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Neuroprotective mechanisms of standardized extracts of *Bacopa monniera* and their relevance in the treatment of Alzheimer's and Parkinson's diseases.

Par
Manjeet Singh

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Comité d'évaluation

Président du jury et examinateur interne	Prof. Thomas Sanderson INRS - Institut Armand-Frappier
Directeur de recherche	Prof. Charles Ramassamy INRS - Institut Armand-Frappier
Examinateur externe	Prof. Edith Hamel MNI - McGill University
Examinateur externe	Prof. Richard J. Wagner L'Université de Sherbrooke

RÉSUMÉ

Les maladies d'Alzheimer (MA) et de Parkinson (MP) sont les deux formes les plus communes de maladies neurodégénératives liées à l'âge. Elles touchent des millions d'individus à travers le monde. Dans un premier temps, les caractéristiques pathologiques de la MA comprennent la déposition de plaques séniles extracellulaires composées du peptide d'amyloïde beta (Aβ) et des enchevêtrements neurofibrillaires extracellulaires, composés de la protéine tau hyperphosphorylée ainsi que de la perte neuronale. Dans un second temps, la MP est caractérisée par la perte sélective des neurones dopaminergiques au niveau de la substance noire compacta (SNpc) dans le cerveau et la déposition de l'alpha (α)-synucléine contenue dans les corps de Lewy. La composante génétique contribue à <10% des diverses formes familiales de la MA et de la MP et prés de 90% de MA et de MP des formes sporadiques.

En Inde, de nombreuses plantes médicinales sont utilisées comme agents nootropes et toniques pour le cerveau dans le but de restaurer le déclin mental lié à l'âge. Le taux d'incidence entre l'âge et la MA dans les milieux ruraux d'Inde sont au moins trois fois plus faibles que les taux des populations américaines de référence. Cependant, la prévalence de la MP chez les indiens asiatiques est aussi trois fois inferieure à celle des caucasiens britanniques. Contrairement aux américains, il n'existe pas de lien entre l'âge et la perte des neurones du SNpc chez les indiens asiatiques. La *Bacopa monniera (BM)* est l'une des plus importante plante médicinale utilisée dans le système de médecine traditionnelle en Inde. Elle est utilisée comme agent nootrope et comme stimulant pour le cerveau dans le but de restaurer les déclins cognitifs liés à l'âge. Il a été rapporté que les *bacosides*, saponines triterpénoïdes, sont les constituants responsables de l'activité

biologique de *BM*. Cependant, en dépit, de sa large utilisation comme stimulant cérébral, les détails des mécanismes neuroprotecteurs sur des modèles *in vivo* de la MA et de la MP restent à être étudiés. Le but du projet proposé était de cribler les mécanismes neuroprotecteurs de l'extrait de *BM* enrichi en composés *Bacosides* dans diverses toxines en fonction des modèles *in vitro* de la MA et de la MP. Notre hypothèse était que l'extrait de *BM* contenant des extraits de *Bacosides* permettrait d'augmenter la survie cellulaire, de réduire le stress oxydatif et les radicaux libres, de préserver les fonctions mitochondriales et de moduler de nombreuses voies de signalisation redox contre différentes neurotoxines.

Notre premier objectif de recherche était de déterminer, si les extraits de *BM* peuvent protéger contre le peroxyde d'hydrogène (H_2O_2), l'acroléine, le 1-methyl-4-phenyl-pyridinium iodide ($MPP^{+)}$ et le paraquat (PQ)/diquat (DQ) toxines qui induisent la mort neuronale. La toxicité du peptide d' Aβ est médiée par une variété de voies de signalisation incluant la génération d'H_2O_2 et des produits de la peroxydation lipidiques. L'acroléine est l'un des sous-produits de la peroxydation lipidique le plus réactif. Le MPP^+ est un inhibiteur du complexe I mitochondrial. Le PQ et le DQ, sont des herbicides bipyridyl avec une structure similaire au MPP^+, ils sont communément utilisés dans les études réalisées sur des modèles *in vitro* et *in vivo* de la MP. L'utilisation de nombreux tests de cytotoxicité et de survie cellulaire tels que les tests LDH, XTT et Résazurin nous ont permis de montrer l'effet neuroprotecteur d'extraits standardisés de *BM* la toxicité contre de l'H_2O_2, l'acroléine, le MPP^+ et le PQ molécules sur les cellules de neuroblastome humain, les SK-N-SH et sur les cellules dopaminergiques de rat, les PC12. Dans le but de faire la différence entre les effets toxiques et proliférateurs des extraits de *BM*, nous avons réalisé le test de la lactate déshydrogénase (LDH) qui permet de mesurer l'intégrité de la membrane cellulaire

et ainsi de quantifier la moyenne des cellules mortes. Nos résultats montrent que le prétraitement avec 50.0 µg/ml de *BM*, protège la lignée cellulaire dopaminergique SK-N-SH contre la toxicité induite par le MPP^+ et le PQ dans de nombreux test de survie cellulaire. Par ailleurs, nous avons aussi montré que les composés *Bacosides* sont impliqués dans la neuroprotection contre ces nombreuses toxines.

Notre second objectif de recherche était d'étudier l'activité antioxydante de l'extrait de *BM*. Pour cela nous avons utilisé de nombreux tests fluorescents et des détections électrochimiques, nous avons démontré que l'extrait de *BM* dégrade l'H_2O_2 mais aussi réduit le niveau des espèces réactives à l'oxygène (EROs). L'extrait de *BM* protège aussi de la formation des anions superoxyde induits par le PQ dans les cellules SK-N-SH et les PC12. De plus, l'extrait de *BM* (2.5 µg/ml) protège de la réduction du glutathion (GSH) induite par le MPP^+ en plus de réduire le niveau des protéines oxydées induites par l'H_2O_2 et l'acroléine. L'adaptation et la survie neuronale en réponse à un stress oxydatif dépendent de nombreux facteurs de transcription régulés par le potentiel redox.

Ainsi, pour notre troisième objectif de recherche nous avons évalué la capacité de l'extrait de *BM* à moduler différentes voies de signalisation redox. L'utilisation de western-blot et de tests enzymatiques nous a permis de montrer que l'extrait de *BM* active la protéine kinase B (AKT) et le facteur nucléaire erythroid 2 (Nrf2) avec des concentrations respectives de 40.0 µg/ml et 10.0 µg/ml, et augmentent l'activité de nombreuses enzymes antioxydantes de phase 2. Un prétraitement avec l'extrait *BM* (40.0 µg/ml) protège aussi de la diminution de la régulation des systèmes de défense antioxydants induite par le PQ et le DQ tel que γ-glutamylcysteine synthetase (γ-GCS) et de thioredoxine 1 (Trx1). Par ailleurs, un prétraitement avec l'extrait *BM* protège contre l'activation du facteur nucléaire kappaB

(NF-κB), des protéines kinases activées par des agents mitogènes (MAPKs) et p66Shc et préserve le niveau de sirtuine 1, induit par l'H_2O_2 et l'acroléine dans les cellules SK-N-SH. Ces résultats indiquent que l'extrait de *BM* peut moduler différentes voies de signalisation redox.

De plus, il a été montré que les dysfonctions mitochondriales jouent un rôle important dans la pathophysiologie des MA et MP. D'ou pour notre dernier objectif nous avons étudié l'effet de l'extrait de *BM* sur différentes fonctions mitochondriales. À l'aide de tests fluorescents nous avons démontré que l'extrait de *BM* protège contre le déclin du potentiel membranaire mitochondrial induit par l'H_2O_2, l'acroléine, et le MPP^+. Nous avons montré par la méthode de semi-purification mitochondriale que l'extrait de *BM* (50.0 μg/ml) protège l'activité du complexe 1 mitochondrial contre le traitement MPP^+. De plus, l'extrait de *BM* augmente l'activité de la NADH déshydrogénase mitochondriale. Ces résultats suggèrent que l'extrait de *BM* peut moduler et préserver les fonctions mitochondriales durant un stress oxydatif.

En conclusion, notre étude contribue à une meilleure compréhension de nombreux mécanismes neuroprotecteurs de l'extrait de *BM* contre différentes neurotoxines, ce qui peut favoriser davantage possibilité dans son application thérapeutique en ciblant et modulant de nombreux mécanismes physiopathologiques impliqués dans la progression de la MA et de la MP.

Manjeet Singh
Étudiant

Dr. Charles Ramassamy
Directeur de Recherche

ABSTRACT

Alzheimer's disease (AD) and Parkinson's disease (PD) are the two most common age-related neurodegenerative disorders affecting millions of people worldwide. Pathological hallmarks of AD include the deposition of extracellular senile plaques composed of beta amyliod peptides (Aβ), and intracellular neurofibrillary tangles, composed of hyperphosphorylated tau proteins and neuronal loss. On the other hand PD is characterized by the selective loss of dopaminergic neurons in the substantia nigra pars compacta (SNpc) of the brain and deposition of alpha (α)-synuclein containing lewy's bodies (LBs). Genetic components contribute to < 10% of familial variants of AD and PD and around 90% of AD and PD cases are sporadic.

In India, several medicinal plants are used as nootropic agents and brain tonics to restore age-related decline in mental abilities. The incidence rates of the age-specific AD in rural Indian are at least three times lower than those of an age-matched American reference population. Moreover, prevalence of PD in Asian Indians is also at least three times lower than British Caucasians and unlike the Americans there is no age-related loss of SNpc neurons in Asian Indians. *Bacopa monniera* (*BM*) is one of the most important medicinal plants used in the Indian system of traditional medicine as a nootropic agent and brain tonic to restore age-related decline in cognitive abilities. *Bacosides*, the triterpenoid saponins have been reported to be active constituents responsible for the biological activity of *BM*. However, in spite of widespread use of *BM* extract as a brain tonic, the detailed neuroprotective mechanisms in *in vitro* models of AD and PD remain to be investigated. The aim of proposed investigation was to screen the neuroprotective mechanisms of *BM* extract enriched in *Bacosides* compounds in various toxins based *in vito* models of AD and

PD. Our hypothesis was that *BM* extract containing *Bacosides* compounds will enhance cell survival, decrease oxidative stress and free radicals, preserve mitochondrial functions and modulate various redox-signaling pathways against various neurotoxins.

Our first research objective was to determine whether *BM* extract can protect against hydrogen peroxide (H_2O_2), acrolein, 1-methyl-4-phenyl-pyridinium iodide (MPP^+), and paraquat (PQ)/diquat (DQ)-induced neuronal cell death. Aβ peptide toxicity is mediated through a variety of downstream pathways including the generation of H_2O_2 and lipid peroxidation products. Acrolein is one of the most reactive by-products of lipid peroxidation. MPP^+ is an inhibitor of the mitochondrial complex I. PQ and DQ are bipyridyl herbicides and with a similar structure to MPP^+, are commonly used both in *in vitro* and *in vivo* models of PD. Using XTT and Resazurin based cell survival assays, we demonstrated the neuroprotective effects of the standardized extracts of *BM* against H_2O_2, acrolein, MPP^+ and PQ/DQ-induced toxicity in human neuroblastoma SK-N-SH and rat dopaminergic PC12 cell lines. To distinguish between toxicity and proliferation effects of the *BM* extracts, lactate dehydrogenase (LDH) assay which measures the cellular membrane integrity and is a mean of quantifying dead cells was also done. Our results show that a pre-treatment with 50.0 μg/ml *BM* extract protected the dopaminergic SK-N-SH cell line against MPP^+ and PQ-induced toxicity in various cell survival assays. Furthermore, we showed that *Bacosides* compounds were involved in neuroprotection against various toxins.

In the second research objective we investigated the antioxidant activity of *BM* extract. Towards this, using various fluorescent dyes and electrochemical detection, we demonstrated that *BM* extract degraded H_2O_2 and decreased H_2O_2 and PQ/DQ-induced

intracellular reactive oxygen species (ROS) levels. *BM* extract also prevented PQ/DQ-induced superoxide anions generation in SK-N-SH and PC12 cells. Additionally, *BM* extract (2.5 μg/ml) also prevented MPP^+-induced intracellular reduced glutathione (GSH) depletion. Besides, *BM* extract also decreased H_2O_2 and acrolein-induced protein oxidation. Adaptation and neuronal survival in response to oxidative stress depend on various cellular redox-regulated transcription factors. Hence, in the third research objective we assessed the ability of the *BM* extract to modulate different redox-signaling pathways. Using western lot analysis and enzymatic assays, we demonstrated that *BM* extract alone activated the activated protein kinase B (AKT) and nuclear erythroid 2-related factor 2 (Nrf2) at 40.0 μg/ml and 10.0 μg/ml respectively, and increased the activities of various phase 2 antioxidant enzymes. *BM* pre-treatment (40.0 μg/ml) also prevented the PQ/DQ-induced down regulation of the antioxidant defence systems such as γ-glutamylcysteine synthetase (γ-GCS) and thioredoxin1 (Trx1) levels. Moreover, *BM* pre-treatment prevented the H_2O_2 and acrolein-induced activation of the nuclear factor-kappaB (NF-κB), mitogen activated protein kinases (MAPKs) and p66Shc and preserved Sirtuin1 level in SK-N-SH cells. These results indicate that *BM* extract can modulate different redox-signaling pathways.

Since mitochondrial dysfunctions have also been shown to play a pivotal role in AD and PD pathophysiology, in the final objective we investigated the effect of *BM* extract on various mitochondrial functions. Using various fluorescent dyes, we demonstrated that *BM* extract prevented the H_2O_2, acrolein, and MPP^+-induced decline in mitochondrial membrane potential (MMP) levels. Using semipurifed mitochondrial fraction, we showed that *BM* extract (50.0 μg/ml) also preserved mitochondrial complex1 activity against MPP^+ treatment. Additionally, *BM* extract increased mitochondrial NADH dehydrogenases

activity. These results suggest that *BM* extract can modulate and preserve mitochondrial functions during oxidative stress.

Taken together our work contributes to a better understanding of the various neuroprotective mechanisms underlying *BM* extract against various neurotoxins and this may further open up the possibility of its therapeutic application in targeting and modulating the various pathophysiological mechanisms involved in the progression of AD and PD.

Manjeet Singh
Student

Dr. Charles Ramassamy
Director of Research

List of articles included in the thesis

Research Articles:

Singh M, Murthy V and Ramassamy C (2010). Modulation of hydrogen peroxide and acrolein induced oxidative stress, mitochondrial dysfunctions and redox regulated pathways by the *Bacopa monniera* extract. *Journal of Alzheimer's disease.* 21 (1): 229-247.

Singh M, Murthy V and Ramassamy C (2012). Standardized extracts of *Bacopa monniera* extract protect against Paraquat and MPP^+- induced toxicities by modulating mitochondrial, proteasomal functions and redox pathways. *Toxicological Science.* 125 (1): 219-232.

Singh M, Murthy V and Ramassamy C (2013). Neuroprotective mechanisms of the standardized extract of *Bacopa monniera* in a paraquat/diquat-mediated acute toxicity on PC12 cells. *Accepted in Neurochemistry International.*

Reviews:

Singh M, Arseneault M, Sanderson T, Murthy V and Ramassamy C (2008). Challenges for research on polyphenols from food in Alzheimer's disease: bioavailability, metabolism, and cellular and molecular mechanism. *Journal of Agricultural and food chemistry.* 56 (13): 4855-4873.

Singh M, Nam DT, Arseneault M, and Ramassamy C (2010). Role of by-products of lipid oxidation in Alzheimer's brain. *Journal of Alzheimer's disease.* 21 (3): 741-756.

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LIST OF ABBREVIATIONS

ABAD	Aβ-binding alcohol dehydrogenase
AD	Alzheimer's disease
AKT	Actiavted protein kinase B
ALDH	Aldehyde dehydrogenase
APOE	Apolipoprotein E
APP	Amyloid precursor protein
ARE	Antioxidant response elements
ASK1	Apoptosis signal-regulating kinase
ATP	Adenosine triphosphate
Aβ	Amyloid-β peptide
BACE	β Secretase
BM	*Bacopa monniera*
COMT	Catechol-*O*-methyltransferase
COX	Cytochrome *c* oxidase
COX-2	Cyclooxygenase-2
CypD	Cyclophilin-D
DA	Dopamine
DAT	Dopamine transporter
DNA	Deoxyribonucleic acid
DOPAC	3,4-dihydroxyphenylacetic
DOPAL	Dihydroxyphenylacetaldehyde
ER	Endoplasmic Reticulum
ERKs	Extra cellular signal regulated kinases
FAD	Familial form of AD
fAb	Fibrillary Aβ
GCS	γ-glutamyl-cysteine-synthase
GPx	Glutathione peroxidase
GR	Glutathione reductase
GSH	Reduced glutathione

GSSG	Oxidized glutathione
GST	Glutathione *S*-transferases
H_2O_2	Hydrogen peroxide
HO-1	Heme oxygenase-1
HSPs	Heat shock proteins
HVA	Homovanillic acid
4-HNE	4-Hydroxy-2-trans-nonenal
iNOS	Inducible nitric oxide synthase
JNK/SAPK	C-Jun N-terminal kinase/stress activated protein kinase
Keap1	Kelch-like ECH-associated protein
LBs	Lewy bodies
LDLR	Low density lipoprotein receptor
LOAD	Sporadic late onset form of AD
LOX-5 & LOX-12	Lipooxygenase 5 and 12
MAO-B	Monoamino oxidase B
MAPKs	Mitogen activated protein kinases
MCI	Mild cognitive impairment
MDA	Malondialdehyde
Mitochondtial	Nicotinamide adenine dinucleotide (NADH)
complex1	dehydrogenase- ubiquinone oxidoreductase
MMP	Mitochondrial membrane potential
MnSOD/SOD2	Manganese superoxide dismutase
MPP^+	1-methyl-4-phenyl-pyridinium ion
MPTP	1-methyl-4-phenyl-1,2,3,4-tetrahydropyridine
NAD	Nicotinamide adenine dinucleotide
NFTs	Neurofibrillary tangles
3-NPA	3-nitropropionic acid
NF-κB	Nuclear factor-kappa B
NOX	NADPH oxidase
NO/NO•	Nitric oxide

NQO1	NAD (P) H: quinone oxidoreductase
Nrf2	Nuclear erythroid-2 related factor 2
4-ONE	4-oxo-2-nonenal
$O_2^{\bullet -}$	Superoxide anion
OH•	Hydroxyl radical
$ONOO^-$	Peroxynitrite
4-ONE	4-oxo-2-nonenal
8-OHdG	8-hydroxy-2'-deoxyguanosine
PD	Parkinson's disease
PDH	Pyruvate dehydrogenase
PGC-1 alpha	peroxisome proliferator-activated receptor gamma coactivator-1 alpha
PI3-K	Phosphatidylinositol3-phosphate kinase
PKC	Protein kinase C
PQ/DQ	Paraquat/Diquat
PSEN1/2	Presenilin 1/2
PTP	Permeability transition pore
PUFAs	Polyunsaturated fatty acids
RAGE	Receptor for advanced glycation end products
RNS	Reactive nitrogen species
ROS	Reactive oxygen species
Sirt	Sirtuins
SNpc	Substantia nigra pars compacta
SOD	Superoxide dismutase
SOD1	Copper/zinc superoxide dismutase
TH	Tyrosine hydroxylase
TNF-α	Tumour necrosis factor alpha
Trx	Thioredoxins
UPS	Ubiquitin-proteosomal system
VDAC	Voltage-dependent anion channel

I- Cellular redox homeostasis: free radicals production and antioxidant defense systems

Free radicals can de defined as molecules or molecular fragments containing one or more unpaired electrons in atomic or molecular oribitals. These unpaired electrons give a considerable degree of reactivity to free radicals. Reactive oxygen species (ROS), as well as reactive nitrogen species (RNS), are products of normal cellular metabolism. Both reactive species are now well recognized as signaling molecules in addition to being toxic metabolites. Although there are several intracellular sites of ROS production, mitochondria are the single largest source of hydrogen peroxide (H_2O_2) and superoxide anion ($O_2^{\bullet -}$) generation. Colocalization of ROS production (specifically superoxide) at sites of nitric oxide (NO•) production can lead to formation of RNS, the proximal species being peroxynitrite. Aging is associated with accumulation of these oxidative-induced damages in brain, owing to an imbalance between antioxidant defenses and intracellular generation of ROS. The overall rationale of oxidative stress in aging brain is based on the following premise: (a) brain contains high levels of unsaturated fatty acids which are vulnerable to oxidation (particularly high in 20:4 and 22:6 fatty acids); b) brain consumes high amounts of oxygen (about 20% of the total amount used in the body; c) although the levels of antioxidants such as reduced glutathione (GSH) and vitamin C are much higher in brain but may decrease with age; and d) brain contains high concentrations of transition metals such as iron (Fe^{2+}) that are key catalysts of oxidative-induced damages (Emir *et al.*, 2011; Halliwell, 1992; Meredith *et al.*, 2011). For scavenging these free radicals cells have an extensive antioxidant system in place comprising both enzymatic and nonenzymatic

substances, which is differentially distributed within various cellular compartments. Endogenous enzymes including superoxide dismutase (SOD), glutathione peroxidase (GPx), glutathione reductase (GR), catalase, peroxiredoxins, and thioredoxins can scavenge ROS thereby mitigating their toxicity (Fig. 1).

