence protein (GFP) tagged with LC3 when expressed in cells and tissues; it shows green puncta which is an indicator of autophagosome formation [37]. In general the developing phagophore membrane interacts with particular protein aggregates and organelles; but LC3-II acts as a receptor on the phagophore membrane and takes up the protein aggregates and spoiled mitochondria leading to their degradation [30]. The multiadaptor molecule p62/SQSTM1 combines with Atg8/LC3 present on the phagophore membrane for their degradation [38, 39]. Likewise Atg32 protein in yeast promotes the mitochondrial engulfment which is known as mitophagy [40]. In the last step of autophagy, the autophagosome fuses with the lysosomes forming autolysosomes. The acidic

enzymes secreted from lysosome helps in the degradation of cargo which on the other hand provides energy to the cells to act against the stress [39, 41].

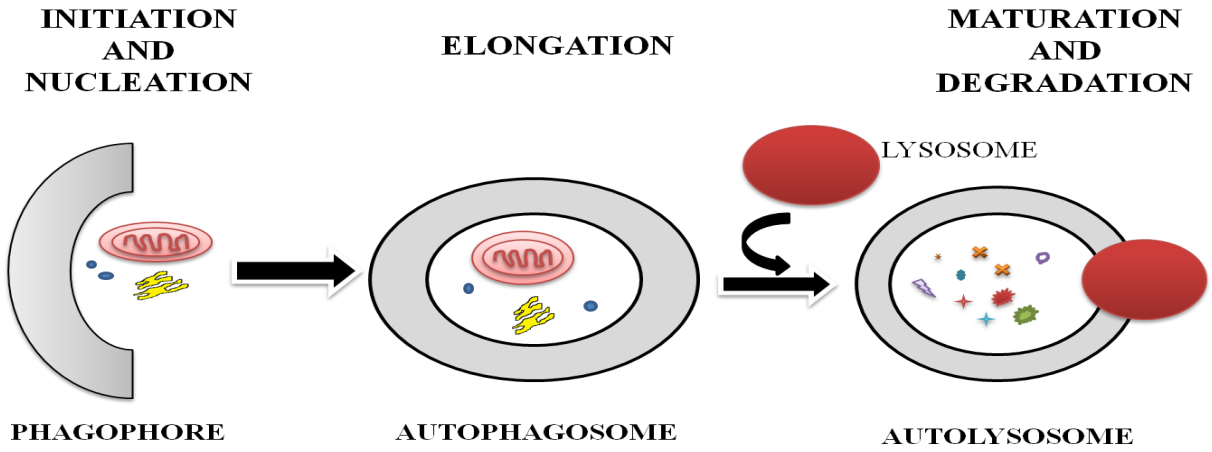

Fig. 2. Three steps of Autophagy i.e. Initiation or nucleation, Elongation and Maturation/Degradation.

In this way autophagy has a vital prosurvival and housekeeping function during cellular and metabolic stresses and degrades the unnecessary misfolded proteins and damaged cell organelles whose accumulation may cause inflammatory response and may show adverse effect on the cell. In contrast it has been found that the dysregulation or over activation of autophagic process induce nonapoptotic cell death [42, 43, 44, 45]. The increased formation of autophagic vesicles (AV) and their accumulation results in autophagic cell death [10]. It differs from apoptotic cell death in many ways; apoptosis involves chromatin condensation and caspase activation but autophagic cell death lacks caspase activation and chromatin condensation [46, 47].

2. REVIEW OF LITERATURE

2.1) BENZO [a] PYRENE (B[a]P) INDUCING CYTOTOXICITY

Benzo[a]pyrene (B[a]P) (Fig.3) is a Polycyclic aromatic hydrocarbons (PAH) as well as a potent air pollutant. These compounds are generated during incomplete combustion of organic materials like fossil fuels, coal, oil, cigarettes [48, 49] and also from industrial waste, diesel exhaust and charcoal- broiled food. B[a]P is the first pure compounds whose carcinogenicity has been demonstrated in mice [50]. B[a]P is also involved in interrupting the endocrine system [51]. The role of B[a]P in enhancing the level of apoptosis has been studied in many cells like wise Hepa1c1c7 hepatoma cells [52], human ectocervical cells [53] and primary human macrophages [54]. But the detailed mechanism of apoptosis induction by B[a]P has been studied in Hepa1c1c7 cells [55, 56]. The effects of B[a]P vary from cell to cell. In some cells it causes enhanced cell proliferation [57] and in some other it changes the energy metabolism [58] and it is also involved in inducing necrotic and apoptotic cell death [59]. These kinds of PAH are also involved in increasing the level of phosphorylation and aggregation of the tumor suppressor protein p53 leading to the formation of DNA adduct [59, 60]. They result in DNA damage by inducing apoptosis [61]. In the cell a transcription factor known as aryl-hydrocarbon receptor (AhR) is present to which the ligand B[a]P binds [62]. This ligand- receptor complex moves into the nucleus and dimerizes with the aryl hydrocarbon receptor nuclear translocator (ARNT). Then this large complex combine specific promoter sequence by which the genes required for the metabolism are transcribed. These genes are cytochromes P450 CYP1A1, CYP1A2, and CYP1B1 [62–65]. The oxidation of B[a]P is carried out by many cytochrome P450 enzymes such as 7,8-epoxidation of B[a]P and 9,10-epoxidation of B[a]P-7,8-diol. CYP1A1 which has been reported as the most potent oxidants in case of mammals [66–68]. It activates B[a]P into

B[a]P- 7,8-oxide. This newly formed oxide is hydrated by the enzyme epoxide hydrolase to form (_/_)-B[a]P-*trans*-7,8-dihydrodiol (B[a]P- 7,8-DHD). This B[a]P- 7,8-DHD which is the substrate for another Cytochrome P450-dependent oxidation reaction is responsible for the production of a carcinogenic compound termed as, B[a]P-7,8-dihydroxy-9,10-epoxide (BPDE). BPDE has the ability to bind to the nuclear DNA covalently and this binding result in the mutation, replication erroe and apoptosis mediated cell death [69]. The enzymes responsible for B[a]P activation are present inside the uterine endometrium [70] which has been described as a possible target tissue for B[a]P action [71].

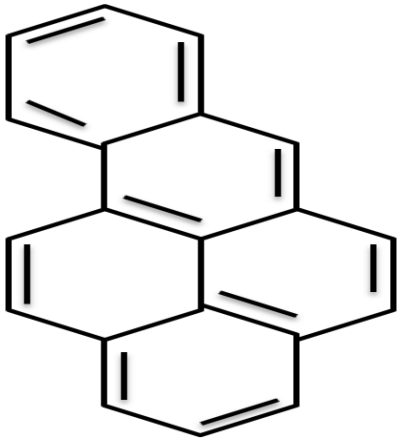

Fig. 3. Structure of Benzo[a]pyrene

2.2) ROLE OF AUTOPHAGY IN CYTOPROTECTION

Autophagy is involved in various biological processes, such as development, differentiation, aging, and immunity [72, 73]. It takes part in the cellular metabolism and protects the cell from starvation due to nutrient deprivation [72]. Various research studies have proved the cytoprotective role of autophagy during stresses like starvation [74]. In an experiment on mice whose Atg5 was genetically removed (Atg52/2) to stop the action of autophagy were found to be sensitive to cardiac dysfunction leading to starvation [75]. It has also been shown that knockdown of beclin 1 or LC3, or by 3-methyladenine (3MA, chemical inhibitors of autophagy)

in starved HeLa cells, mediated by small interfering RNA (siRNA) were found to inhibit autophagy following the activation of caspase-3 and initiation of apoptosis [76]. The recent studies have proved the interrelation between autophagy and apoptosis [77, 78]. In the extrinsic apoptotic pathway, one component of death inducing signal complex (DISC) i.e. FADD (proapoptotic protein) is associated with Atg5 to regulate the pathway [79]. The complex formed by beclin1 and Bcl-2, an inhibitor of apoptosis [80] can induce cell survival by the over expression of beclin1 and inhibition of apoptosis or can induce autophagic death by inhibition of Bcl-2 [80, 81]. The cell protective activity of autophagy has been noticed in mammals, plants, yeast, and flies [31]. During starvation, the application of autophagy inhibitors like hydroxyl chloroquine, 3-methyladenine, bafilomycin A1 etc and knocking down the Atg genes like Atg5, Atg10, Atg12, and Beclin 1 results in apoptosis [82]. During the development of a mice embryo, the autophagic level is very less but it suddenly increases and persist this high level for 3 to 12 hours after birth. Moreover it comes back to the original level within 1 to 2 days [31]. It has been shown that neuron specific Atg genes (Atg 5 and 7) deficient mutant mice affected with neuron degeneration [83, 84]. During the release of cytochrome *c* with lack of caspase activation in apoptosis, Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) raises the autophagic level [85]. Autophagy is activated by nuclear GAPDH which enhances the expression of Atg12 [31]. As many types of stresses trigger apoptosis, the cytoprotective mechanism of autophagy is evident in cells that lack the apoptotic cell death pathway. Recent reports have suggested that mitochondrial autophagy (mitophagy) is particularly important for inhibition of cell death. The damaged mitochondria is removed by autophagy which is induced by mitochondrial permeability transition induces autophagy. All the above examples reflect the cytoprotective action of autophagy.

2.3) DYNAMIC EFFECTS OF *Bacopa monnieri* (BRAHMI)

Since from the ancient era Indians, Chinese, Egyptians and others have been utilizing a diversity of plants and their products as therapeutics against various diseases [86]. Pharmacopoeia has reported that about 25% drugs are obtained from plants and their products [87]. The World Health Organization (WHO) predicted that for basic health requirements, about 80% of the developing countries population depends on conventional medicines mainly obtained from plants [88]. Out of these plants, *Bacopa monnieri* (Brahmi) is one of the potent ancient medicinal plants. For about 3000 years *Bacopa monnieri* (Brahmi, BM) has been showing its cognitive impact to mankind as a traditional ayurvedic medicine [89]. It is a wetland macrophyte with creeping stem. BM is considered as a drug for memory enhancement and for nervous system related disorder [90, 91]. Besides its role in memory enhancement, it is also found to be very effective against various disorder related to respiratory, cardiac and nervous system like anxiety, anti-amnesic, depression, epilepsy, insomnia, psychosis and stress [92]. No fallouts have been reported regarding the use of BM [92]. Numerous triterpenoid saponins such as Bacosides have been recognized as the active components of BM [93, 94]. Bacoside A and B are considerd to be the main reason for the memory-boosting action of BM [95]. The BM extract made by using alcohol has shown the presence of many other phytochemicals like bacosides A1, A2 and A3 [96], bacopasaponins A to G [97], and bacopasides I to V [98] triterpenoid saponins (e.g. dammarane-type and jujubogenin) and aglycones (e.g.pseudo-jujubogenin). Components of BM having many applications in drug preparation include triterpenoid saponins, saponins (D-mannitol and hersaponin), alkaloids (brahmin and herpestine) and sterols [99]. Other active compounds have been recognized are stigmastarol, betulic acid and beta-sitosterol. The beneficial outcomes of BM against the toxicity caused due to paraquat/diquat have been studied