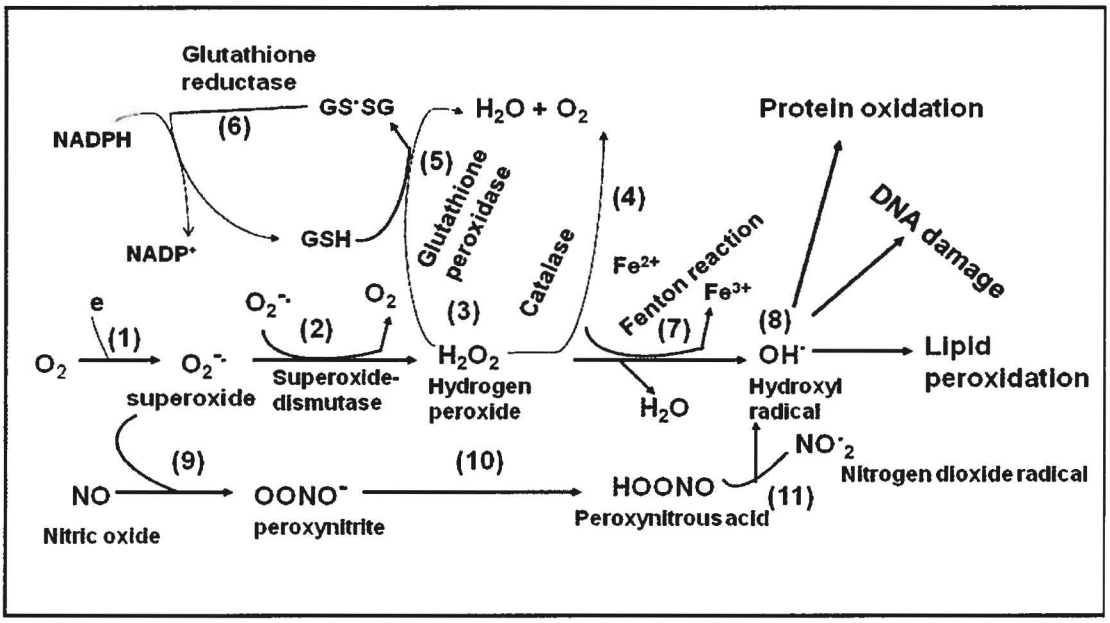

Fig.1 Generation and detoxification of ROS and RNS. (1, 2, 3) Univalent reduction of oxygen results in the formation of superoxide ($O_2^{\bullet -}$) primarily in mitochondria that is further converted into hydrogen peroxide (H_2O_2) by the superoxide dismutases (SOD). (4, 5, 6) Hydrogen peroxide is further catabolized by Catalase and Glutathione peroxidase and oxidized glutathione (GS•SG) is recycled back to reduced form (GSH) by the enzyme Glutathione reductase. (7, 8) On the other hand H_2O_2 in the presence of redox active ferrous iron (Fe^{2+}) may generate highly toxic hydroxyl (OH•) radicals that may further damage cellular macromolecules. (9, 10, 11) Peroxynitrite is formed by the combination of nitric oxide and superoxide; and other RNS are formed from the peroxynitrite after sequential degradation. Modified from (Singh and Ramassamy, 2008).

II- Redox imbalance and oxidative modifications of macromolecules in brain during aging

A decline in the antioxidant enzymes with aging may compromise cellular redox homeostasis resulting in high concentrations of ROS and RNS (Suh *et al.*, 2004), that may further disrupt cellular redox circuits and induce damage to nucleic acids, lipids and proteins in brain, thereby contributing to age-associated neurodegenerative diseases such as Alzheimer's disease (AD) or Parkinson's disease (PD) (d'Ischia *et al.*, 2011; Ramalingam and Kim, 2012). The most frequent oxidative modification in proteins is the formation of carbonyl groups, which may lead to inactivation, proteolysis, or formation of intra-/intermolecular cross-links. Oxidative-induced damage to proteins can affect virtually all amino acids, with sulfur- containing amino acids and aromatic amino acids being the most susceptible (Berlett and Stadtman, 1997). In proteins, thiol group in cysteine can be oxidized to both reversible [sulfenic acid (SOH), disulfide bond formation (-S-S-)] and irreversible oxidative states [sulfinic (SO_2H) and sulfonic acids (SO_3H)] (Fig. 2) (Eaton, 2006; Thomas and Mallis, 2001).

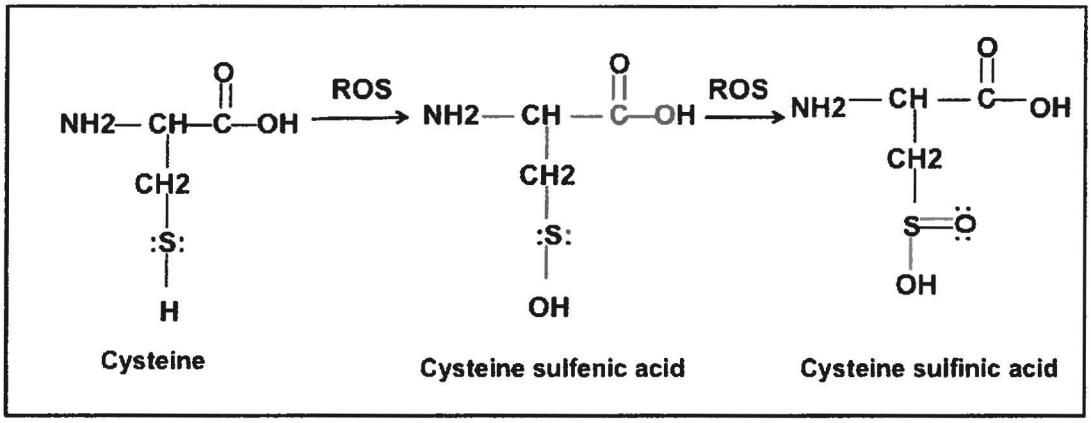

Fig. 2 ROS induced formation of Cysteine sulfenic and Cysteine sulfinic acid. Adapted from (Reed, 2011: *Free Radic Biol Med* 51, 1302-19).

Protein oxidation may be considered to be the most important functionally, because proteins act as cellular receptors, transporters, enzymes, and transcription factors. In fact accumulation of oxidized proteins in brain increases with age could be nearly two- and four-fold greater in human and rats, respectively (Smith *et al.*, 1991). Moreover, the levels of protein carbonyls, 3-nitrotyrosine, and 4-hydroxy-nonenal adducts were elevated with age in mitochondria of the brain cortex of rats (Gilmer *et al.*, 2010). Protein stability and resistance to oxidative stress are key determinants of longevity in the longest-living rodent, the naked mole-rat, and collapse of proteostasis is an early event in *Caenorhabditis elegans* during aging (Ben-Zvi *et al.*, 2009; Pérez *et al.*, 2009).

Lipid peroxidation is another consequence of decreased antioxidant mechanisms with aging. Reaction of ROS in the presence of redox active metals with the double bond of polyunsaturated fatty acids (PUFAs) produces oxidized lipids which may further result in a large number of reactive electrophilic aldehydes including malondialdehyde (MDA), 4-hydroxy-nonenal (4-HNE), 4-oxo-2-nonenal (4-ONE) and acrolein (Fig. 3) (LoPachin *et al.*, 2009).

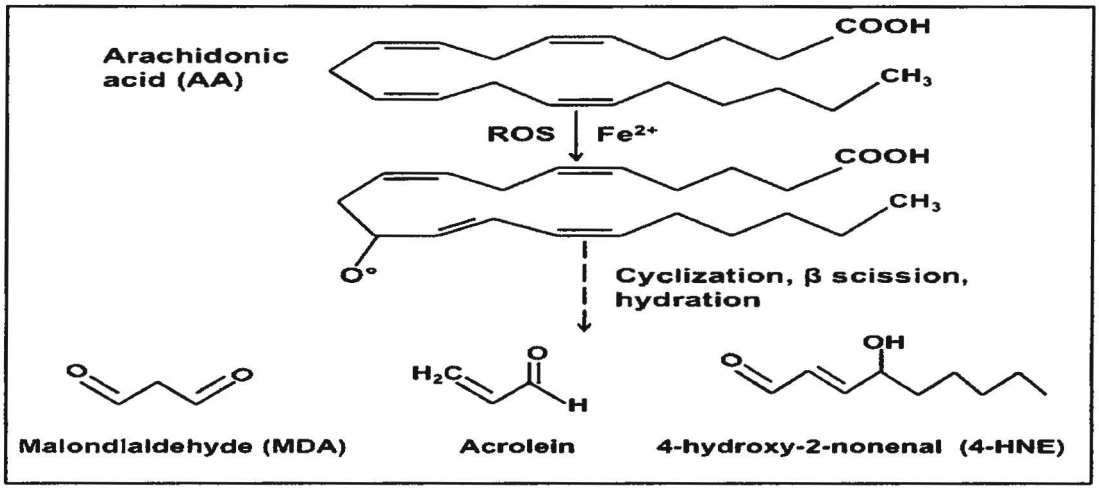

Fig. 3 ROS induced generation of lipid peroxidation products from arachidonic acid.

Lipid peroxidation has been reported to be elevated in brain with age. MDA was increased in the cytoplasm of neurons and astrocytes in normal aging, but was barely detected in normal young subjects (Dei *et al.*, 2002). All these biologically active aldehydes e.g. acrolein, 4-HNE and 4-ONE are capable of gene induction and cytotoxicity; as they can react with many biological molecules including various amino acids, proteins, and bases in deoxyribonucleic acid (DNA) to produce further damage in aged brains (LoPachin *et al.*, 2009; Poon *et al.*, 2004). Activation of DNA damage by these reactive aldehydes has been shown to induce apopotosis in human neuroblastoma cells through activation of p53 pathway (Shibata *et al.*, 2006).

In normal tissues, 10,000 oxidative interactions occur between DNA and endogenously generated free radicals per human cell per day (Collins, 1999) resulting in damaged nucleotides and strand breaks and these oxidative DNA lesions can block genome replication if not repaired properly. One of the most common lesions is 8-oxo-7, 8-dihydroguanine (8-oxoG); a hydroxyl radical-induced modification of guanine, and its level is elevated four times in old brains when compared to young brains (Hamilton *et al.*, 2001). Mitochondrial DNA (mtDNA) is more susceptible to oxidative stress than nuclear DNA (nDNA) owing to its close proximity to the ROS generating site and the lack of protective histones combined with a lower capacity for DNA repair. The rate of increase in the 8-oxoG levels was 10 times more in mtDNA than in the nDNA in the cerebral cortex and cerebellum from humans with age (Mecocci *et al.*, 1993). Moreover, mitochondrial imports of 8-oxyguanine-DNA glycosylase, an enzyme responsible for the repair of 8-oxoG lesions, declined with aging thereby further impairing mitochondrial DNA repair capacity and functions (Szczesny *et al.*, 2003). Additionally, mtDNA damage also enhanced ROS

production, which further induced telomeric damage (Passos *et al.*, 2007). Telomeres are specialized nucleoprotein structures and protect chromosome ends during replication and by promoting neurogenesis and neuritogenesis may preserve neuronal functions during aging (Ferrón *et al.*, 2007). Although the relationship between the magnitude of modifications of multiple biomolecules by oxidative damages and age-related functional losses is not a linear one, still it may affect brain health and functions in the long run. These changes observed in normal aging are exacerbated in various neurodegenerative diseases related to aging such as AD and PD as shown in Fig. 4.

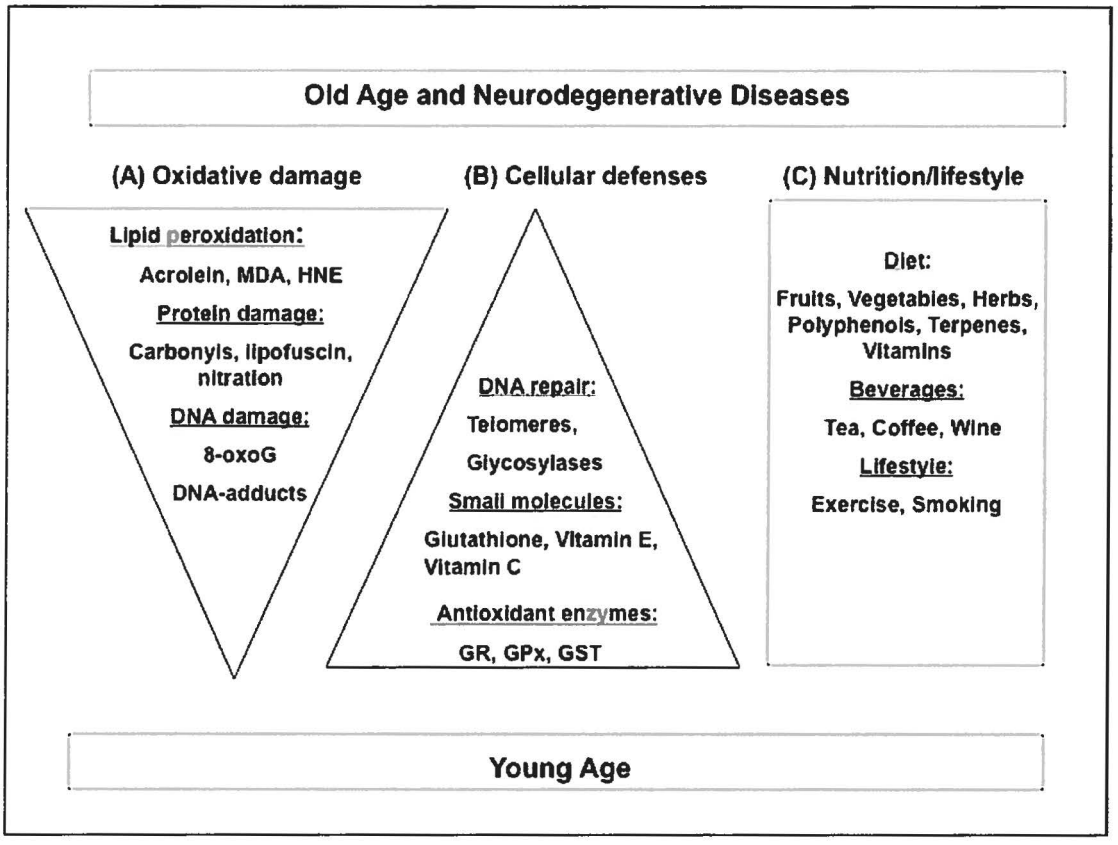

Fig. 4 Interplay between cellular ROS levels, antioxidant system during aging and their modulation by nutrition and lifestyle changes. Modified from (Singh and Ramassamy, 2008).

Specific nutrients and dietary factors can affect cognitive processes and influence neuronal functions and synaptic plasticity. Recent studies have revealed some of the vital mechanisms that are responsible for the action of diet on brain health. Dietary antioxidant compounds, due to their pivotal role in the modulation of cellular redox mechanisms, might help preserve neuronal functions that are perturbed in aging. One major component of fruits, vegetables, herbs and spices is the antioxidant polyphenols. Increased consumption of antioxidant-rich foods in general and of polyphenols in particular is associated with better cognitive performance in elderly (Valls-Pedret *et al.*, 2012). In addition to the direct antioxidant action polyphenols have the ability to modulate various signaling systems (Singh *et al.*, 2008). Their effects on the neuronal glutathione system and the activation of several transcription factors including cyclic AMP response element-binding (CREB) protein, increasing the levels of the brain-derived neurotrophic factor (BDNF), modulating energy sensing pathways, mitochondrial functions and anti-inflammatory effects may preserve cellular antioxidant defenses and enhance neuronal survival during aging (Andrade and Assunção, 2012; Schaffer *et al.*, 2012; Williams and Spencer, 2012). Current epidemiological, preclinical and clinical data suggest that omega-3 polyunsaturated fatty acids (n-3 PUFAs), vitamin B(12), and vitamin C may attenuate cognitive impairment in aging and mitigate age-related neurodegenerative changes (Luchtman and Song, 2013; Murakami *et al.*, 2011; Selhub *et al.*, 2011). Caloric restriction (CR) and physical exercise have been shown to either decrease or prevent the progression of several age-related pathological effects in brain and extended the lifespan in many species, including nonhuman primates (Fontana *et al.*, 2010; Maalouf *et al.*, 2009; Mattson, 2012). Thus both nutrition and lifestyle changes can determine healthy and disease free brain aging.

III- Redox regulated transcription factors

The adaptation and survival of cells in response to ROS depend on their ability to co-ordinate and up regulate the various cellular protective redox regulated transcription factors and antioxidant enzymes, which represent ancient and highly conserved cytoprotective mechanisms. The cellular response to ROS, mediated by these redox regulated transcription factors, is determined by their expression, stability, nuclear targeting, or DNA-binding affinity. These factors are regulated by the level and duration of ROS and may either mediate the toxic effects of ROS or result in detoxification of oxidants thereby resulting in cell survival (Ma, 2010). In the past few decades some of these redox regulated transcription factors and signalling pathways along with their specific roles in cellular adaptive as well as toxic response have been identified and delineated. Some of these redox transcription factors and proteins including the mitogen- activated protein kinases (MAPKs), nuclear factor-kappaB (NF-κB), Sirtuins, nuclear erythroid 2-related factor 2 (Nrf2), and p66Shc have been shown to play important roles in cell survival and death pathways in response to oxidative stress (Leonarduzzi *et al.*, 2010; Ma, 2010). Nrf2 has been shown to preserve the cellular redox homeostasis by inducing the transcription of antioxidant response elements (ARE) containing genes and promoting cell survival during oxidative stress (de Vries *et al.*, 2008). ROS mediated NF-κB induced cyclooxygenase-2 (COX-2) and NADPH oxidases induction further potentiates inflammation and oxidative stress (Barakat *et al.*, 2012; Lee *et al.*, 2006). Sirtuins, MAPKs and p66Shc, have been implicated in mitochondrial metabolism and ROS generation (Giorgio *et al.*, 2005; He and Aizenman, 2010; Zhang *et al.*, 2011). The activation of these key redox sensors and their roles in ROS homeostasis and cell survival is summarized below.