[89]. Its defensive action against rotenone and cell death due to oxidative stress and mitochondrial impairment had been shown using the model like Drosophila [100]. This herb has assured the organism to protect against oxidation, lipid peroxidation [101, 102], fatigue [103], inflammation [104], fertility [105], cancer [106] and ulcer [107]. It has also the potential to reduce the action of the enzyme lipoxygenase and the divalent metals leading to ROS deactivation [108]. Aquatic plants have the ability to cumulate the heavy metals [109]. So as an wetland plant BM also counter act the effect of heavy metals by enhancing the generation of antioxidants [110, 111]. BM chelates the metal ions and disrupts oxidative chain reaction [112], which ultimately ameliorate the action of superoxide dismutase, catalase and glutathione peroxidase like antioxidant enzymes [113]. Damaged neurons renovation by improving the kinase activity, re-establishment of synaptic activity with neuronal synthesis and conduction of nerve impulse are also carried out by BM [114]. The stress relieving capacity of BM in animal models is due to the alteration of Hsp70 expression levels and cytochrome P450. The protective action of BM against brain injury by ischemia has been studied in an animal [115]. Increased spatial learning potential and memory [116] has been reported in rats by the medication of BM. Previously the alcoholic extract of BM had demonstrated the suppression of scopolamine induced amnesia and inhibition of stress in rats [117]. The BM extract had also reduced the β-amyloid activity in a transgenic mouse model which in reality induces oxidative stress leading to Alzheimer's disease [117]. The defending actions of BM against the neurotoxic effect of Methyl Mercury (MeHg) have been reported in rats [118].

Kingdom---------- Plantae

Subkingdom ------- Tracheobionta

Superdivision------ Spermatophyta

Division---------- Magnoliophyta

Class---------- Magnoliopsida

Subclass---------- Asteridae

Order---------- Scrophulariales

Family---------- Scrophulariaceae

Genus----------*Bacopa*

Species---------- *monnieri*

Fig. 4.1 Classification and picture of *Bacopa monnieri* Plant

Fig. 4.2 Flower of Brahmi

Fig. 4.3 Brahmi capsule from Himalaya Herbal Healthcare

3. MATERIALS AND METHODS

3.1) REAGENTS:

3-[4,5-Dimethylthiazol-2-yl]-2,5-diphenyltetrazolium (MTT), dimethyl sulfoxide (DMSO), trypsin, were purchased from Sigma Aldrich, India. The fetal bovine serum (FBS), Dulbecco's modified Eagle Medium (DMEM) was purchased from Invitrogen, India. B[a]P and Chloroquine were purchased from Sigma Aldrich, India. BM capsules from Himalaya Herbal Healthcare were used for the experiment. Caspase Glo 3/7 assay was carried out using promega kit.

3.2) SOURCE OF *Bacopa monnieri* (BM):

BM capsules were dissolved in 10X PBS and the stock concentration of 10mg/ml was made under sterile condition.

3.3) CELL LINE:

The immortalized human keratinocyte cell line (HaCaT) was obtained from National Centre for Cell Science, Pune, India and cultured in Dulbecco's modified Eagle's medium with high glucose (DMEM/high glucose), supplemented with 10% heat-inactivated fetal bovine serum (FBS) containing and 1% penicillin–streptomycin. The cells were maintained at 37^{o}C in a humidified atmosphere at 5% CO_2. All media, supplements and antibiotics were purchased from Invitrogen.

3.4) MTT ASSAY:

Principle:

This is a colorimetric cell viability and proliferation assay. By the action of mitochondrial succinate dehydrogenase enzyme the metabolically active cell converts the yellow coloured 3-(4, 5-dimethythiazol-2-yl)-2, 5-diphenyl tetrazolium bromide (MTT) into purple coloured formazan product (fig.5). The MTT enters into the cells and passes into the mitochondria where it is reduced to an insoluble, dark purple coloured formazan product. The cells are then solubilised with DMSO and this solubilised formazan product is measured spectrophotometrically at a wavelength typically between 500 and 600 nm. Only the viable cell can reduce MTT and the capacity to reduce MTT is the estimation for viability of the cells.