1- Nrf2 pathway and modulation of intracellular ROS

One of the most important redox regulated pathway e.g. the antioxidant response element (ARE)-mediated gene activation is coordinated by the nuclear factor E2-regulated factor2 (Nrf2), which, upon exposure to electrophiles or ROS, translocates to the nucleus where it binds ARE and activates the expression of phase2 detoxification enzymes or GSH (de Vries *et al.,* 2008). Under normal physiological conditions, Nrf2 is retained in inactive form in cytoplasm by binding to its inhibitory protein, Kelch-like ECH-associated protein (Keap1) and Cullin-3 protein that facilitates its degradation by the ubiquitin-proteasome system (UPS) (Jaiswal, 2004). Human Keap1 is a 70-kDa cysteine-rich protein (624 amino acids) and contains 27 cysteines that act as cellular redox sensor (Ahn *et al.*, 2010; Dinkova-Kostova *et al.*, 2002). Oxidation or alkylation of different thiol groups on Keap1 facilitate its dissociation from Nrf2 and permit Nrf2 translocation in the nucleus for activation of the ARE genes (Wakabayashi *et al.*, 2004; Zhang and Gordon, 2004). Under basal conditions, the half-life of Nrf2 is short (~15 min) whereas in presence of oxidants or electrophiles, Nrf2 half-life is increased to ~60 min and Nrf2 has been shown to upregulate a variety of both antioxidant and anti-inflammatory proteins (Calkins *et al.*, 2009; Lee and Johnson, 2004; Lewis *et al.*, 2010). It activates transcription of genes encoding antioxidant enzymes such as hemeoxygenase 1 (HO-1), γ-glutamylcysteine synthetase (γ-GCS), thioredoxins (Trx) and phase II detoxification enzymes, such as NAD(P)H:quinone oxidoreductase1 (NQO1), glutathione S-transferases (GST), GR and GP_X and downregulates inflammatory enzymes like cycloxygenase-2 (COX-2), inducible nitric oxide synthase (iNOS) as shown in Fig. 5 (Dinkova-Kostova *et al.*, 2005; Itoh *et al.*, 1999; Kobayashi *et al.*, 2006; Lewis *et al.*, 2010).

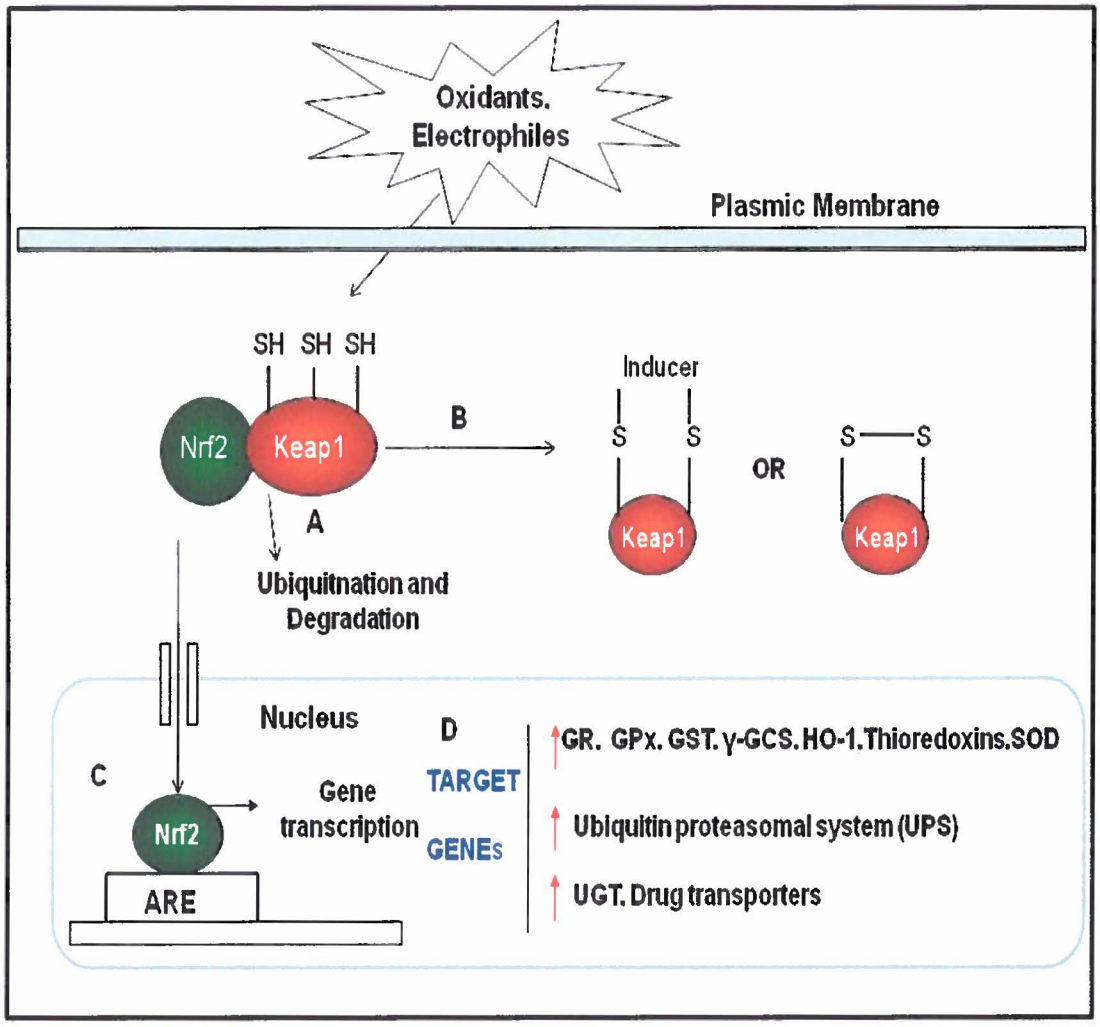

Fig. 5 Keap1-dependent regulation of Nrf2 activity by electrophiles. (A) Under normal conditions, Keap1 binds to Nrf2 in the cytosol through the Kelch domain, promoting cullin 3-dependent Nrf2 ubiquitination. (B, C) In the presence of electrophiles, reaction with target cysteines in Keap1 occurs, destabilizing the interaction between Keap1 and Nrf2 and switching the ubiquitination reaction from Nrf2 to Keap1. (C, D) Nrf2 accumulates in the nucleus, activates the expression of phase II antioxidant gene and drug transporters and Ubiquitin proteasomal system (UPS).

γ-GCS, is a rate-limiting enzyme involved in the synthesis of GSH, the most abundant nonprotein thiol, which protects cells against exogenous and endogenous toxins, including ROS, RNS, lipid peroxide products and electrophiles (Dickinson and Forman, 2002). Both neurons and astrocytes have the capacity to synthesize GSH in an adenosine triphosphate (ATP)-dependent two step process: in the rate limiting first step, γ-glutamylcysteine is formed from conjugation of cysteine and glutamate, catalyzed by the enzyme γ-GCL; then glycine is added by GSH synthase (Dickinson and Forman, 2002). GST is a major enzyme involved in the regulation of oxidative stress by conjugation and detoxification of electrophiles including acrolein with reduced GSH (He *at al.*, 1998; Hubatsch *et al.*, 1998). On the other hand GR regulates the intracellular GSH level by converting oxidized glutathione (GSSG) to reduced GSH using NADPH as a reducing cofactor (Carlberg and Mannervik, 1985). Recently it has been shown that Nrf2 induced GR regulated glutathione recycling is more effective for cell survival under oxidative stress as compared to *de novo* GSH synthesis (Harvey *et al.*, 2009). A family of GPx enzymes is responsible for the enzymatic degradation of H_2O_2, organic hydroperoxides and peroxynitrite (Arthur, 2000). Disruption of Nrf2 in knockout mice rendered neuronal tissues more susceptible to death due to oxidative stress and mitochondrial dysfunctions (Burton *et al.*, 2006; Calkins *et al.*, 2005; Shih *et al.*, 2005). For many decades, chemical substances from plants, called phytochemicals, have been shown to act as Nrf2 inducers (Kelsey *et al.*, 2010; Surh *et al.*, 2008). The potent Nrf2 inducer compounds can be listed as sulforaphane from cruciferous vegetables, curcumin, epigallocatechin-3-gallate from green tea, resveratrol from grape, caffeic acid phenethyl ester, cafestol, kahweol, cinnamon based compounds, garlic organosulfur compounds, lycopene, carnosol, and avicins (Kelsey *et al.*, 2010; Surh *et al.*,

2008). So, up regulation of Nrf2 and ARE genes in neurons by dietary factors may strengthen cellular defenses and protect them against oxidative stress associated with aging and neurodegenerative diseases.

2. Phosphatidylinositol3-phosphate kinase (PI3-K)-AKT

Activated protein kinase B (AKT) is a member of a larger class of serine/threonine kinases called AGC [protein kinase A (AMP protein kinase), PKG (GMP protein kinase), and PKC]. AKT has an N-terminus pleckstrin homology domain that mediates the interaction of AKT with a plasma membrane phospholipid, phophatidylinositol 3, 4, 5-triphosphate (PIP3). Recruitment of AKT to the plasma membrane, and its association with PIP3 is crucial for its activation (Klippel *et al.*, 1997; Kohn *et al.*, 1996). Several studies have shown that the AKT signaling pathway exerts a neuroprotective function against oxidative stress by inactivating downstream substrates of Akt such as Bad, procaspase-9, and Forkhead transcription factors (Cantley, 2002). Pharmacological activation of AKT pathway with small compounds and dietary flavanones protected cells against oxidative stress induced toxicity (Liu *et al.*, 2009; Picone *et al.*, 2011; Williams and Spencer, 2012; Zeng *et al.*, 2011). Thus modulation of PI3-K/AKT pathway could provide neuronal survival during oxidative stress as shown in Fig. 6.

3. Sirtuins

The sirtuin enzymes represent a phylogenitically conserved family of enzymes that catalyze the nicotinamide adenine dinucleotide (NAD)-dependent protein deacetylation and have been reported to play an important role in the pathophysiology of aging, metabolic and neurodegenerative disorders (Donmez and Guarente, 2010; Haigis and Guarente, 2006; Haigis and Sinclair, 2010; Longo and Kennedy, 2006). This family comprises seven

members (Sirt1 to Sirt7) that has been shown to localize to the nucleus (Sirt1, 6, and 7), the cytoplasm (Sirt2) and the mitochondria (Sirt3, 4, and 5) and some of these can shuttle between these compartments as shown in Fig. 6.

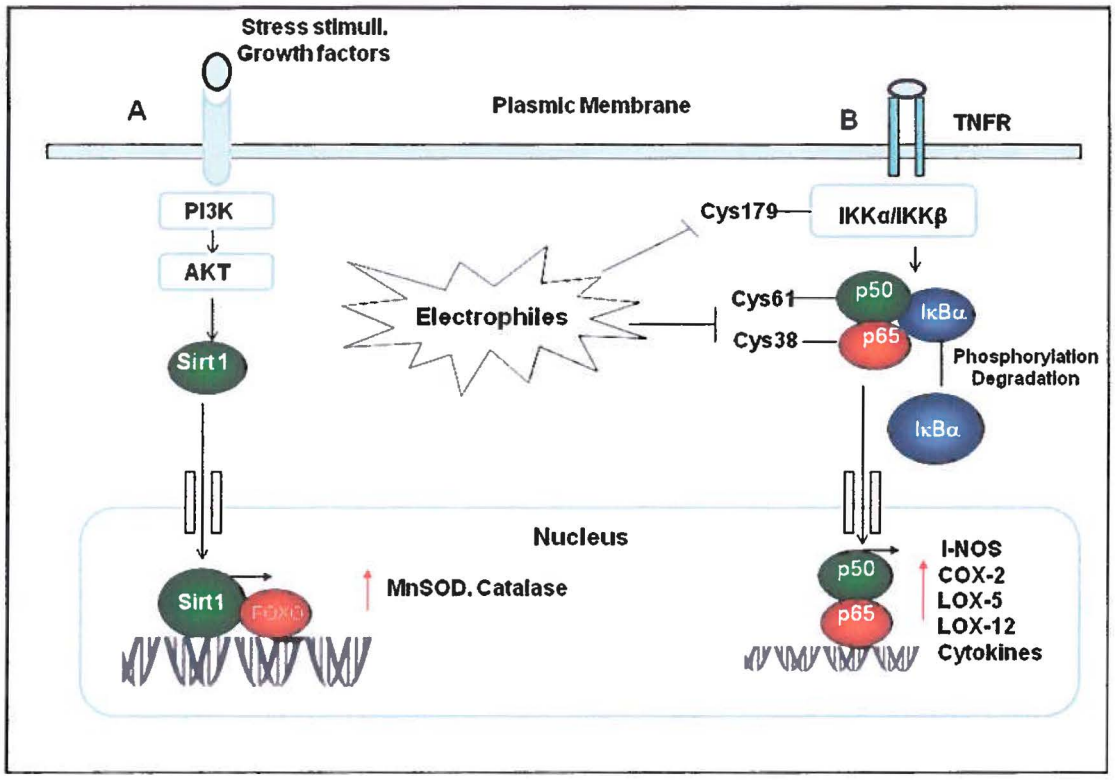

Fig. 6 Phosphatidylinositol3-phosphate kinase (PI3-K)-AKT, Sirtuins and NF-κB pathways.

(A) Upon stimulation with growth factors or oxidative stress conditions, PI3-K-AKT are activated which in turn promote nuclear localizaion of SIRT1. In the nucleus, SIRT1 activates transcription of FOXO-dependent antioxidant genes, MnSOD and catalase.

(B) Under pro-inflammatory conditions, IKKs phosphorylates IκB, releasing the heterodimer p50/p65. Upon nuclear translocation, p65 activates the transcription of a variety of cytokine and inflammatory enzyme-coding genes. Adduction of IKKs and p50/p65 by electrophiles results in its inhibition, impairing NF-κB activation.

Their decacetylase activity consists in the removal of the lysine- linked acetyl group of a target protein (Sauve, 2010). Since their activity is regulated by the intracellular concentrations of NAD they have been proposed as cellular metabolic sensors (Yu and Auwerx, 2010). The beneficial effects of caloric restriction (CR) including an increase in life span in lower organisms and mice have also been shown to be mediated through the Sirt1 (Bordone *et al.*, 2007; Cohen *et al.*, 2004). Sirt1 deacetylates numerous transcription factors such as forkhead-box (FOXOs), p53, NF-κB, peroxisome proliferator-activated receptor gamma coactivator-1 alpha (PGC-1α) that mediates the antioxidant, anti-apoptosis, anti-inflammatory and mitochondrial biogenesis (Aquilano *et al.*, 2010; Brunet *et al.*, 2004; Solomom *et al.*, 2006; Yeung *et al.*, 2004; Wang *et al.*, 2010). Deacetylation of these transcription factors results into a robust protective cellular response and may tilt the balance towards cell survival. Recent findings indicate that Sirt1 is also target of resveratrol, a polyphenol associated with many health benefits found in grapes and red vine and activators of sirtuins such as resveratrol, butein and piceatannol can be used to extend lifespan and overcome a variety of stresses in higher organisms. This has fuelled a flurry of scientific interest in the activators of Sirt1 from natural sources as a novel approach in the treatment of diseases of aging (Baur 2010; Blum *et al.*, 2011). In recent studies Sirt1 has been shown to protect cardiomyocytes and heart against age related, paraqaut and antimycin A induced oxidative stress by increasing the expression of antioxidant enzymes i.e. catalase and mitochondrial manganese superoxide dismutase (MnSOD/SOD2) (Alcendor *et al.*, 2007; Tanno *et al.*, 2010) as shown in Fig. 6.

4. NF-κB pathways

NF-κB is another redox regulated transcription factor that regulates numerous physiological functions, and is involved in the pathogenesis of various diseases. In mammals, the NF-κB/Rel family of transcription factors comprises five members, p50, p52, p65 (Rel-A), c-Rel and Rel-B. These proteins form homo and hetero-dimers that are retained in inactive form in the cytoplasm by inhibitory molecules, called IkBs, comprising of IκBα, IκBβ, IκBγ, IκBε, Bcl-3, p100 and p105 (Hayden and Ghosh, 2008; Morgan and Liu, 2011). Upon cell stimulation by multiple stimuli including inflammation, infection, injury and oxidative stress, the IκB proteins are phosphorylated by Ik Kinases (IKK) and degraded, and the freed NF-κB translocates into the nucleus to regulate the expression of multiple target genes (Hayden and Ghosh, 2008, 2012; Morgan and Liu, 2011) as shown in Fig. 6. A dual role has been suggested for the NF-κB in neurodegneration, its activation in neurons being prosurvival, whereas in glial cells it may be apoptotic through the induction of inflammation (Camandola and Mattson, 2007; Mattson and Meffert, 2006). In glial cells tumour necrosis factor alpha (TNF-α)-induced ROS generation mediates the activation of NF-κB which further induces the production of various proinflammatory cytokines such as interleukin-1 beta (IL-1β) and IL-6 and various inflammatory proteins such as COX-2, iNOS and lipooxygenase 5 and 12 (LOX-5 & LOX-12) (Clark *et al.*, 2010). Interestingly, some phytochemicals and electrophiles can also interact and oxidize Cys-179 of IKKβ and Cys-38 of p65 protein thereby inhibiting NF-κB signaling and generation of ROS and RNS (Salminen *et al.*, 2008). On the other hand constitutive form of NF-κB in neurons mediates the actions of growth factors such as nerve growth factor and glutamate on calcium influx, synpatogenesis and membrane depolarization (Albensi and Mattson, 2000; Boersma *et al.*,

2011; Lilienbaum and Israel, 2003; Scholzke *et al.*, 2003). Constitutively expressed NF-κB is indispensible and essential for the activation of mitochondrial MnSOD/SOD2 and cytosolic copper/zinc superoxide dismutase (SOD1) enzymes (Dhar *et al.*, 2004; Maehara *et al.*, 2000; Rojo *et al.*, 2004). Thus, modulation of NF-κB may induce cell survival by regulating ROS, RNS and different antioxidant enzymes.

5. Mitogen activated protein kinase (MAPK) and p66Shc in pathways and oxidative stress

Mitogen activated protein kinases (MAPK) comprise an important group of signalling molecules that play a prominent role in regulating cell differentiation, proliferation, synaptic plasticity, memory and abundantly expressed in brain (Thomas and Huganir, 2004; Sweatt, 2001). The three best-characterized MAPK pathways are extracellular signal regulated kinases (ERKs), C-Jun N-terminal kinase/stress activated protein kinase (JNK/SAPK) and p38 pathways (Sweatt, 2001; Thomas and Huganir, 2004). ERKs are essential for neuronal development and functional plasticity of the central nervous system leading to learning and memory (Kelleher et al., 2006). In cultured cells, ERKs are activated by insults, such as ROS, glutamate receptors agonists, or calcium influx, which mimics aspects of different neurodegenerative diseases (Chu *et al.*, 2004; Hetman and Gozdz, 2004, Subramaniam and Unsicker, 2010) as shown in Fig. 7.

The mitochondrial adaptor protein p66Shc is emerging as a novel link between mitochondria, oxidative stress, and longevity as shown in Fig. 7. p66Shc is a member of the Shc protein family, the isoforms encoded by the mammalian ShcA locus are called p46Shc, p52Shc, and p66Shc, based on their molecular weight, and they promote opposite cellular fates, growth (p46Shc/p52Shc) or apoptosis (p66Shc) (Luzi *et al.*, 2000).