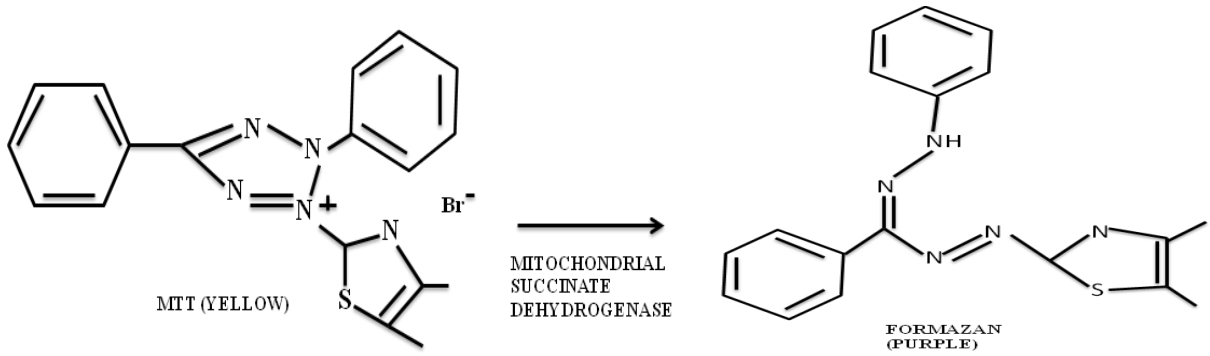

Fig.5. Reduction of MTT by Mitochondrial Succinate Dehydrogenase into Fomazan product.

Procedure:

In this study cytotoxic effect of B[a]P and cytoprotection activity of BM of varying concentration was demonstrated in HaCat cell line by MTT assay. The cells were re harvested in T25 culture flask (BD) and trypsinised when they reached at 80-90% confluency. The cells were

seeded in 96 well plates. After 24 hrs cells were treated with varying concentration of BM and again after 3hrs cells were retreated with B[a]P. After 72hrs of incubation MTT was added and kept for about 4hrs. After that DMSO (SIGMA) was added to dissolve the formazan and O.D was taken at 595nm in an Elisa plate reader (Perkin Elmer).

3.5) ACRIDINE ORANGE STAINING:

Principle:

It is an acidotropic nucleic acid selective stain which intercalates DNA and RNA. When it intercalates DNA it fluoresces green (525nm) and when RNA it fluoresces red (>630nm). It enters into the lysosome and gets protonated. This protonated form fluoresces red colour. More red intensity corresponds to more formation of autophagic vesicles. This dye stains the late autophagic vesicles only.

Procedure:

HaCaT cells were cultured with varying concentration of BM and B[a]P for 24 h and then washed with PBS. Then the cells were stained with 0.5 µg/ml of acridine orange for 15 min to detect the late autophagic vesicles. After that the media was discarded and the cells were washed with PBS for three times and observed under inverted fluorescent microscope.

3.6) CASPASE ACTIVITY ASSAYS:

Principle:

The Caspase activity was measured by Caspase-Glo 3/7 kit (Promega). It is a luminescent assay that measures the activitities of two most important executioners of apoptosis i.e. caspase-3 and -7. These are cysteine rich protease enzymes which cleave the proteins at the aspartic acid residue

site. The kit provides a luminogenic caspase-3/7 substrate. This contains a tetrapeptide sequence DEVD. This reagent is added to cell lysate and kept for 8-10 hr. If the cell is undergoing apoptosis then the caspase will cleave the DEVD sequence and will generate a "glow-type" luminescent signal, produced by luciferase (Fig.6). Luminescence or the glow is proportional to the amount of caspase activity present.

Procedure:

In the cell lysate this caspase Glo 3/7 reagent was added and kept for some hrs. The readings were taken under Promega luminometer.

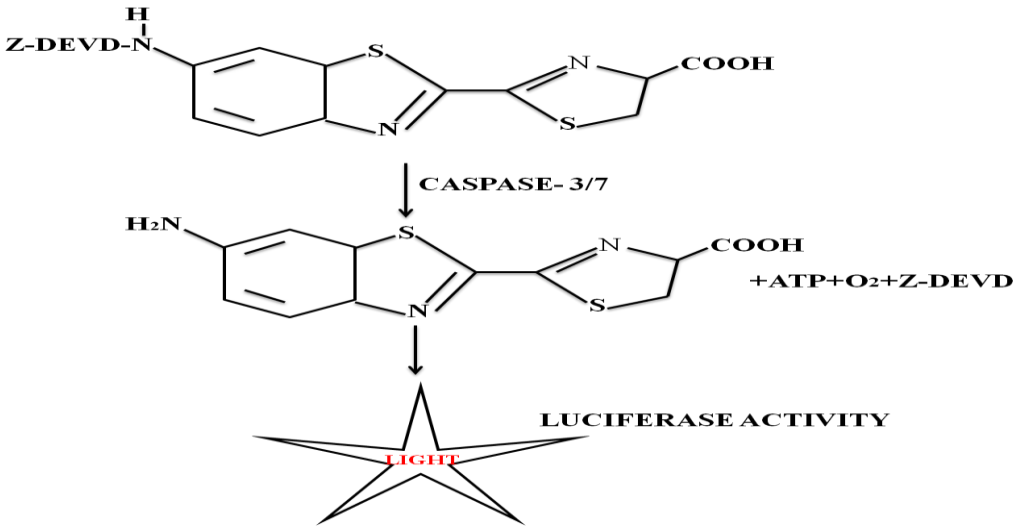

Fig.6. Caspase Glo 3/7 assay showing the cleavage of DEVD by caspase which results in light luminescence.

3.7) AUTOPHAGY INHIBITION ASSAY USING CHLOROQUINE:

Principle:

Chloroquine is an autophagy inhibitor. It enters into the acidic parts of the cell like endosomes and lysosomes and inhibits endosomal acidification [119]. They actually inhibit the lysosomal enzymes which have an acidic pH, and finally prevent the fusion of autophagosome and lysosomes and lysosomal protein degradation [120]. Chloroquine inhibits autophagy by raising the lysosomal pH.