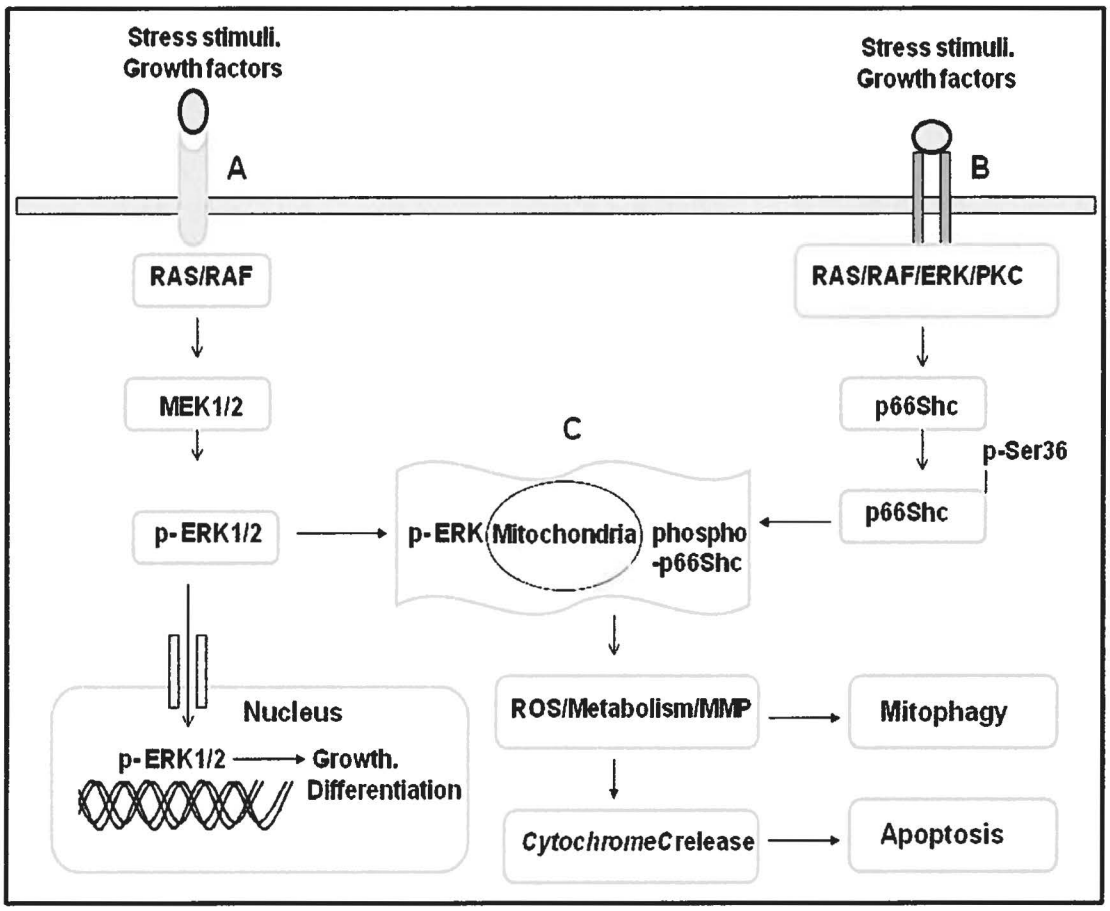

Fig. 7 Activation of extra cellular signal regulated kinases and p66Shc pathways.

(A) Under growth factors or oxidative stress conditions, RAS/RAF are activated which in turn promote the phosphorylation of MEK1/2 and ERK1/2 and nuclear localizaion of pERK1/2. In the nucleus, pERK1/2 activates transcription of genes regulating growth and differentiation.

(B) Under stress conditions, different kinases RAS/RAF/ERK/PKC are activated which in turn phosphorylate p66Shc at serine36, thereby facilitating its mitochondrial localization.

(C) Upon mitochondrial translocation, p66Shc and ERK regulate mitochondrial ROS, metabolism and MMP levels and activate either the mitophagy or induce cytochrome *C* release that in turn induce autophagy.

Mice lacking p66Shc genes have less ROS generation, live 30% longer, are resistant to paraquat induced oxidative stress, and are also protected from age related endothelium dysfunctions and diabetes (Camici et al., 2009; Migliaccio *et al.*, 1999). Phosphorylation and mitochondrial translocation of the redox active protein p66Shc plays an important role in mitochondrial ROS generation and apoptosis through the opening of mitochondrial permeability transition pore (PTP) (Giorgio *et al.*, 2005) as shown in Fig. 7. p66Shc has been shown to determine cellular O_2 consumption, NADH metabolism, aerobic glycolysis and also decreased expression of several ROS scavenging enzymes such as catalase and MnSOD (Nemoto and Finkel, 2002, Nemoto *et al.*, 2006). However, recent studies have also shown p66Shc to be highly expressed in fibroblasts from centenarians and p66Shc derived ROS is required as a preconditioning signal to confer neuroprotection (Brown *et al.*, 2010; Pandolfi *et al.*, 2005). Thus modulation of ERK and p66Shc pathways by natural antioxidants could be important mechanisms to ameliorate mitochondrial dysfunctions and oxidative stress associated with different neurodegenerative diseases.

IV- Alzheimer's disease (AD)

Alzheimer disease (AD) is a progressive, neurodegenerative brain disorder characterized by the loss of cognitive and memory decline, loss of lexical access, impairment of judgement (Mattson, 2004) and is the leading cause of dementia in the Western world, affecting up to 24.3 million people worldwide. This number could reach 34 million and 100 million by 2025 and 2050, respectively, due to the aging population (Alzheimer's Association, 2010; Brookmeyer *et al.*, 2007; Mount and Downton, 2006). Aging may be regarded as the major risk factor for all forms of dementia and particularly for AD (Herrup, 2010). Approximately 5-10% of people above the age of 65 years suffer from AD and its prevalence doubles approximately every five years after the age of 60, but dramatically increases to nearly half of those over 85 being affected by the disease. As the absolute numbers of old persons living in developing countries increase; the cases of AD in developing countries like India, China, and other south Asian countries will grow by a whooping 300% as compared to almost doubling in the developed countries between 2001 and 2040 (Ferri *et al.*, 2005). Owing to aging of the world population, AD poses one of the greatest threats to the future of healthcare systems, with respect to healthcare financing and delivery costs both in developed and developing countries. The annual worldwide healthcare cost of AD is over $ 200 billion and expected to be about $ 100 billion in the United States of America (USA) alone (Alzheimer's Association, 2009; Wimo and Winblad, 2008; Wimo *et al.*, 2010).

1- Neuropathology of AD

AD is characterized by a massive degeneration of synapses and death of neurons in brain regions involved in learning and memory processes, including the temporal and frontal lobes as well as the hippocampus (Arendt, 2009). Histopathological hallmarks of AD

lesions observed in the brains of AD patients at autopsy include: intracellular neurofibrillary tangles (NFTs) and extracellular senile plaques in the neocortex, hippocampus, and other subcortical regions of brains essential for cognitive function (Mattson, 2004). NFTs are formed from paired helical filaments composed of neurofilaments and hyperphosphorylated tau protein in the somatodendritic regions of neurons (Grundke-Iqbal *et al.*, 1986; Buee *et al.*, 2000). In AD, the hierarchical pattern of NFTs degeneration starts early in the entorhinal/perirhinal cortex, then hippocampus, followed by the cortices and subcortical structures, including the amygdala and the nucleus basalis of Meynert and finally primary neocortex. According to the National Institute of Ageing-Reagen Institute (NIA-RI) diagnostic criteria (NIA-RI Consensus 1997), Braak and Braak have described six stages of neurofibrillary degeneration in three entorhinal, limbic, and isocortical regions of affected brain providing the basis for distinguishing six stages of disease progression: the transentorhinal Braak stages I–II represent clinically silent cases; the limbic stages III–IV, incipient AD; the neocortical stages V–VI, fully developed AD (Braak and Braak, 1997; Braak *et al.*, 2006). On the other hand, extracellular senile plaques are composed mostly of amyloid-β peptide (Aβ) that results from the cleavage of the amyloid precursor protein (APP) by various secretases (Selkoe, 2001) and owing to its higher rate of fibrillization and insolubility, $A\beta_{1-42}$ is more abundant than $A\beta_{1-40}$ within the plaques. On the basis of their morphological classification and staining with dyes such as Congo Red and Thioflavin-S, senile plaques have been categorized into diffuse and dense-core plaques and the later have been implicated in the neuronal deleterious effects including increased dystrophic neurites, synaptic and neuronal loss, activation of both astrocytes and microglial cells (Urbanc *et al.*, 2002; Vehmas *et al.*, 2003). Unlike NFTs, amyloid plaques

tend to be diffuse throughout the isocortex, allocortex and neocortex including entorhinal cortex and hippocampus with little region specificity and diffuse deposits of amyloid also occur in the striatum and cerebellar cortex (Arnold *et al.*, 1991; Braak and Braak, 1991, 1990; Joachim *et al.*, 1989; Thal *et al.*, 2002). Presently, it is generally accepted that overall amyloid plaque burden correlates poorly with disease severity and symptoms and conversely the spatial and temporal progression of NFTs is a better indicator of progression of clinical symptoms (Giannakopoulos *et al.*, 2003; Iqbal and Grundke-Iqbal, 2002).

2- Genetics and familial form of AD (FAD)

AD is multifactorial disease with a complex combination of genetic and non-genetic components. The early-onset familial form of AD (FAD) represents only a small fraction of all cases (≤5%) and typically presents itself with age of onset younger than 65 years, while non-genetic or sporadic form represents the majority of AD cases. To date mutations on 3 genes have been reported to cause early-onset familial form of AD. These include the genes coding for the amyloid precursor protein (APP) on chromosome 21, presenilin 1 (PSEN1) on chromosome 14, and presenilin 2 (PSEN2) on chromosome 1 (Chartier-Harlin *et al.*, 1991; Sherrington *et al.*, 1995; Levy-Lahad *et al.*, 1995). There are 32 APP, 179 PSEN1 and 14 PSEN2 gene mutations that result in early- onset, autosomal dominant, fully penetrant AD and these mutations can be examined in detail at the Alzheimer Disease and Frontotemporal Dementia Mutation Database (http://www.molgen.ua.ac.be/ADmutations/). Although these FAD-causing mutations occur in 3 different genes located on 3 different chromosomes, they all share a common biochemical pathway, e.g., the altered production of the Aβ peptide with an increase production of $A\beta_{1-42}$, which has increased propensity to aggregate and generally considered to be responsible for neuronal death and dementia

(Citron *et al.*, 1997; Shen and Kelleher 3rd, 2007). APP is a transmembrane glycoprotein and is known to have role in neuroprotection, synaptic transmission, signal transduction, axonal transport, brain apolipoprotein E and cholesterol metabolism, and cellular iron metabolism (Liu *et al.*, 2007; O'Brien and Wong, 2010; Zheng and Koo, 2011). APP is important to all forms of AD because its proteolytic cleavage by different proteases/ secretases is known to generate both protective as well as toxic fragments (Turner *et al.*, 2003) as shown in Fig. 8. In non- amyloidogenic processing, the initial extracellular cleavage of APP is catalyzed by a group of proteases called alpha (α) secretases. Four members of a disintegrin and metalloproteinase (ADAMs), ADAM-9, ADAM-10, ADAM-17 and ADAM-19 have been proposed as the α- secretases (Allinson *et al.*, 2003; Asai *et al.*, 2003; Kuhn *et al.*, 2010). These α-secretases induce APP proteolysis producing a soluble secretory extracellular fragment called sAPPα, and a membrane associated 83-residue C-terminal fragment called C83. Several studies have shown sAPPα to exert multiple and important roles in neuronal plasticity/survival against excitoxicity besides regulating neuronal stem cell proliferation (Caille *et al.*, 2004; Freude *et al.*, 2011; Taylor *et al.*, 2008). On the other hand during the amyloidogenic processing, Aβ peptide is formed and released after the sequential cleavage of APP by beta (β) secretase (BACE) and gamma (γ) secretase, respectively. BACE1 has been identified as an aspartic protease and is the primary β-secretase and therefore the key rate-limiting factor in the production of Aβ peptide in AD (Vassar *et al.*, 2009). APP undergoes proteolystic cleavage by BACE to produce a soluble extracelluar fragment called sAPPβ, and a membrane associated 99-residue C-terminal fragment called C99 (Vassar *et al.*, 2009). BACE1 expression is increased in various paradigms of cellular stress e.g. energy deprivation, hypoxia, ischemia,

and oxidative stress and in AD patients (Guglielmotto *et al.*, 2009; Holsinger *et al.*, 2002; Jo *et al.*, 2010; O'Connor *et al.*, 2008; Yang *et al.*, 2003).

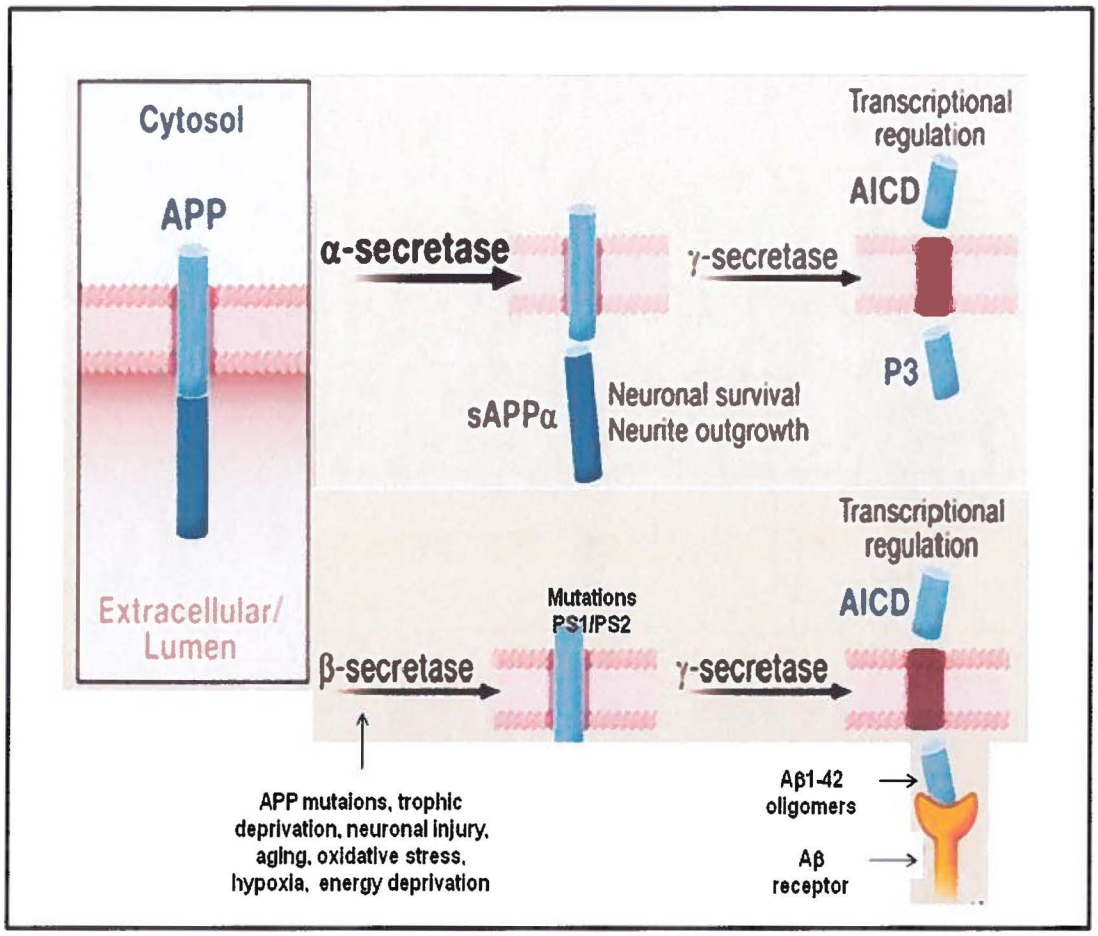

Fig. 8 Generation of amyloid beta (Aβ) peptide from APP metabolism. APP is a transmembrane glycoprotein and in non- amyloidogenic processing, the initial extracellular cleavage of APP is catalyzed by a group of proteases called as alpha α-secretases followed by γ-secretase and this process generates an extracellular neuroprotective fragment called as sAPPα. On the other hand during the amyloidogenic processing, $A\beta_{1-42}$ peptide, which is more fibrillogenic, is formed and released after the sequential cleavage of APP by β-secretase and γ-secretase, respectively. Modified from (Kim and Tsai: 2009, *Cell* 137, 997).

The γ-secretase complex can further cleave C99 at different sites, giving rise to Aβ peptides that are 39 to 43 amino acids long. The exact location of C-terminal cleavage is critical, because generation of the more amyloidogenic peptides (such as $A\beta_{1-42}$) is strongly correlated with AD development. In comparison to α- and β secretases, the γ-secretase activity involves a large multimeric protein complex consisting of at least four major protein components including PSEN1, PSEN2, which form the catalytic core along with Presenilin enhancer-2 (PEN2) and Anterior pharynx-defective 1 (APH1) (Bergmans and De Strooper, 2010; De Strooper, 2003; Takasugi *et al.*, 2003). Mutations in the PSEN1 and PSEN2 are causative in majority (50-70%) of FAD (Sherrington *et al.*, 1995). In addition to APP, more than 90 other proteins have been proposed as independent substrates for γ-secretase (De Strooper and Annaert, 2010; McCarthy *et al.*, 2009), which makes its targeting in AD pathophysiology a challenging task.

3- Sporadic late onset form of AD (LOAD)

The vast majority of AD cases are late-onset and both genetic and environmental risk factors may play a role in LOAD. One strong genetic risk factor for LOAD is the presence of the ε4 allele of the apolipoprotein E (*APOE*) gene, located on chromosome 19q13 (Poirier *et al.* 1993; Strittmatter *et al.* 1993; Strittmatter and Roses 1995) which encodes a glycoprotein ApoE with a molecular weight of 35 kDa containing 299 amino acids with crucial roles in cellular cholesterol metabolism (Mahley, 1988). The three different common isoforms of ApoE e.g. ApoE2, ApoE3 and ApoE4, are encoded by three alleles (ε2, ε3 and ε4, respectively) of a single gene and in the human population, the *APOE* ε3 genotype is the predominant isotype typically present in 50–70% of the population, the ε4 allele accounts for 10–20% and the ε2 allele for 5–10% (Mahley, 1988). Although the three

common isoforms of ApoE: ApoE2 (cys112, cys158), ApoE3 (cys112, arg158), and ApoE4 (arg112, arg158) differ by only one or two amino-acids at residue 112 or 158, these differences have profound affect on apoE structure and function (Mahley *et al.*, 2006). Although the *APOE* ε4 allele is present in 10%–20% of population (Singh *et al.*, 2006), but may account for 65–80% of all AD cases, thereby highlighting the crucial importance of ApoE4 in AD pathogenesis (Farrer *et al.*, 1997; Saunders *et al.*, 1993). The allele *APOE* ε4 is known to increase the risk for AD threefold in heterozygous individuals and 12 to 15-fold for homozygous individuals (Bertram *et al.*, 2007; Roses, 1996).