Procedure:

1mg Chloroquine (CQ) was dissolved in 1ml of PBS and the final concentration of 2mM was used for the experiment. For 1ml of media 20µl of chloroquine (2mM) is used. First CQ was added and after 2-4 hrs B[a]P (5µM) was added and finally after 2-4 hrs BM (1mg/ml) was treated in HacaT cells. After 24hrs the cells were observed under bright field microscope.

4. RESULT

4.1) MTT ASSAY

B[a]P with different concentrations were treated on HaCat cell lines for 72 h and the MTT assay was carried out. The data showed that viability of cells declined in a dose dependent manner (Fig.7).

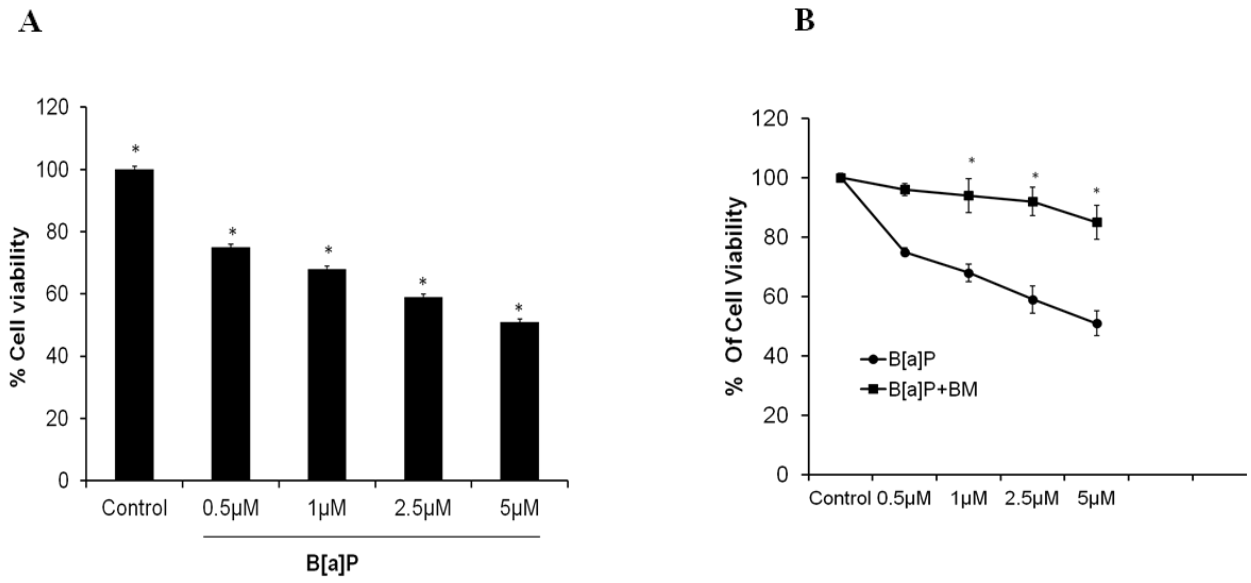

Fig. 7. Effect of B[a]P and Brahmi on cell viability and growth. (A) Different concentrations of B[a]P were treated on HaCaT cell lines and cell viability was measured by MTT assay. Data are reported as the mean ± SD of four different observations and compared against control by using Student's t-test. P values < 0.05 were considered significant (*significant compared to the control). (B) Cell viability comparison between B[a]P and B[a]P+BM showing the significant increase in the cell viability on addition of BM into B[a]P.

This indicates that B[a]P is a cytotoxic chemical and at a higher dose it is inhibiting the proliferation of normal human cells *in vitro*.

4.2) CELL VIABILITY VISUALIZATION UNDER BRIGHT FIELD MICROSCOPE

BM, BM+B[a]P and only B[a]P were treated on HaCaT cell lines and observed under Bright Field Microscope. Cell death is clearly visible in B[a]P treated cells. In BM+B[a]P the cell viability was found to increase (fig.8). This is because of the cytoprotective action of BM against B[a]P.

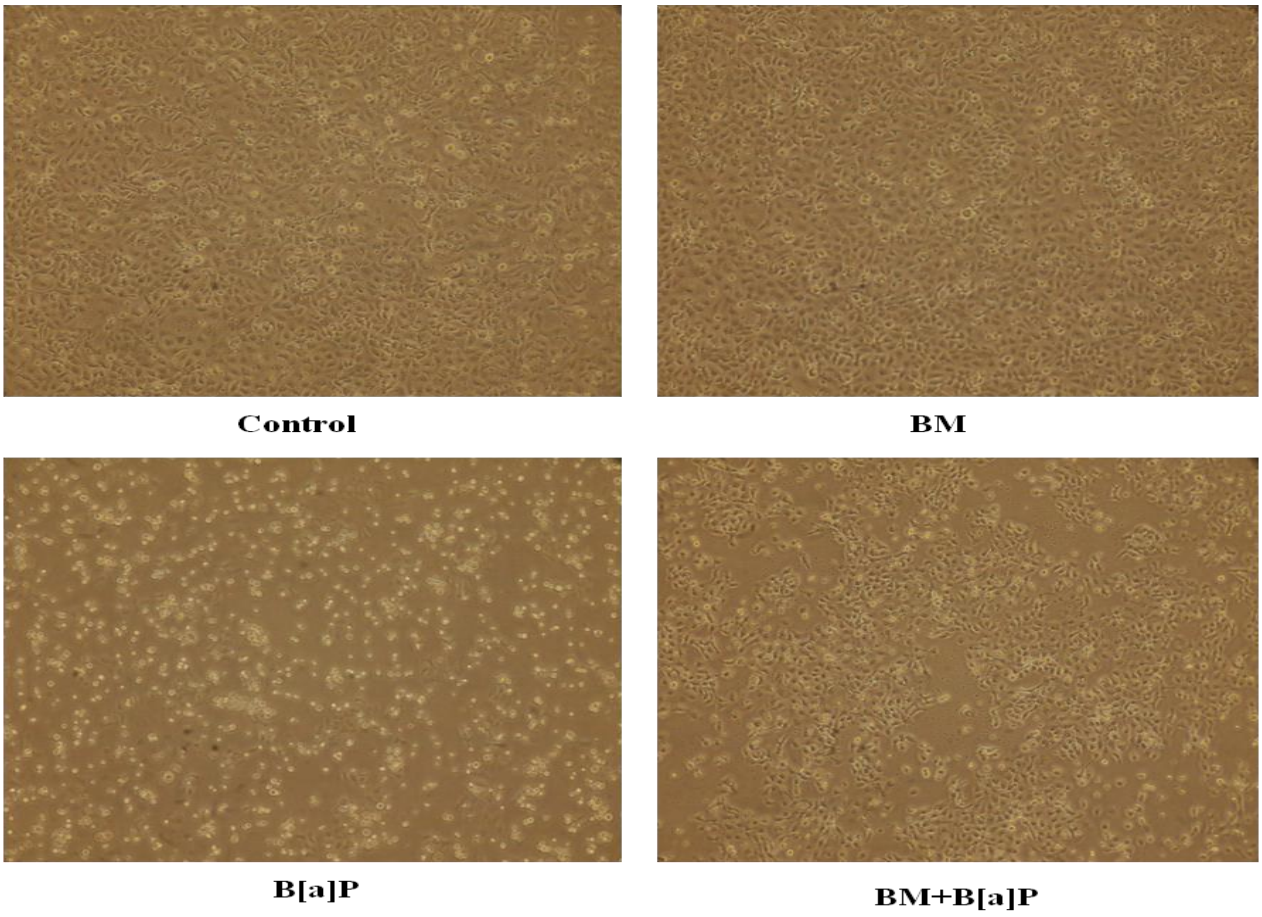

Fig. 8. The figures showing the morphological changes in the cells treated with BM, BM+B[a]P and only B[a]P. There is no change between control and BM. But the change in cell viability between B[a]P and BM+B[a]P is clearly visible reflecting that BM is reducing the cytotoxic action of B[a]P.

4.3) MEASUREMENT OF AUTOPHAGY BY ACRIDINE ORANGE STAINING

To reveal the enigma behind the cytoprotective action of BM against B[a]P, acridine orange staining was carried out. In this experiment, with increase in the concentration of BM red intensity was found to increase (Fig.9) reflecting the increase in the autophagy level with BM concentrations. This has indicated that BM is protecting the cell from the toxic effect of B[a]P through inducing autophagy.