ApoE is the predominant and major apolipoprotein in the brain synthesized and secreted mainly by the astrocytes and microglia and involved in the transportation of lipids and cholesterol to neurons (Xu *et al.*, 2000, 2006). ApoE is lipidated prior to secretion in astrocytes and microglia principally by the ATP-binding cassette transporter A1 (ABCA1) and lipidated ApoE is a high affinity ligand for the low density lipoprotein receptor (LDLR) and LDL receptor related protein 1 (LRP1) on the neurons, astrocytes and microglia (Herz and Bock 2002; Herz and Chen, 2006). These receptors can also bind Aβ peptide directly (Basak *et al.*, 2012; Deane *et al.*, 2004) or indirectly *via* Aβ chaperones and ApoE is one of the best-characterized Aβ chaperones and plays crucial roles in regulating brain Aβ peptide levels by determining their deposition, trafficking, Aβ peptide oligomers stablization and phagocytic clearance (Cerf *et al.*, 2011; Holtzman, 2001; Lee *et al.*, 2012; Terwel *et al.*, 2011; Tokuda *et al.*, 2000; Zlokovic *et al.*, 2005). In APP transgenic mice, ApoE isoform-specific effects on the propensity of Aβ to be deposited in the brain are goverened by E4>E3>E2 order (Holtzman, 2004). Recent studies have shown that ApoE can facilitate the clearance of Aβ peptides by microglia and this effect of ApoE on

microglial Aβ clearance is dose-dependent (Castellano *et al.*, 2011; Jiang *et al.*, 2008). While all isoforms of human ApoE increased clearance of soluble Aβ, ApoE2 exhibited the strongest effect whereas ApoE4 was significantly less efficient in promoting the degradation of soluble Aβ (Castellano *et al.*, 2011; Jiang *et al.*, 2008). Morover, fibrillar Aβ peptide burden in different brain areas in cognitively normal older people is also associated and correlated with *APOE* ε4 gene dose (Reiman *et al.*, 2009; Rodrigue *et al.*, 2012) and *APOE* isotype modifies the association between Aβ load and cognition in cognitively normal older adults (Kantarci *et al.*, 2012). Mounting evidence also demonstrates that ApoE4 may contribute to AD pathogenesis by modulating amyloid independent mechanisms by directly regulating oxidative stress, mitochondrial functions and glucose metabolism (Chen *et al.*, 2011; Ramassamy *et al.*, 2000; Reiman *et al.*, 2005), tau hyperphosphorylation (Harris *et al.*, 2004), hippocampus neurogenesis (Li *et al.*, 2009), neuronal survival against excitotoxicity (Buttini *et al.*, 2010) and synaptic functions through the modulation of receptor trafficking (Chen *et al.*, 2010). Thus, ApoE4 may have pleiotropic determinental effects in the pathophysiology of LOAD.

4- 'Amyloid cascade hypothesis' of AD: pitfalls and shortcomings

A number of genetic and cell biology studies have implicated the production of amyloidgenic Aβ peptides in familial AD (FAD)-linked mutations has led to a popular hypothesis of AD known as the 'amyloid cascade hypothesis' (Citron, 2004; Hardy and Selkoe, 2002) as shown in Fig. 9. This hypothesis proposed that aggregates of Aβ peptides were responsible a series of downstream events ranging from synaptic loss to plaque deposition, neuroinflammation, tau hyperphosphorylation and ultimately to the death of susceptible neurons. The original amyloid cascade hypothesis claimed that the fibrilized

form of Aβ (fAβ) was the main component of senile plaques was proposed to be responsible for toxicity (Hardy and Higgins 1992). Since many processes of AD were not explained by fAβ, there is still no clear consensus on the precise nature of the toxic form of Aβ, but recent attention has focused on early protein assemblies (protofibrils, soluble oligomers, Aβ-derived diffusible ligands (ADDLs), or globular neurotoxins) and therefore the effects of Aβ could depend on the aggregation state (Tamagno *et al.*, 2006).

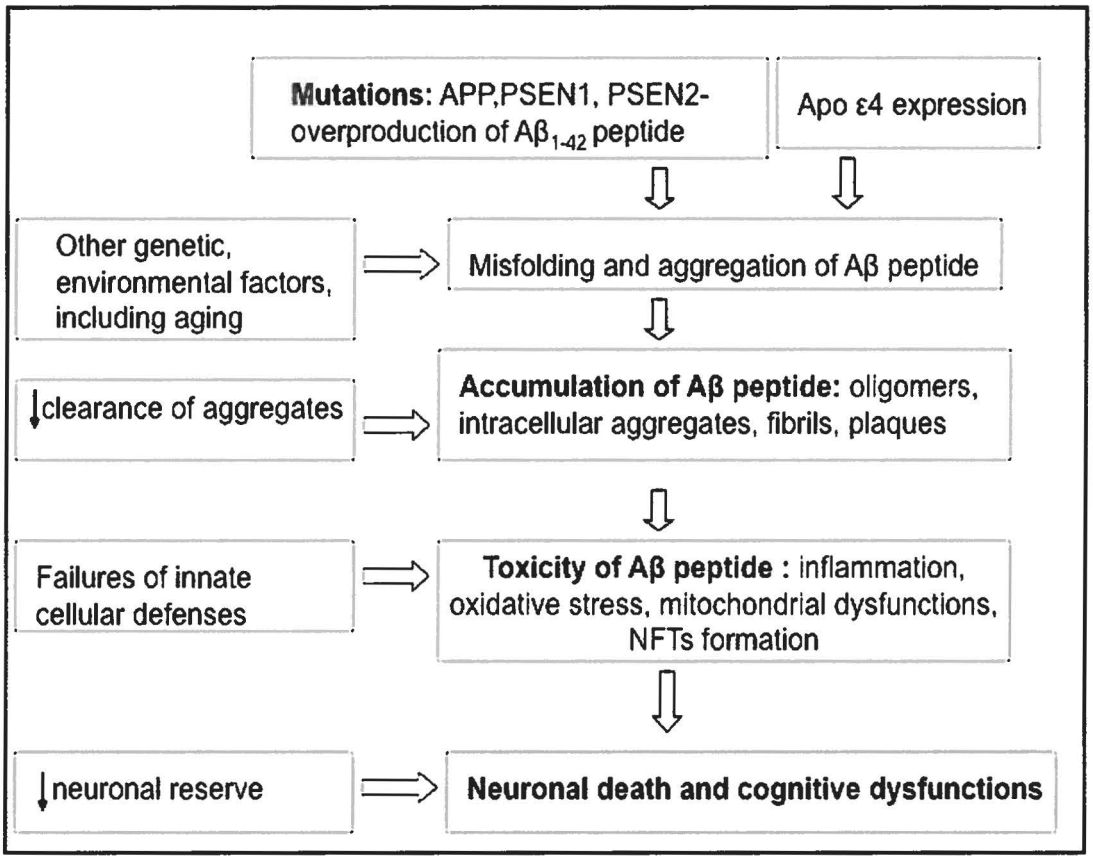

Fig. 9 'amyloid cascade hypothesis' of AD, which posits that aggregates of Aβ peptides are responsible for a series of downstream events ranging from synaptic loss to plaque deposition, neuroinflammation, tau hperphosphorylation and ultimately to the death of susceptible neurons leading to cognitive dysfunctions.

Based on these findings, it has been proposed that Aβ peptide acts through a biphasic neurotoxic mechanism that is conformation dependent. According to the most popular model, both oligomeric and fibrillary Aβ (fAβ) deposits have been proposed to be eventually responsible for neuronal degeneration involving disruption of synaptic functions leading to the characteristic cognitive deficits (Hardy and Higgins, 1992; Hardy and Selkoe, 2002; Larson and Lesné, 2012; Li *et al.*, 2009; Walsh and Selkoe, 2004). In addition to soluble oligomers recent studies have identified that even Aβ peptide dimmers, trimers and tetramers can induce neurotoxicity by binding to the lipid membranes, stabilizing the formation of fibrillar intermediates and impairing synaptic plasticity and memory (Hung *et al.*, 2008; O'Nuallain *et al.*, 2010; Shankar *et al.*, 2008). However, in recent years because of the near total failure of Aβ peptide-focused clinical trials coupled with the broadening of AD knowledge has prompted researchers to revisit the role of Aβ peptides and amyloid cascade hypothesis in AD (Herrup, 2010; Pimplikar *et al.*, 2010). There are two schools of thought regarding the role of Aβ peptide in the neurodegenerative process in AD. In one, Aβ peptide has been proposed to rather act as a protective response to neuronal insult (Lee *et al.*, 2007). Alternatively, Aβ peptide has been proposed to initiate the disease process once produced in excess through ROS generation and mitochondrial dysfunctions (Butterfield *et al.*, 2012; Mao and Reddy, 2011).

5- Oxidative stress and mitochondrial dysfunctions in AD

Several *in vitro* and *in vivo* studies have implicated the role of Aβ peptide induced mitochondrial dysfunctions, ROS generation, protein oxidation and lipid peroxidation in the pathophysiology of AD (Belkacemi and Ramassamy, 2012; Butterfield, 2002; Butterfield *et al.*, 2002, 2012; Perluigi *et al.*, 2012; Reed *et al.*, 2011; Sultana and

Butterfield, 2010). In addition to extracellular toxic effects of Aβ peptide as plaques, the role of intracellular accumulation of Aβ peptide is also being considered to be a critical component in AD. Intracellular accumulation of Aβ peptide occurs before development of neurofibrillary tangles and extracellular Aβ plaques (Gouras *et al.*, 2010). Several studies have shown that extracellular Aβ peptide can be transported to the intracellular space by various cell surface receptors including the receptor for advanced glycation end products (RAGE) (Takuma *et al.*, 2009). Once inside Aβ peptide has been shown to be transported into mitochondria *via* translocase of the outer membrane (TOM) machinery (Hansson Petersen *et al.*, 2008). Recent studies have shown that intracellular Aβ peptide can be toxic to neuronal mitochondria (Reddy and Beal, 2008) as shown in Fig. 10. In the mitochondrial compartment, Aβ peptide has been shown to interact with Aβ-binding alcohol dehydrogenase (ABAD) and cyclophilin-D (CypD) (Muirhead *et al.*, 2010). CypD is an integral part of mitochondrial permeability transition pore (PTP), whose opening is induced by Aβ peptide-CypD interactions leading to the depolarization of mitochondria, release of cytochrome C, generation of ROS and finally induction of neuronal death (Du *et al.*, 2008; Du and Yan, 2010). On the other hand Aβ peptide interaction with ABAD, a short chain inner mitochondrial dehydrogenase, has been shown to generate free radicals, caspase 3 activation and apoptosis (Lustbader *et al.*, 2004; Takuma *et al.*, 2005). Mitochondrial dysfunctions e.g. a drop in mitochondrial membrane potential (MMP) and respiration, reduced adenosine triphosphate (ATP) levels, and deficits in pyruvate dehydrogenase (PDH) and cytochrome *c* oxidase (COX) level and activity were observed in transgenic mouse models of AD before the deposition of Aβ plaques and an increased production of H_2O_2 was found as early as 3 month of age in the mitochondria from 3xTg-AD mice model

(Hauptmann *et al.*, 2009; Rhein *et al.*, 2009; Yao *et al.*, 2009). Recently mitochondrion-derived ROS was found to be sufficient for triggering Aβ peptide production *in vitro* and *in vivo* (Leuner *et al.*, 2012). Thus, mitochondrial dysfunctions may start a vicious cycle that contributes to the Aβ peptide-induced toxicity in AD pathogenesis.

The toxicity of Aβ peptide assemblies may be mediated through different mechanisms as shown in Fig. 10. Aβ peptide is known to generate H_2O_2 during aggregation through electron transfer interactions involving Aβ bound redox-active metal ions in the presence of reducing agents (Behl *et al.*, 1994; Huang *et al.*, 1995; Tabner *et al.*, 2005). Aβ peptide has high affinity for both copper and zinc and both Aβ peptide and APP display strong copper reductase activity, generating copper (Cu^{+}) from Cu^{2+} that in turn in the presence of dopamine, cholesterol, and biological reducing agents generates neurotoxic H_2O_2 (Opazo *et al.*, 2002; Wang and Colon, 2007). Moreover, Aβ peptide, APP and Aβ-Cu^{2+} complexes have been shown to oxidize cholesterol to form neurotoxic oxysterols i.e. 7β-hydroxycholesterol, 24-hydroxycholesterol which exert potent toxic effects in nanomolar concentrations through generation of ROS especially H_2O_2 and subsequent impairment of cellular redox equilibrium (Gamba *et al.*, 2011; Nelson and Alkon, 2005; Puglielli *et al.*, 2005). Several studies have shown that Aβ peptide alone can generate intracellular ROS level and increased free cellular iron level which may further give rise to the toxic hydroxyl radical *via* Fenton chemistry (Abramov *et al.*, 2004; Butterfield, 2002; III-Raga *et al.*, 2010, Sultana *et al.*, 2005; Wan *et al.*, 2011). Aggregated and Aβ peptide fibrils can also generate increased production of nitric oxide (NO) (Akama *et al.*, 2000; Keil *et al.*, 2004; III-Raga *et al.*, 2010). NO has been accounted for neuronal death in AD directly or indirectly through

inducing mitochondrial dysfunctions (Cho *et al.*, 2009; Moncada and Bolaños, 2006; Steinert, 2010).

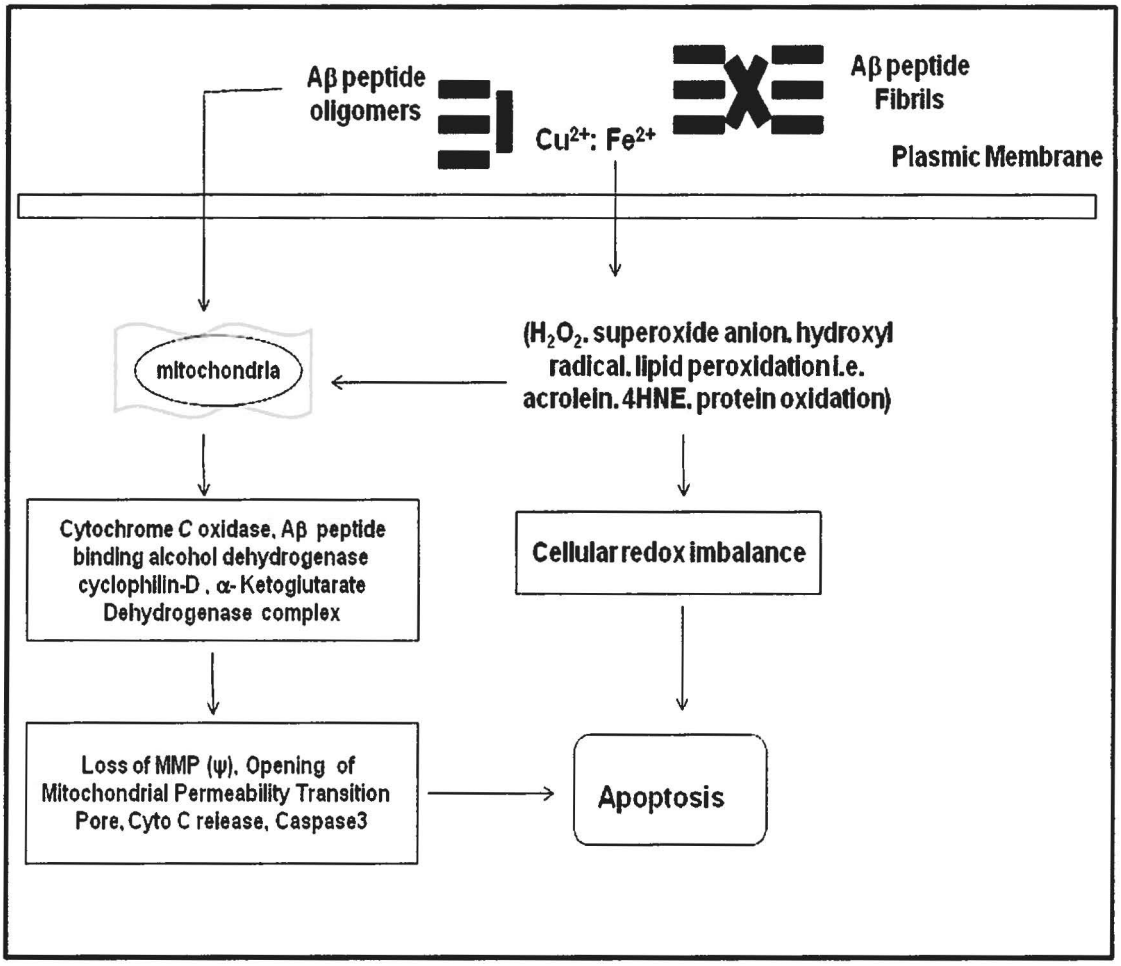

Fig. 10 Mechanisms of toxicity of Aβ peptide in AD. Both Aβ oligomers and fibrils are responsible for toxicity. Extracellular Aβ peptides are known to generate ROS, RNS and lipid peroxidation in the presence of heavy metals thereby compromising the cellular redox homeostasis. On the other hand a number of membrane receptors have been implicated in the intracellular transportation of Aβ peptide, which can interact directly with various components of mitochondria and induce a series of downstream events ultimately leading to apoptosis.

Additionally Aβ peptides can also undergo nitration of tyrosine10 and phophorylation at serine8 which in turn accelerates its aggregation and plaque formation (Kumar *et al.*, 2011, 2012; Kummer *et al.*, 2011). Moreover, excessive production of ROS and NO may increase the formation of peroxynitrite ($ONOO^-$), which is more toxic to cells than either ROS or NO (Groves, 1999). In nutshell Aβ peptide is capable of inducing nitro-oxidative stress in neuronal cells.

The major ROS considered responsible for cell damage are $O_2^{\bullet -}$, H_2O_2, and hydroxyl radical (OH•). ROS on the other hand generates highly electrophilic α, β-unsaturated carbonyl derivatives including acrolein, 4-hydroxy-nonenal (4-HNE), 4-oxo-2-nonenal (4-ONE) from the peroxidation of membrane lipids (LoPachin et al., 2009). Morever, in a comparative toxicity study between acrolein and ROS, acrolein was found to be more toxic than H_2O_2 and hydroxyl radical (OH•) (Yoshida *et al.*, 2009). Current evidence suggests that *in vivo* acrolein is formed either in the metal-catalyzed oxidation of PUFAs including arachidonic acid (AA) (Uchida *et al.*, 1998) or as a toxic metabolite of polyamines metabolism, especially from spermine, by polyamine oxidase (Kimes and Morrios, 1971; Saiki *et al.*, 2009). Amongst all the α, β-unsaturated aldehydes, acrolein is the most reactive with GSH and reacts 110–150 times faster with GSH than HNE or crotonal (Hamann and Shi, 2009) and has increasingly been implicated in the pathogenesis of AD (Dang *et al.*, 2010, 2011; Singh *et al.*, 2010). In the different brain regions such as hippocampus/parahippocampal gyrus and cerebellum of subjects with mild cognitive impairment (MCI) and patients with early AD, exhibit high levels of acrolein and 4-HNE that localize to Aβ aggregates (Bradely *et al.*, 2010; Williams *et al.*, 2006). In primary hippocampus cultures, acrolein was found to be more toxic than 4-HNE at 5.0 μM

concentration in a time and concentration dependent manner (Lovell *et al.*, 2001). Moreover, acrolein was also more potent in impairing brain mitochondrial functions than 4-HNE (Vaishnav *et al.*, 2010). Both acrolein and 4-HNE have been shown to impair mitochondrial functions directly and differentially especially by targeting the pyruvate dehydrogenase and mitochondrial complexI-associated proteins (Picklo and Montine, 2007; Vaishnav *et al.*, 2010). Recent studies from our lab and others have shown that acrolein is a potent inducer of GSH depletion, ROS generation, protein oxidation and dysregulated redox-senstitive pathways in astrocytes and human neuroblastoma SK-N-SH cells (Ansari *et al.*, 2008; Dang *et al.*, 2010, 2011; Singh *et al.*, 2010). Thus, acrolein could disrupt neuronal functions and synaptic homeostasis by provoking oxidative stress and mitochondrial dysfunctions.

Excessive protein oxidation may render the unfolding and accumulation of proteins, thereby compromising their biological functions. Protein oxidation has been reported to be elevated in AD (Sultana *et al.*, 2009; 2010). Protein carbonyls are produced by the oxidant attack on several amino acids side chains (Lys, Arg, Pro, Thr, Trp etc.) or formation of Michael adducts between His, Lys, and Cys residues and reactive electrophiles such as 4-HNE and acrolein (Butterfield and Stadtman, 1997). Protein carbonyls have been detected both in tangles- and non-tangles-bearing neurons, in frontal lobe or hippocampus (Aksenov *et al.*, 2001; Pamplona *et al.*, 2005). Using redox proteomics approach, several carbonylated proteins have been identified in the hippocampus and the parietal lobe of the MCI and AD brains as shown in Table 1, and some of these oxidized proteins are directly or indirectly involved in cellular energy production while others are important components of cellular antioxidant, ubiquitin-proteosomal system (UPS), heat shock proteins (HSPs)

and synaptic system, and oxidation of these key proteins may compromise their functional integrity (Aluise *et al.*, 2011; Butterfield *et al.*, 2012; Castegna *et al.*, 2002; Martínez *et al.*, 2010). Some of these proteins such as DJ-1 and SOD-1, in addition to exerting antioxidant functions are also known to protect against cell death by directly binding or inducing the expression of anti-apoptotic mitochondrial B-cell lymphoma 2 (Bcl-2) and Bcl-XL proteins (Guareschi *et al.*, 2012; Pedrini *et al.*, 2010; Ren *et al.*, 2011). Thus, protein oxidation and lipid peroxidation products may play an important role in the progression of AD. Thus, protein oxidation and lipid peroxidation products may play an important role in the progression of AD.

Table 1 Examples of oxidized proteins identified during the progression of AD.

Class	Proteins
Energy production	Lactate dehydrogenase (LDH), ATP synthase, glyceraldehyde 3-phosphate dehydrogenase (GAPDH), voltage-dependent anion channel (VDAC), glutamine synthase (GS), α-enolase, aconitase
Antioxidant system	DJ-1, MnSOD, GST, peroxiredoxin6 (Prx6), carbonyl reductase
UPS and HSPs	Ubiquitin carboxy-terminal hydrolase L-1 (UCHL-1), HSP60, HSP70, HSP90
Neurotransmission	CRMP-2, α-tubulin, β-actin, syntaxin binding protein 1 (SBP1)

6-Redox dysregulation of transcription factors in AD

One of the most important redox regulated pathway e.g. Nrf2 is also not activated and retained in the cytoplasmic compartment in the AD brains (Ramsey *et al.*, 2007). Consequently, the levels of different antioxidants such as GSH, GPx, GST, and GR have been shown to be significantly declined in the frontal cortex from the AD brains (Ansari

and Scheff, 2010). Interestingly, depletion of GSH occurred before the deposition of senile plaques and neurofibrillary tangles in transgenic mouse models of AD and GSH supplementation protected neurons against Aβ peptide-induced toxicity (Gosh *et al.*, 2012; Zampagni *et al.*, 2012; Zhang *et al.*, 2012). Recently, studies have shown that intrahippocampal injection of lentiviral vector expressing Nrf2 improved spatial learning in a transgenic AD mice without change in total Aβ peptide levels while boosting the activity of the Nrf2-ARE pathway by tert-butylhydroquinone treatment delayed AD-like pathology in transgenic AD mice (Kanninen *et al.*, 2008, 2009). AKT signaling has been shown to be downregulated both in the AD brain as well as in the hippocampus during aging and activation of AKT preserved memory in animal model of AD (Jackson *et al.*, 2009; Lee *et al.*, 2009; Malm *et al.*, 2007). Recently, sirtuins and particularly Sirt1 has fuelled a flurry of scientific interest in the neuroprotection in AD. A significant reduction of Sirt1 was observed in parietal cortex of AD patients (Julien *et al.*, 2009).

In a mice model of AD, Sirt1 suppressed toxic Aβ peptide production from APP by activating the alpha- secretase gene ADAM10 (Donmez *et al.*, 2010). Resveratrol and other polyphenols-induced Sirt1 activation inhibited the NF-κB signalling in microglia and astrocytes, by deacetylation of RelA subunit at lysine 310, which inactivates NF-κB, and protected AD neurons against Aβ peptide induced toxicity (Chen *et al.,* 2005; Longpré *et al.*, 2006). TNF-α is further thought to play a central role in the self-propagation of neuroinflammation through the activation of transcription factor NF-κB that mediates the production of various proinflammatory cytokines and inflammatory proteins (Yamamoto and Gaynor, 2004). In transgenic mice models of AD, microglial levels of TNF-α were markedly increased and associated with increased Aβ plaques deposition (Hickman *et al.*,

2008; Yamamoto *et al.*, 2007). Activated NF-κB has been detected in degenerating neurons and glial cells in the brain of AD patients (Kaltschmidt *et al.*, 1997) and in the brains of Tg2576 mice (an animal model of AD) compared with wild type-littermates, and this increase was associated with deposition of Aβ and inhibition of NF-κB activation protected neuronal cells against Aβ induced toxicity (Longpré *et al.*, 2006; St-Laurent-Thibault *et al.*, 2011) besides preserving cognition and memory in a transgenic mouse model of AD (Echeverria *et al.*, 2009; Sung *et al.*, 2004). Numerous studies indicate that Aβ peptide can activate NF-κB in neurons and activated NF-κB upregulated the production of Aβ42 peptide by activating BACE1 promoter activity (Bourne *et al.*, 2007; Buggia-Prevot *et al.*, 2008). These studies suggest that deregulated activation of NF-κB in neurons may lead to increased Aβ production and participate in the progression of AD. Additionally, NF-κB also prevented the transcriptional activation of Nrf2-dependent antioxidant genes by preventing the binding of Nrf2 with CREB-binding protein (CBP) (Liu *et al.*, 2008). Many studies have shown that toxicity of Aβ peptide, 4-oxo-2-nonenal (ONE), acrolein and H_2O_2 is mediated through the ERK1/2 (Brewer *et al.*, 2010; Chong *et al.*, 2006; Lee *et al.*, 2009; Ruffels *et al.*, 2004; Tanel and Averill-Bates, 2007). Phosphorylation of p66Shc is critical for Aβ peptide mediated toxicity and under oxidative stress condition, ERK1/2 has been identified as the kinase phosphorylating p66shc on Ser36 (Hu *et al.*, 2005, Smith *et al.*, 2005). Thus modulation of ERK and p66Shc pathways by natural antioxidants could mitigate mitochondrial dysfunctions and oxidative stress associated with AD.

V- Parkinson's disease (PD)

Parkinson's disease (PD) is a chronic progressive neurodegenerative movement disorder characterized by a profound and selective loss of nigrostriatal dopaminergic neurons with presence of eosinophilic, intracytoplasmic, proteinaceous inclusions termed Lewy bodies (LBs), and dystrophic Lewy neuritis in surviving neurons (Forno, 1996; Zipp and Aktas, 2006). PD is a devastating degenerative neurological illness without cure affecting 1-2% of the 'over 50' population with a current estimation of approximately 10 million people worldwide and 1.5 million patients in the US alone (Thomas and Beal, 2007; Parkinson's Disease Foundation, 2010). Although, dopaminergic neuronal loss in substantia nigra pars compacta (SNpc) is pronounced, there is widespread neurodegeration in the other parts of brain including cerebral cortex and striatum (Braak *et al.*, 2003). Dopaminergic SNpc neurons project primarily to the striatum, which is composed of the caudate and putamen nuclei; and associated nigral cell loss results in the depletion of striatal dopamine. On the basis of Lewy neurites and LBs distribution a staging system for PD pathology has been proposed (Braak *et al.*, 2003). In stages I–II, LBs are found within the medulla and pons and only in stage III aggregated α-synuclein affects the SNpc neurons. Stages (IV-V) involve the appearance of LBs in a graded fashion in amygdala, nucleus of Meynert, hippocampus, whereas stage VI ultimately leads to substantial neocortical pathology (Braak *et al.*, 2003). Clinically it is characterized by motor impairments involving resting tremors, bradykinesia, postural instability and rigidity along with non-motor symptoms like autonomic, cognitive and psychiatric problems. By the time PD motor symptoms are clinically recognized, 60% of dopaminergic SNpc cells are lost, resulting in a concomitant 80% depletion of striatal dopamine (Ma *et al.*, 2002). While the etiology of dopaminergic

neurons cell death is elusive, a combination of genetic and environmental factors seems to play a critical role.

1- Genetics of PD and sporadic forms of PD

Over the past decade, a definitive link has been demonstrated between mutation in specific genes and heritable forms of PD. Although genetic components contribute to < 10% of PD while 90% of cases are sporadic. Mutations in Parkin, DJ-1 and PINK1 have been linked to recessively inherited parkinsonism whereas mutations in α- synuclein and LRRK2 have been linked to dominantly inherited parkinsonism (Cookson and Bandmann, 2010). Recent studies have shown that Parkin, PINK1, DJ1, alpha Synuclein and LRRK2 participate and co-ordinate different cellular functions leading to dopaminergic cell survival by modulating cellular redox homeostasis and preserving mitochondrial functions.

Mutations in the PARK6/ PINK1 (phosphatase and tensin (PTEN) homolog-induced putative kinase 1) gene cause early-onset familial PD (Valente *et al.*, 2004). PINK1 is a 581 amino acid protein with mitochondrial localization and contains a highly conserved protein kinase domain similar to serine/threonine kinases of the Ca^{2+} calmodulin family (Gandhi *et al.*, 2006). Mitochondrial dysfunctions such as the loss of mitochondrial membrane potential, impaired oxygen consumption, decreased complex1 activity, increased mitochondrial oxidative stress, destabilization of mitochondrial calcium homeostasis, which in turn triggers compensatory fission, mitophagy and biosynthetic repair pathways along with increased sensitivity and vulnerability to oxidative stress and mitochondrial toxins rotenone and MPP^+ (1-methyl-4-phenyl-pyridinium ion) have been observed in the cellular models of PINK1 deficiency (Cherra *et al.*, 2009; Chu, 2010; Dagda *et al.*, 2009;

Deng *et al.*, 2005; Gautier *et al.*, 2008; Hoepken *et al.*, 2007; Marongiu *et al.*, 2009; Morais *et al.*, 2009; Tang *et al.*, 2006).

The parkin (PARK2) gene encodes a 465 amino acid protein containing an N-terminal ubiquitin like domain and parkin functions as an E3 ubiquitin protein ligase (Shimura *et al.*, 2000; Winklhofer, 2007). Parkin targets misfolded proteins to the ubiquitn-proteasome (UPS) pathway for degradation, and the loss of its E3 ligase activity due to mutations lead to autosomal recessive early-onset PD (Nussbaum, 1998; Shimura *et al.*, 2000; Zhang *et al.*, 2000). Recent studies have shown that parkin is selectively recruited to the impaired and depolarized mitochondria by PINK1 where it promoted mitophagy thereby preserving neuronal mitochondrial homeostasis (Geisler *et al.*, 2010; Narendra and Youle, 2011; Vives-Bauza *et al.*, 2010b). Thus, parkin regulates the degradation of inner and outer membrane proteins through mitophagy and UPS pathways respectively.

DJ-1 (PARK-7) is a small 20-kDa protein and DJ-1 mutations account for 1–2% of all early-onset PD (Hedrich *et al.*, 2004). DJ-1 is a highly conserved protein of 189 amino acids that is expressed in a variety of mammalian tissues including brain and localized to mitochondria (Bandopadhyay *et al.*, 2004; Zhang *et al.*, 2005). DJ-1 functions as an antioxidant by eliminating and scavengeing H_2O_2 and ROS by oxidation of its Cys106 residue, which appears to facilitate its mitochondrial association where it has been shown to be protective (Blackinton *et al.*, 2009; Canet-Aviles *et al.*, 2004; Junn *et al.*, 2009; Lev *et al.*, 2008; Taira *et al.*, 2004; Wilson, 2011). Overexpression of wild-type DJ-1 in cell culture and *in vivo* models protects against a wide variety of oxidative stress, proteasome inhibitors and mitochondrial toxins (Inden *et al.*, 2006; Liu *et al.*, 2008; Mullett and Hinkle, 2011; Paterna *et al.*, 2007; Taira et *al.*, 2004; Zhou *et al.*, 2011) and this may be partially

mediated through the ability of DJ-1 to stabilize the Nrf-2, a master regulator of cellular antioxidant gene battery (Clements *et al.*, 2006). Additionally, DJ-1 has been shown to induce thioredoxin1, SOD1 and elevated intracellular glutathione levels by increasing the transcription and enzymatic activity of glutamate cysteine ligase (GCL), a rate-limiting enzyme in glutathione biosynthesis (Im *et al.*, 2012; Wang *et al.*, 2011; Zhou and Freed, 2005). Thus, DJ-1 preserves mitochondrial functions and facilitates cell survival in response to oxidative stress.

In contrast to Parkin, DJ-1 and PINK1, mutations in α-synuclein and LRRK2 have been linked to dominantly inherited PD (Cookson and Bandmann, 2010). α-Synuclein (PARK-1) is a natively unfolded 140 amino acid presynaptic protein widely distributed throughout the brain; is highly expressed in neurons where it can reach concentrations of 0.5–1% of total proteins (e.g. 30–60 μM) and is involved in synaptic vesicle recycling, storage and compartmentalization of neurotransmitters (Abeliovich et al., 2000; Bodner *et al*, 2009; Spillantini *et al*, 1997; Yavich *et al.*, 2004, 2006). Three missense mutations in α-synuclein gene (A53T, A30P and E46K) (Kruger *et al.*, 1998; Polymeropoulos *et al.*, 1997; Zarranz *et al.*, 2004), along with genomic duplications and triplications of a region of α-synuclein gene are associated with autosomal dominant PD (Ross *et al.*, 2008; Singleton *et al.*, 2003). Transgenic mice expressing human A53T mutation develop mitochondrial dysfunctions indicating a role for α-synuclein in modulating mitochondrial functions in dopaminergic neurons (Martin *et al.*, 2006; Stichel *et al.*, 2007). A53T and A30P α-synuclein mutations have been shown to promote oligomerization but not fibrillization of α-synuclein and enhancing α-synuclein fibrillization protects against neuronal degeneration (Conway *et al.*, 2000; Smith *et al.*, 2010). Although the pathophysiology of α-synuclein gene duplications

and triplications is not very clear, but with gene triplication (which results in an approximate doubling of plasma α-synuclein levels) age of onset is younger and disease progression is faster (Farrer *et al.*, 2004; Miller *et al.*, 2004; Ross *et al.*, 2008). These studies indicate that both overexpression and mutations of α-synuclein are deleterious for dopaminergic neurons. Mutations in the leucine-rich repeat kinase 2 (LRRK2) or PARK8 genes are now the most common known cause of autosomal dominant familial PD worldwide (Paisan-Ruiz *et al.*, 2004; Webber and West, 2009; Zimprich *et al.*, 2004). LRRK2 encodes a 2527 amino acid multidomain, 280 kDa protein belonging to ROCO protein family that includes a Rho/Ras-like GTPase domain, a protein kinase domain of the MAPKKK family, as well as a WD40-repeat and a leucine-rich repeat domains suggesting its multiple functions (Deng *et al.*, 2008; Liu *et al.*, 2010; Mata *et al.*, 2006; Xiong *et al.*, 2010). More than 80 missense mutations have been identified in the entire LRRK2 protein and unlike other PD-causative genes; mutations in *LRRK2* have also been detected in up to 2% sporadic PD patients (Correia Guedes *et al.*, 2010). Gly2019Ser, the most frequent mutation in *LRRK2*, has been detected worldwide with an average frequency of 1% in patients with sporadic PD and 4% in patients with hereditary PD (Healey *et al.*, 2008; Paisan-Ruiz *et al.*, 2009).

LRRK2 protein is expressed in majority of forebrain structures including nigrostriatal dopaminergic neurons and wildtype LRRK2 has been shown to be protective against oxidative stress-induced cell death (Liou *et al.*, 2008). Transgenic mice and rat expressing Gly2019Ser mutant LRRK2 exhibited degeneration of nigrostriatal dopamine neurons, reduced neurite outgrowth and autophagic abnormalities (Dusonchet *et al.*, 2011; Ramonet *et al.*, 2011). Similarly, overexpression of mutant LRRK2 in model organisms *Drosophila*

and *C. elegans* caused dopaminergic neuronal death and increased sensitivity to rotenone and paraquat (Liu *et al.*, 2008, 2011; Ng *et al.*, 2009; Saha *et al.*, 2009; Venderova *et al.*, 2009). Identification of physiological substrates of LRRK2 and characterization of its functions *in vivo* models will help in understanding both its physiological and pathological functions.

In nutshell the discovery of genes linked to rare familial forms of PD have provided vital clues and information in understanding molecular pathogenesis underlying PD and helped us to identify probable targets for developing neuroprotective therapies, which may revolutionize the treatment of this debilitating disorder.

2- Alpha (α) Synulein in sporadic PD

α-Synuclein has an increased propensity to aggregate and the fibrillar α-synuclein is a major structural component of Lewy bodies (LBs) in PD thereby suggesting a role of aggregated α-synuclein in disease pathogenesis (Spillantini *et al.*, 1998). In LBs, α-synuclein is arranged in fibrils with a β-sheet like structure (Chen *et al*, 2007; Der-Sarkissian *et al*, 2003). Presently, it is unclear whether accumulation of misfolded proteins that lead to LBs-like inclusions are toxic or protective in PD. α-Synuclein aggregation has been shown to be promoted by numerous factors including mitochondrial complex I inhibitors paraquat and rotenone, oxidative and nitrosative stress, lipid peroxidation products, metals, interactions with other amyloidogenic proteins such as tau or amyloid-β peptide which can promote the aggregation and accumulation of each other and accelerate cognitive dysfunction (Clinton *et al.*, 2010; Cole *et al.*, 2005; Giasson *et al.*, 2000, 2003; Leong *et al.*, 2009; Manning-Bog *et al.*, 2002; Masliah *et al.*, 2001; Ostrerova-Golts *et al.*, 2000; Qin *et al.*, 2007; Sherer *et al.*, 2002, 2003). Additionally, phosphorylation of Ser129

in α-synuclein promoted its aggregation, and this is a major component of LBs (Anderson *et al.*, 2006; Fujiwara *et al.*, 2002; Smith *et al.*, 2005). Approximately 90% of α-synuclein deposited in LBs is phosphorylated at serine 129, whereas as only 4% of total α-synuclein is phosphorylated in normal brain, suggesting that accumulation of Ser129-phosphorylated α-synuclein is involved in the pathogenesis of PD (Anderson *et al.*, 2006; Smith *et al.*, 2005). Although the normal function of Ser129-phosphorylated α-synuclein remains unclear, Ser129 phosphorylation is reported to reduce the ability of α-synuclein to regulate the tyrosine hydroxylase (TH) activity and rapid elevation of Ser129-phosphorylated α-synuclein monomers may cause transient damage to dopaminergic neurons in an aggregation-independent manner (Lou *et al.*, 2010; Machiya *et al.*, 2011). Moreover, enhancing α-synuclein phosphatase activity protected against α-synuclein-mediated neurotoxicity and improved motor functions in wild type α-synuclein overexpressing mice model of PD, suggesting a causal detrimental role of a-synuclein phosphorylation in the disease process (Lee *et al.*, 2011).