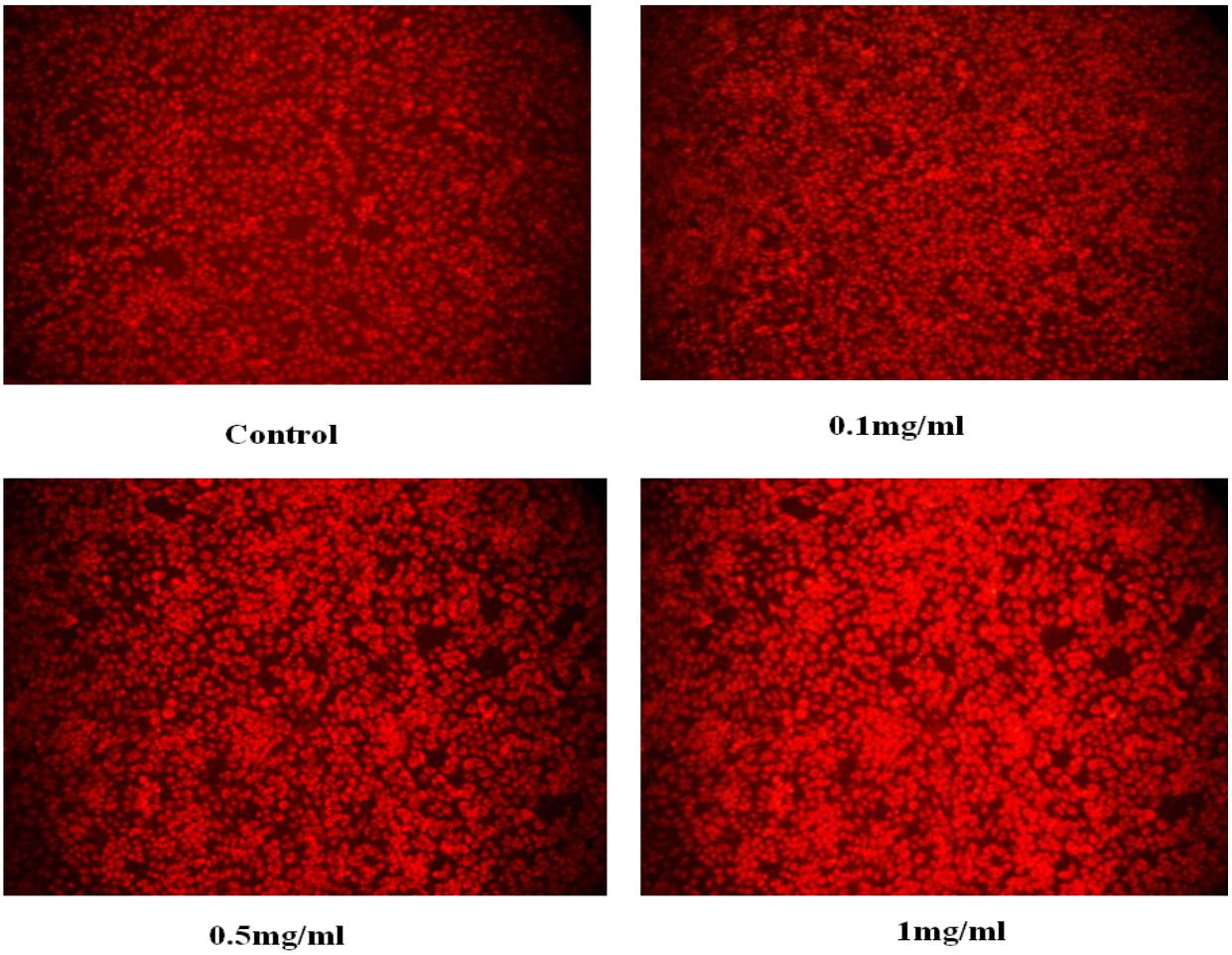

Fig. 9. Effect of BM on autophagy induction in HaCaT cells. HaCaT cells were treated with different concentrations (0.1, 0.5, and 1 mg/ml) of BM for 24h followed by staining with acridine orange and then visualized in inverted fluorescence microscope (Olympus IX71; 200×). Increase in red intensity indicates sharp increment in late autophagic vesicles.

4.4) DETERMINATION OF CASPASE ACTIVITY BY CASPASE 3/7 GLO ASSAY

Caspase activity for apoptosis was quantified in cell lysate of B[a]P, BM and BM+B[a]P using caspase-Glo 3/7 assay. In this assay high caspase activity was observed in the cell treated with B[a]P. This indicates that the cytotoxic nature of B[a]P is due to its ability to induce apoptosis. But in case of BM and B[a]P the caspase activity of B[a]P has decreased a lot indicating the cytoprotective action of BM against B[a]P (Fig. 10).

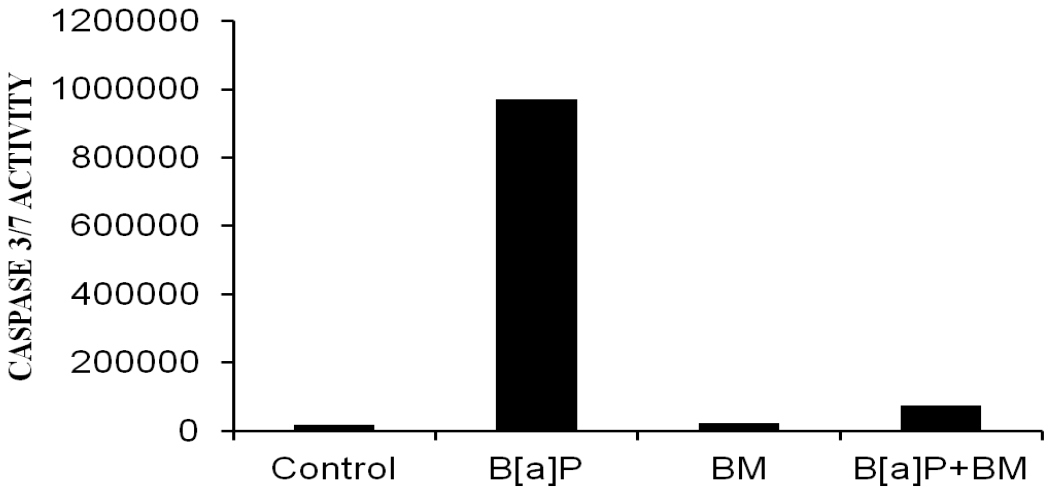

Fig. 10. Effect of B[a]P on apoptosis induction and effect of BM in reducing this apoptosis induction by B[a]P in HaCaT cells were studied by caspase-Glo 3/7 assay. In case of B[a]P high caspase activity is observed and in case of BM+B[a]P the caspase activity is found to decrease significantly due to the action of BM. Control and BM showed nearly similar effect.

4.5) INHIBITION OF AUTOPHAGY BY CQ REDUCED THE PROTECTIVE EFFECT OF BM ON B[a]P INDUCED CYTOTOXICITY

To further verify the cytoprotective action of BM due to autophagy induction against B[a]P, CQ was used. HaCaT cells treated with B[a]P showed maximum cell death under bright field microscope. Similarly in case of BM+B[a]P the cell viability was found to increase significantly. The CQ treated in BM+ B[a]P cells showed cell death (Fig. 11) indicating that CQ has inhibited the autophagy induced by BM against the toxic effect of B[a]P.