Several studies have indiacted that the pathogenicity of α-synuclein is associated with aggregation of the protein, which involves formation of small neurotoxic oligomers that eventually mature to larger insoluble deposits (Haass and Selkoe, 2007; Kramer and Schulz-Schaeffer, 2007; Kostka *et al*, 2008; Kayed *et al*, 2009; Li *et al*, 2004; Winner *et al.*, 2011). Recent studies have implicated α-synuclein oligomer induced endoplasmic reticulum (ER) stress in synucleinopathy (Colla *et al.*, 2012a,b). Aggregates of α- synuclein have also been shown to activate stress-signaling protein kinases (Klegeris *et al.*, 2008), and accumulation of α-synuclein in mitochondria is accompanied by impairment of mitochondrial complex1, mitochondrial fragmentation and inhibition of mitochondrial

fusion (Devi *et al.*, 2008; Kamp *et al.*, 2010; Nakamura *et al.*, 2011). These altered pathophysiological attributes are detrimental to normal functioning of dopaminergic neurons and implicate α-synuclein-in PD.

3- Oxidative stress and mitochondrial dysfunctions in PD

Multiple *in vivo* and *in vitro* studies suggest a pathogenic role of oxidative damage and mitochondrial dysfunction in causing PD. A deficit (25-30%) in the activity and oxidative damage of various subunits of mitochondrial reduced nicotinamide adenine dinucleotide (NADH) dehydrogenase-ubiquinone oxidoreductase (complex I of the electron transport chain, containing approximately 46 subunits) in blood platelets and SNpc neurons of PD patient is a common finding (Beal, 2005; Keeney *et al.*, 2006; Parker *et al.*, 1989; Schapira *et al.*, 1990). Accidental discovery of 1-methyl-4-phenyl-1,2,3,4-tetrahydropyridine (MPTP) whose toxic metabolite 1-methyl-4-phenyl-pyridinium (MPP^+), by selective uptake in dopaminergic neurons caused Parkinsonism in designer-drug abusers due to mitochondrial dysfunctions (Langston *et al.*, 1983) further cemented the role of mitochondrial dysfunctions in PD. Partial deficiency of mitochondrial complex I is accompanied by increased vulnerability of dopamine neurons to MPTP in mice (Sterky *et al.*, 2012), which further strengthen the link between complex I dysfunctions and PD. The enzyme monoamino oxidase B (MAO-B) present on astrocytes and microglia converts MPTP into MPP^+, a free radical oxidant that is taken up by DA transporters (DAT) in neurons as shown in Fig. 11. MPP^+ accumulates in mitochondria where it inhibits complex 1 function, thereby disrupting electron transport chain-1 (ETC-1) and triggering a cascade of events leading to oxidative stress and activation of mitochondrial cell death machinery (Przedborski and Vila, 2003).

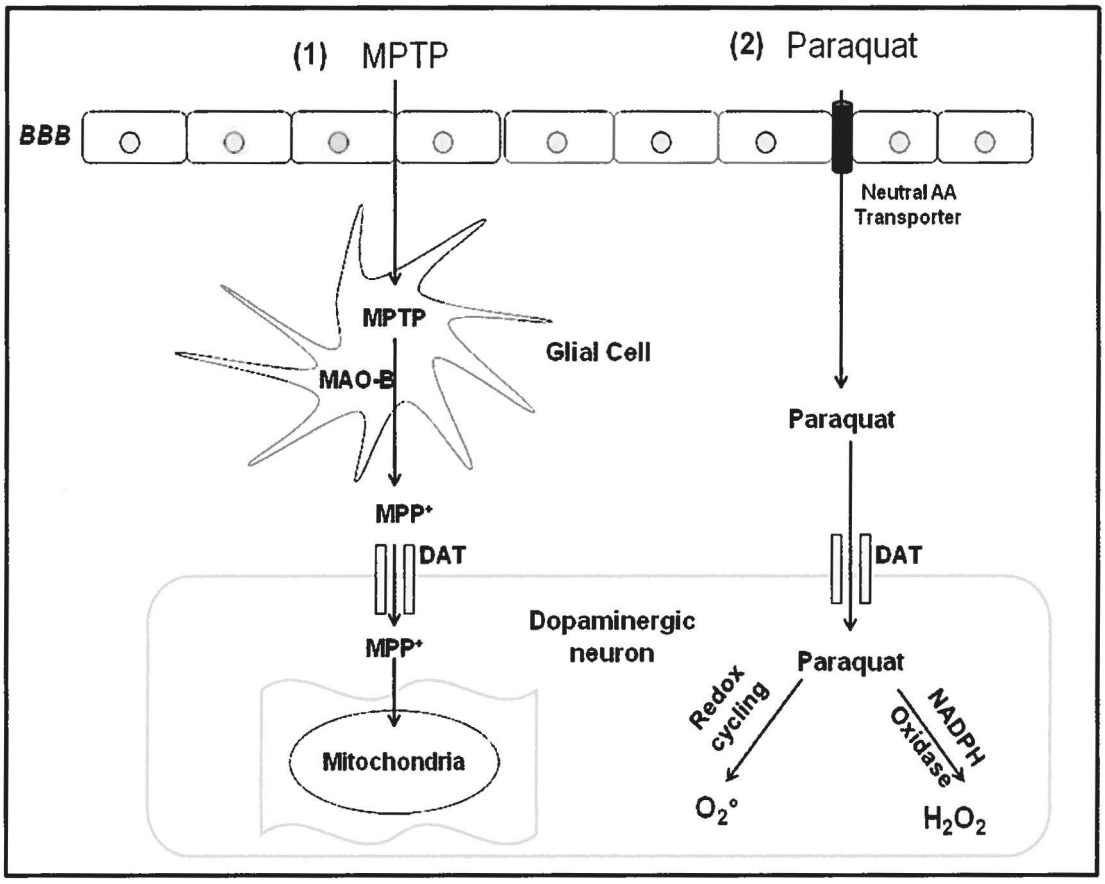

Fig. 11 Schematic of the MPTP and Paraquat metabolic pathway.

(1) Following systemic administration, MPTP readily crosses the blood-brain barrier (BBB). Within the brain, MPTP is metabolized into the toxic MPP^+ species by the enzymatic action of monoamine oxidase-b (MAO-B) within glial cells. The MPP^+ is then released into the extracellular space where it is selectively taken up into DAergic neurons by the dopamine transporter (DAT). Once inside the DAergic neurons, the MPP^+ is taken up by and concentrated within mitochondria, where it inhibits mitochondrial complex I. (2) Paraquat is absored through neutral amino acid transporter at BBB, taken up into DAergic neurons by the DAT. Once inside the DAergic neurons it undergoes redox cycling and generates oxidative stress.

One of the earliest expected consequences of impaired mitochondrial functions will be a reduction in adenosine triphosphate (ATP) production and loss of mitochondrial membrane potential (MMP) leading to subsequent cellular bioenergetic failure. High levels of ATP are required for synaptic transmission and to regulate cellular calcium (Ca^{2+}) homeostasis during intense synaptic activity (Keating, 2008). MPP^+ has been shown to induce loss in MMP (Chen *et al.*, 2005), a rapid increase in mitochondrial fragmentation (Wang *et al.*, 2011) and a profound depletion of ATP levels in isolated hepatocytes, in brain synaptosomal preparations and in whole mouse brain tissues (Chan *et al.*, 1991; Di Monte *et al.*, 1986; Scotcher *et al.*, 1990). In mice, however, MPTP caused only a mild (~20%) and transient reduction in striatal and midbrain ATP levels (Chan *et al.*, 1991). Although complex I activity should be reduced by more than 50% to induce significant ATP depletion in nonsynaptic brain mitochondria (Davey and Clark, 1996), this threshold in synaptic mitochondria, is lowered to 25%, which is within the range of complex I impairment observed in PD patients (Davey *et al.*, 1998). Additionally, owing to the high metabolic demands for neurotransmission in DA neurons, mitochondria have to travel between cell bodies and axon terminals (Vives-Bauza *et al.*, 2010a). MPP^+ has been shown to impair kinesin-mediated anterograde fast axonal transport (FAT) in isolated squid axoplasm, squid giant synapse and in murine mesencephalic cultures through the activation of caspase3 and protein kinase C (PKC) (Kim-Han *et al.*, 2011; Morfini *et al.*, 2007; Serulle *et al.*, 2007). Additional mechanisms are also involved in the loss of DA neurons in MPTP models, which may be associated with glial activation after single exposure in mice (Sugama *et al.*, 2003), monkeys (McGeer *et al.*, 2003) and humans (Langston *et al.*, 1999). Also brain samples from humans or mice exposed to MPTP have elevated cycloxygenase-2

(COX-2) levels and extensive DA-quinone formation (Teismann *et al.*, 2003). Reactive oxygen and nitrogen species, neuroinflammatory cascade and endoplasmic reticulum (ER) stress, loss of cellular antioxidant defence systems and impairment of neurogenesis have been implicated in the MPTP models of PD (Egawa *et al.*, 2011; L'Episcopo *et al.*, 2012; Schildknecht *et al.*, 2009; Yokoyama *et al.*, 2008).

Another intriguing factor contributing towards increased susceptibility of SNpc neurons to various stresses is that they operate under a pro-oxidative state relative to other parts of the brain even in healthy individuals. DA is a very reactive metabolite and its natural degradation produces oxidative stress (Graham, 1978). For instance, during the spontaneous enzymatic oxidation of DA by the monoamine oxidase, H_2O_2 and the reactive intermediate 3,4-dihydroxyphenylacetaldehyde (DOPAL), which is further oxidized to 3,4-dihydroxyphenylacetic (DOPAC) acid via mitochondrial aldehyde dehydrogenase (ALDH) (Stokes *et al.*, 1999; Marchitti *et al.*, 2007) as shown in Fig. 12. DOPAC is then predominantly *O*-methylated by catechol-*O*-methyltransferase (COMT), to homovanillic acid (HVA), the major metabolite of dopamine (Dedek *et al.*, 1979).

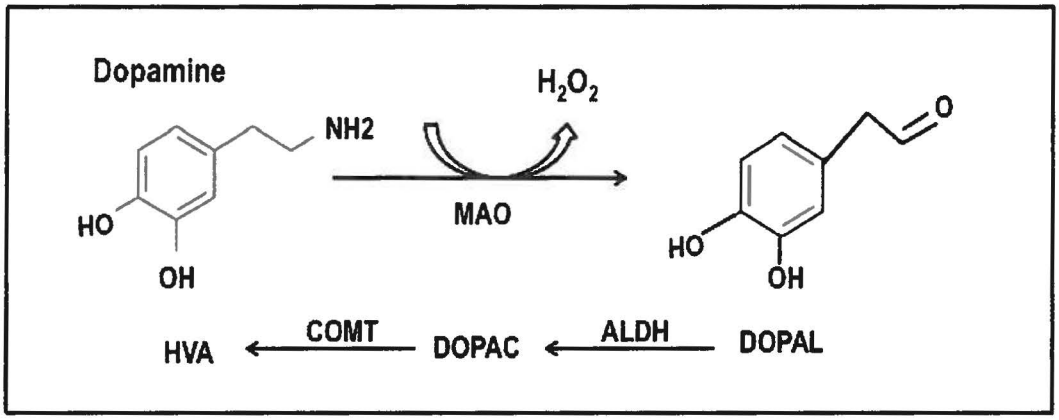

Fig. 12 Dopamine metabolism in neurons.

DOPAL, the reactive aldehyde has been shown to be toxic to dopaminergic cells both *in vitro* and *in vivo* studies through the formation of free radicals, opening of mitochondrial transition pore, promoting release of DA and α-synuclein besides inducing protein modifications (Burke *et al.*, 2008; Hashimoto and Yabe-Nishimura, 2002; Kristal *et al.*, 2001; Li *et al.*, 2001; Rees *et al.*, 2009; Panneton *et al.*, 2010).

In addition, non-enzymatic and spontaneous auto-oxidation of DA produces superoxide radicals and reactive quinones (Graham, 1978). Superoxide radicals are further converted to H_2O_2 by superoxide dismutase (SOD) or may react with nitric oxide radicals ($NO^{\cdot}$) to consequently generate peroxynitrite, which in turn can react with tyrosine residues and form 3-nitrotyrosine-modified proteins (Jenner, 2003). In addition to high levels of DA and DA-derived free radicals, SNpc dopaminergic neurons also contain neuromelanin and high concentrations of iron (Fe^{2+}). Moreover, an increase in the iron content in the SN region of humans brain has been observed with aging (Zecca *et al.*, 2004). Therefore, H_2O_2 can react with metals, especially iron, to form the most cytotoxic radical e.g. hydroxyl radicals via the Fenton and Haber-Weiss reactions, which further increase the oxidative stress in dopaminergic cells (Lotharius and Brundin 2002). Interestingly, the SN and striatum have lower levels of GSH relative to other regions of the brain, which include, in the increasing order: SN, striatum, hippocampus, cerebellum, and cortex (Abbott *et al.*, 1990; Kang *et al.*, 1999). In the SNpc of PD, the depletion of GSH is one of the earliest biochemical changes prior to selective loss of complex I activity and dopamine loss (Jenner, 1998; Riederer *et al.*, 1989). Secondly, the complex I inhibition with subsequent ATP depletion may affect GSH synthesis, as it is an ATP-dependent process (Dickinson and Forman, 2002; Pöltl *et al.*, 2012). Moreover, SNpc neurons have higher basal metabolic rate and mitochondrial

oxidative stress because of the activation of L-type calcium channels during spontaneous depolarization (Guzman *et al.*, 2010). Thus, the SNpc neurons have a high metabolic rate combined with a high content of oxidizable species, including DA, DA-derived ROS, neuromelanin, polyunsaturated fatty acids, iron, and a low content of antioxidants (glutathione in particular) all of which render this brain region highly vulnerable to oxidative and nitrosative stress (Marshall *et al.*, 1999).

4- Role of pesticides in PD

Reinforcing a potential role for complex I defects in PD, are the recent epidemiological studies positively linking PD with the pesticides paraquat and rotenone, which are known to increase oxidative stress and mitochondrial dysfunctions (Tanner *et al.*, 2011). Epidemiological studies have recently reported that exposure to several specific pesticides including dieldrin, maneb, paraquat, and rotenone correlates with increased incidence of PD (Ascherio *et al.*, 2006; Brown *et al.*, 2006; Dhillon *et al.*, 2008; Kamel *et al.*, 2007; Ritz *et al.*, 2009). The structural similarity between paraquat (1,1′-dimethyl-4,4′-bypyridinium), a common herbicide, and MPP^+, prompted speculation that paraquat might be a dopaminergic neurotoxicant and exposure to paraquat may be related to the development of PD. Paraquat (PQ), is a bipyridyl compound widely used as herbicide and commonly used both in *in vitro* and *in vivo* models of PD (Cannon and Greenamyre, 2010) as shown in Fig. 13. Several epidemiological studies have identified PQ exposure as a potential risk factor for the onset of PD (Costello *et al.*, 2009; Hatcher *et al.*, 2008; Tanner *et al.*, 2011). PQ was first produced in 1961 and gained considerable attention because of its extreme toxicity in human and its systemic exposure has been shown to be able to cross the blood-brain-barrier through the neutral amino acid transporter and to selectively damage the nigrostriatal

dopaminergic system in mice causing a 20%–30% selective dopamine neuronal loss in the SNpc (McCormack and Di Monte, 2003).

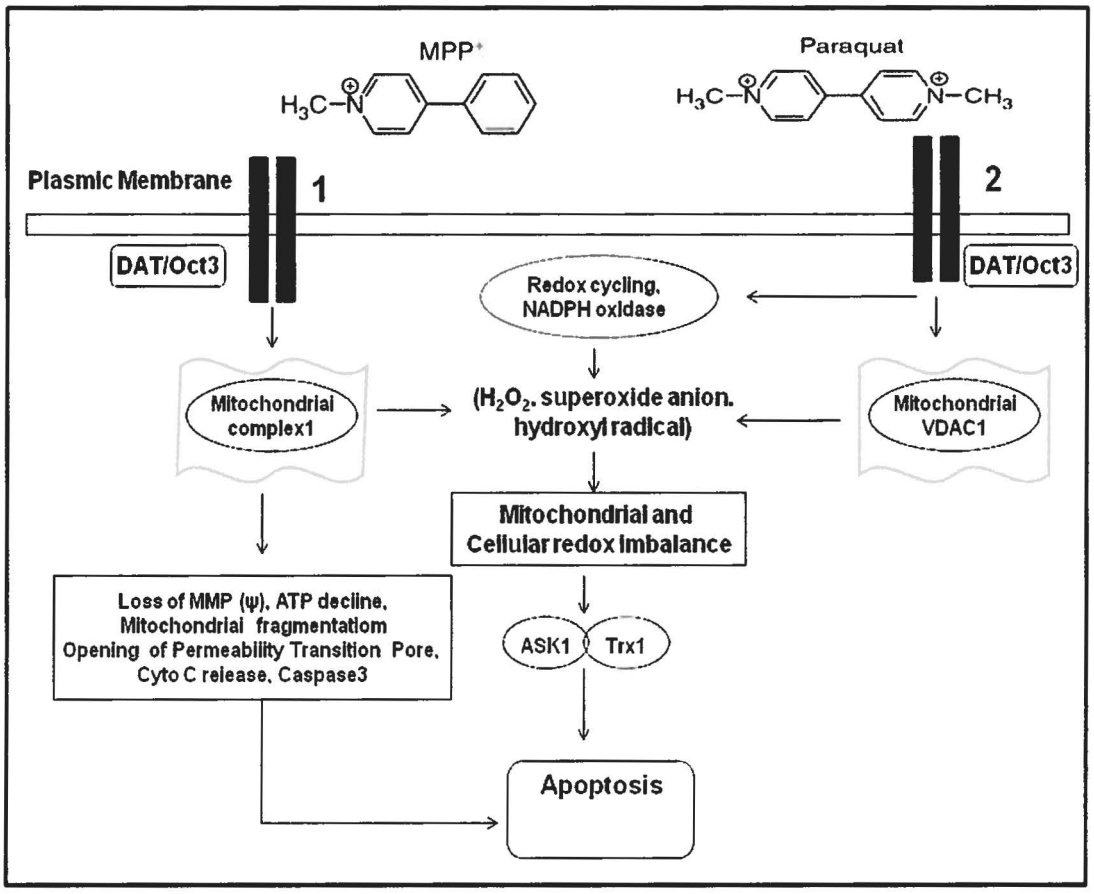

Fig. 13 Mechanisms of toxicity of MPP^+ and Paraquat in toxins based models of PD. The dopaminergic cells take up both MPP^+ and Paraquat from extracellular space through DAT/Oct3 transporters. Both mitochondrial dysfunctions and ROS are responsible for their toxicity. MPP^+ is a potent inhibitor of mitochondrial complex1 activity and induces a series of downstream events ultimately leading to cell death. On the other hand Paraquat generates ROS both in mitochondrial and cytoplasmic compartments through different mechanisms leading to oxidative stress induced cell death.