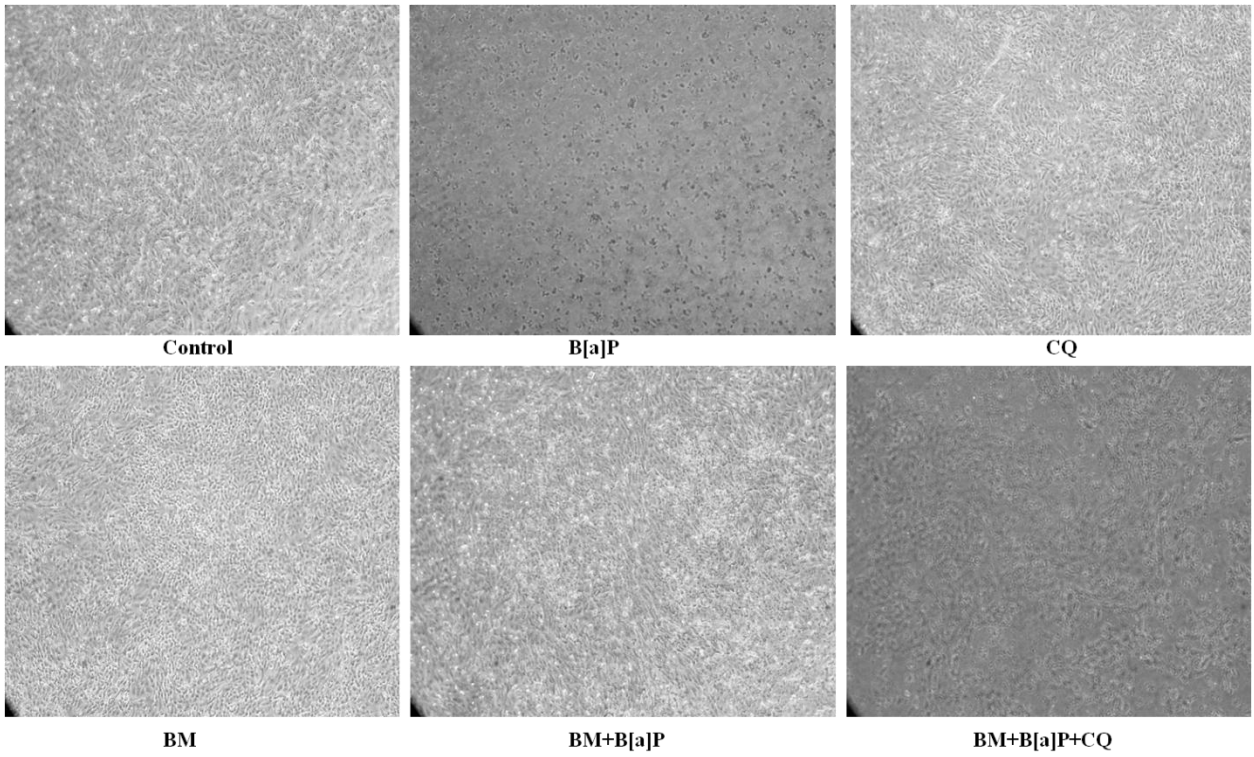

Fig. 11. Showing Autophagy inhibiting action of chloroquine (CQ). B[a]P has showed the maximum cell death potential. In case of BM+B[a]P the cytotoxic effect of B[a]P was reduced by the autophagic inducuction of BM. But in case of BM+B[a]P+CQ cell death was significantly high due to the autophagy inhibiting ability of CQ.

5. DISCUSSION

Natural molecules having the capacity to reduce the toxic effects of air pollutants would be a novel approach for the environment. This study has deciphered the cytoprotective role of *Bacopa monnieri (BM)* against the toxic effect of a potent air pollutant i.e. Benzo[a]Pyrene (B[a]P), revealing the induction of autophagy by BM against B[a]P. In our pursuance for a natural molecule, BM has been proved as an ideal plant against B[a]P due to its wide range of availability. The cytotoxic effect of B[a]P was validated in HaCaT cell lines by MTT assay reflecting dose dependent increase in the inviability. Along with this the increase in the cell viability on addition of BM into B[a]P treated cell was also demonstrated. More over the morphology of cells treated with BM, B[a]P and BM+B[a]P were observed under bright field microscope showing the maximum cell death in case of B[a]P and the cell viability was found to increase on treatment of BM. How B[a]P is causing cell death was demonstrated by Caspase-Glo 3/7 assay which has demonstrated the high caspase 3/7 activity which is the main executioner of apoptosis. The autophagy induction by BM was explained by acridine orange staining. To further prove the autophagy induction of BM against B[a]P, an autophagy inhibitor i.e. Chloroquine (CQ) was added in BM+B[a]P treated cells revealing the inhibition of BM induced autophagy by CQ resulting in cell death.This study highlights the BM induced autophagy which has the potential to reduce the cytotoxic effect of B[a]P.

6. FUTURE WORK PLAN

- Study of detail mechanism of autophagy induction by BM.
- Deciphering mechanism of cytoprotective effect of BM on B[a]P mediated cytotoxicity.
- In vivo demonstration of cytoprotective effect of BM on B[a]P.

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