Although exact mechanisms by which PQ induces PD in animal models are still unknown, several studies have shown that chronic exposure is associated with the production of oxidative stress and inflammatory cascade which may be involved in the degeneration of nigral dopaminergic neurons (Kuter *et al.*, 2010; McCormack *et al.*, 2002). PQ cellular uptake is accomplished through the dopamine transporter (DAT) and cellular toxicity is determined by the $PQ(^{2+})/PQ(^{+})$ redox cycling, a process in which it accepts an electron from an appropriate donor and subsequently reduces O_2 to produce superoxide anion radical ($O_2^{\cdot-}$) (Bonneh-Barkay *et al.*, 2005; Drechsel and Patel, 2009; Mangano *et al.*, 2011; Purisai *et al.*, 2007; Rappold *et al.*, 2011; Wu *et al.*, 2005). PQ exposure in rats and mice increased mitochondrial H_2O_2 production, depolarized mitochondria, induced mitochondrial oxidative damage, and inactivated the mitochondrial iron sulfur (Fe-S) containing proteins such as aconitase (Cantu *et al.*, 2009, 2011; Chen *et al.*, 2012; Czerniczyniec *et al.*, 2011). Recent studies have identified different enzymes such as NADPH oxidase (Nox1) and mitochondrial Voltage-dependent anion channel 1 (VDAC1) responsible for the generation of superoxide anion (Cristovao *et al.*, 2009; Shimada *et al.*, 2010). Additionally, several redox regulated proteins such as thioredoxin-1 (Trx1), mitochondrial thioredoxin-2 (Trx2) and peroxiredoxin-3 have been implicated in PQ-induced toxicity (Niso-Santano *et al.*, 2010; Ramachandiran *et al.*, 2007; Roede *et al.*, 2011). Thioredoxin/peroxiredoxin systems constitute the main enzymes involved in the GSH homeostasis and H_2O_2 degradation in mitochondria (Drechsel and Patel, 2010). Activation of the c-Jun N-terminal kinase (JNK) and p38 mitogen-activated protein kinase (MAPK) has been observed in PD models and appears to be critical in mediating DA cell death (Choi *et al.*, 2004, 2010; Karunakaran *et al.*, 2008; Klintworth *et al.*, 2007). While the

precise upstream mechanism leading to JNK and p38 activation in DA neurons is not known, PQ and MPTP induced activation of apoptosis signal-regulating kinase (ASK1) is emerging as a potential key player (Karunakaran *et al.*, 2007; Niso-Santano *et al.*, 2010). Therefore, modulation of oxidative stress and mitochondrial dysfunctions by natural antioxidant may mitigate MPP^+ and PQ-induced toxicity and promote cell survival and may be of therapeutic use as neuroprotectants in PD.

5- Redox dysregulation of transcription factors in PD

In past years several reports have highlighted the protective role of Nrf2 in PD. For instance, in SNpc of PD, it has been shown that neuromelanin-containing neurons exhibited strong nuclear Nrf2 staining (Ramsey *et al.*, 2007). Consequently, the expression of Nrf2 regulated protein heme oxygenase-1 (HO-1) and GST also increased in the SNpc neurons containing LBs and synaptosomal fraction from the brain tissue from PD patients (Schipper *et al.*, 1998; Shi *et al.*, 2009). Recently decaffeinated coffee and nicotine-free tobacco extracts conferred neuroprotection in Drosophila models of PD through a Nrf2-dependent mechanism (Trinh *et al.*, 2010). Nrf2 deficiency in mice increased MPTP toxicity whereas Nrf2 overexpression protected them from neuronal death induced by MPTP. Besides various markers of neuroinflammation such as COX-2, iNOS, interleukin-6 (IL-6), and TNF-alpha were also increased in Nrf2- knockout mice (Chen *et al.*, 2009; Rojo *et al.*, 2010). Activation of Nrf2/thioredoxin1 (Trx-1) axis also protected cells against PQ-induced toxicity (Niso-Santano *et al.*, 2010). Trx-1 can inhibit the activation of ASK1 in a redox-dependent manner (Niso-Santano *et al.*, 2010). Additionally, AKT activation in addition to having trophic effect also protected dopaminergic neurons through suppression of macroautophagy (Cheng *et al.*, 2011; Chung *et al.*, 2011; Ries *et al.*, 2006). Cytoplasmic

hybrid (cybrid) cell lines containing mitochondria from PD subjects had reduced Sirt1 phosphorylation, PGC-1α activation along with an increased NF-κB activity (Esteves *et al.*, 2010). Activated NF-κB has been detected in dopamineric cells in the brain of PD patients respectively (Hunot et al., 1997; Kaltschmidt *et al.*, 1997). MPP^+ has also been shown to activate NF-κB in glial cells and in the brains of MPTP intoxicated mice. Selective inhibition of NF-κB in mice by NBD (NEMO-binding domain) peptides and simvastatin, protected dopaminergic neurons from the MPTP induced toxicity (Ghosh *et al.*, 2007, 2009). Moreover, the SNpc neurons from PD patients have abnormal phospho-ERK accumulations in the mitochondria and autophagosomes in LBs (Zhu *et al.*, 2003). Mitochondria-localized ERKs have been shown to regulate mitophagy and autophagic cell stress response and are involved in the 6-OHDA and MPP^+ induced cell death through autophagy (Dagda *et al.*, 2008 Kulich *et al.*, 2007; Zhu *et al.*, 2007). Phospho ERKs also mediated cell death induced by glutathione depletion in dopaminergic neurons and dopamine toxicity in striatal neurons (Chen *et al.*, 2004; Chen *et al.*, 2009; de Bernardo *et al.*, 2004). Moreover, recent studies have linked mutations in the LRRK2 gene with enhanced basal autophagy that in turn triggered cell death via ERK pathway (Bravo-San Pedro *et al.*, 2012). In PINK1 deficient cell lines phosphorylation of p66Shc at Ser36 is significantly increased under normal tissue culture conditions, with a further increase during oxidative stress (Maj *et al.*, 2010). Thus modulation of ERK and p66Shc pathways by natural antioxidants could provide an important arsenal to ameliorate mitochondrial dysfunctions, autophagy and oxidative stress associated with PD.

VI- Neuromodulatory and neuroprotective effects of medicinal plants

Herbs and spices have a long history of both culinary and medicinal uses in different cultures e.g. ancient Egyptian, Sumerians, Greece, Roman, Chinese and Indians. Demonstrating the benefits of herbs and foods by scientific means remain a challenge, particularly when compared with standards applied for pharmaceutical agents. Recent decades have seen an increased use of herbal preparations in western and American societies for psychological states and physical health. Depression and anxiety are two of the most common mental disorders, affecting millions of people worldwide with nearly 55 millions in the United States alone, affecting approximately 18% of the population (Kessler *et al.*, 2005; Somers *et al.*, 2006). Some of the medicinal plants such as *Valeriana officinalis*, or valerian, primarily used to treat insomnia have shown anxiolytic effects in rodents and sedative properties in humans (Donath *et al.*, 2000; Murphy *et al.*, 2010). *Hypericum perforatum* (St. John's Wort) has been approved for use as a sedative and antidepressants by the German Commission E (an expert committee commissioned by the German government in 1978 to evaluate the herbal drugs and preparations from medicinal plants) (Linde *et al.*, 2009). *Passiflora incarnata*, or passionflower, and other species of *Passiflora* genus are widely used all over America and in European countries for their apparent sedative and anxiolytic properties (Dhawan *et al.*, 2001). Since in addition to memory loss, depression is one of the most frequent behavioral symptoms in AD (Aboukhatwa *et al.*, 2010; Modrego, 2010), thus, there is potential for the development of new *Hypericum*, Kava-kava and passion flower extracts and new chemical constituents from these plants as novel antidepressants in rational complimentary strategy for the treatment of AD patients with depression comorbidity.

More recently, herbs from traditional Chinese and Indian medicine systems, such as *Ginkgo biloba*, turmeric (*Curcuma longa*), ginseng (*Panax ginseng*), *Withania somnifera* and *Bacopa monniera* have been advocated for reputed beneficial effects on cognitive processes (Howes and Houghton, 2003; Kumar, 2006; Ramassamy *et al.*, 2007). Although the individual components responsible for the bioactive properties remains to be identified, polyphenols and triterpenoid saponins are the key ingredients in traditional Chinese medicine (TCM) responsible for the most of the observed biological effects (Liu and Henkel, 2002). A total of 86% herbs which are widely used in the TCM were found to contain polyphenols and/ or triterpenoid saponins in significant detectable amounts (Liu and Henkel, 2002). Additionally the herbs used in eight more oftenly used TCM herbal formulae were explicitly rich in polyphenols and/ or triterpenoid saponins (Liu and Henkel, 2002). Polyphenols constitute one of the most numerous and ubiquitous groups of plant metabolites ranging from the simple phenolic molecules to highly polymerised compounds like condensed tannins and their occurrence in fruits, vegetables, herbs and spices is variable. In the past 10 years, research on the neuroprotective effects of dietary polyphenols has developed considerably. These compounds are able to protect neuronal cells in various *in vitro* and *in vivo* models of neurodegenerative diseases through different intracellular targets (Ramassamy *et al.*, 2007; Singh *et al.*, 2008).

Terpenes are the largest and most diverse group of natural products, with more than 50,000 known structures (Conolly and Hill, 1991). Terpenes are classified into several groups according to the number of carbons they contain; the major groups are monoterpenes (C10), sesquiterpenes (C15), diterpenes (C20), triterpenes (C30) and tetraterpenes/ carotenoids (C40) (Kirby and Keasling, 2009) as shown in Table 2. Despite their structural

diversity, all terpenes have a simple unifying feature of isoprene units (C5H8) and are derived from isopentenyl pyrophosphate (IPP) and dimethylallyl pyrophosphate (DMAPP) and their synthesis requires specific enzymes called terpene synthases (TPS) (Kirby and Keasling, 2009).

Table 2 Some examples of terpenoid compounds.

Class	Compounds
Monoterpenes	menthol, 1-8-cineole, geraniol, linalool, genipin, α-pinene
Sesquiterpenes	valernic acid, β-caryophyllene, gossypol, artemisinin, parthenolide
Diterpenes	ginkgolides, taxol, forskolin, kahweol, cafestol, carnosol
Triterpenes	ginsenosides, celastrol, avicins, betulic acid, ursolic acid, lupeol
Tetraterpenes/carotenoids	lycopene, lutein, astaxanthin, β-carotene, zeaxanthin

More than 20,000 triterpenoids exist in nature (Phillips *et al.*, 2006), including in a large variety of vegetarian foods and medicinal herbs, and they have been widely used in Indian and Chinese traditional medicines to treat various ailments due to their anti-inflammatory activity (Liu, 1995). These pentacyclic triterpenes are further categorized into lupane, oleanane and ursane groups. Avicins, a group of electrophilic pentacyclic triterpenoids and oleanolic acid have been shown to possess potent antioxidant properties by upregulating the Nrf2-regulated antioxidant enzymes (Haridas *et al.*, 2004; Reisman *et al.*, 2009). Terpenoids compounds both synthetic and natural ones are known to activate the Nrf2/ARE pathways by forming a Michael adduct with reactive cysteine residues on Keap1 protein (Dinkova-Kostova *et al.*, 2005). In addition to human cells and animals, terpenoids have been shown to modulate the intracellular redox homeostasis in microorganisms especially in bactetia by activating the bacterial redox-responsive transcriptional activator, OxyR,

thereby protecting them against oxidative and nitrosative stress (Bosak and Losick 2008; Haridas *et al.*, 2004). Several triterpenoids saponins, including ursolic and oleanolic acid, betulinic acid, celastrol, lupeol, and the triterpenoids of ginsenosides, have been shown to possess anti-inflammatory activities by inhibiting NF-κB activation (Salminen *et al.*, 2008). Celastrol, a tritrepenoid extracted from *Tripterygium wilfordii Hook* inhibited the production of NO and proinflammatory cytokines in lipopolysaccharide stimulated mice BV-2 microglia cells (Jung *et al.*, 2007) besides reducing the Aβ peptide plaque burden and microglia activation in a transgenic mouse model of AD (Paris *et al.*, 2010). Celastrol is also known to induce various heat shock proteins (HSPs) in human cells by activating the heat-shock transcription factor 1 (HSF1) (Westerheide *et al.*, 2004; Mu *et al.*, 2008). Sominone, the active metabolite of Withanoside IV have shown neuroprotective effect against Aβ peptide and induced the phosphorylation of RET (rearranged during transfection), a receptor for the glial cell-line induced neurotropic factor (GDNF) (Kuboyama *et al.*, 2006; Tohda and Joyashiki 2009). WithanolideA downregulated BACE1 and enhanced the activity of α-secretase ADAM10 in primary rat cortical neurons (Patil *et al.*, 2010). In chronic stress and senescence-accelerated mouse prone 8 (SAMP8) mouse model of aging, Ginsenoside Rg1 decreased cerebral Aβ content, improved cognitive performance, induced neurogenesis and increased the levels of brain derived neurotrophic factor (BDNF) (Shi *et al.*, 2010). Thus triterpenoid saponins by modulating the Nrf2, NF-κB, HSPs, neurotrophic factors, memory enhancing and neurogenesis pathways which are altered in aging and neurodegenerative diseases may be helpful in ameliorating the pathophysiological mechanisms involved in the progression of AD and PD.

VII- Nootropic medicinal plants in the Indian Systems of Traditional Medicine (ISTM)

Ayurveda is a Sanskrit word, which means "the scripture for longevity" represents an ancient system of traditional medicine prevalent in India is based on a holistic view of treatment through establishment and promoting of an equilibrium in the different elements of human life, the body, the mind, the intellect and the soul (Bhatt, 2001). Ayurveda dates back to about 3000 B.C., the period of the Indus Valley civilization and has been passed on through generations of oral tradition (Lodha and Bagga, 2000; Subbarayappa, 2001). Ayurveda encompass eight different subspecialties of medical treatment, named Ashtanga, which included surgery, internal medicine, pediatrics, toxicology, health and longevity, and spiritual healing. Ayurvedic medicines are mainly composed of herbal preparations to rejuvenate different body tissues which are called as Rasyanas, claimed to act as micronutrients. In the Ayurvedic system, the herbs used for promoting brain health to promote antistress, adaptogenic, memory enhancing effects, preventing cognitive deficits and improving brain functions are called as "Medha Rasyanas" ("medhya"=intellect or cognition, and 'rasayana'= rejuvenation in Sanskrit) (Gohil *et al.*, 2010; Kumar, 2006; Singh *et al.*, 2008). Among the most popular plants often used as Medha Rasyanas, in the descending order of importance are: (a) Ashwagandha, (b) Brahmi, (c) Jatamansi, (d) Jyotishmati, (e) Mandukparni, (f) Shankhapushpi, and (g) Vacha (Ven Murthy, *et al.*, 2010). The scientific investigations concerning the different biological activities and different chemical composition of the most extensively investigated of these herbs, and Brahmi will further be discussed in details in this literature review.

1- *Bacopa monniera* (*BM*) or *Brahmi*

The name Brahmi is derived from the word "Brahma", the "supreme creator' according to the Hindu mythology of India and accordingly, since the brain is considered as the central organ for intellectual and creative activities, Brahmi also means "bringing knowledge of the Supreme Reality". *BM* has been used by Ayurvedic physicians in India for almost 3000 years and is classified as a "medhyarasayana" and mentioned in ancient Ayurvedic texts including the Charaka Samhita (6th century AD), as a brain tonic in the management and restorative in certain mental conditions including anxiety, poor cognition and lack of concentration (Gohil *et al.*, 2010; Russo and Borrelli, 2005; Singh *et al.*, 2008).

Extensive research has been carried out to determine the composition of *BM* since 1931, when Bose and Bose isolated an alkaloid, which they named as "brahmine" (Russo and Borrelli, 2005). Following these studies the presence of other alkaloids like nicotine and herpestine from *BM* has been reported (Chopra *et al.*, 1956). The presence of D-mannitol, potassium salts and a saponin hersaponin has also been reported from the *BM.* Since then, several active ingredients have been characterized among which the characteristic saponins termed as "*Bacosides*" are the major constituents. *Bacosides* represent a complex mixture of structurally closely related compounds, (dammarane type triterpenoid Saponins) with jujubogenin or psuedojujubogenin moieties as aglycones (Murthy *et al.*, 2006) and the pharmacological effects of *BM* are mainly attributed to these saponins especially *Bacoside A* and *Bacoside B*, which are therefore, considered as bioactive marker compounds of this species (Deepak and Amit, 2004) as shown in Fig. 14. The composition of *Bacoside A* and *Bacoside B* have been established very recently as a mixture of four triglycosidic and four diglycosidic saponins, respectively (Deepak *et al.*, 2005; Sivaramakrishna *et al.*, 2005). But

the major bioactive putative component mainly responsible for its neuropharmacological effects is *Bacoside A*, which is a mixture of three major components, *Bacoside* A_3 ($C_{47}H_{76}O_{18}$), *Bacopaside II* ($C_{47}H_{76}O_{18}$) and *Bacosaponin C* ($C_{46}H_{74}O_{17}$) and isomer of *Bacosaponin C* (Deepak *et al.*, 2005). *BM* plant extract and active ingredients i.e. *Bacosides*, triterpenoid saponins have been extensively investigated in several laboratories for their neuropharmacolgical effects and a number of reports are available confirming their neuroprotective, cognitive enhancing and nootropic action. *BM* extract has been shown to be well tolerated without side effects (Russo and Borrelli, 2005) and the LD50 in rats was determined to be as high as 2.7 g/kg when administrated orally (Hota *et al.*, 2009).

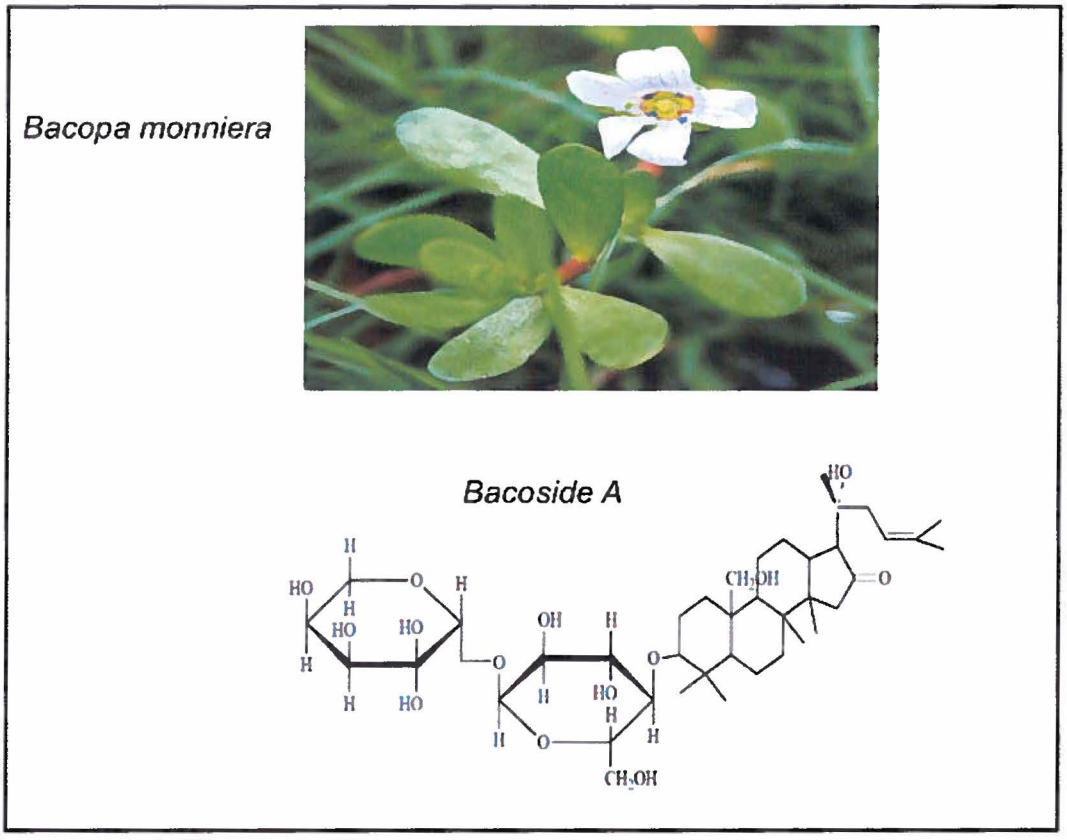

Fig. 14 *Bacopa monniera* plant and chemical structure of *Bacoside A*.

The neuroprotective effects of *BM* extract have been tested against various toxicants including glutamate, aluminium, Aβ peptide, NO, 3-nitropropionic acid (3-NPA), paraquat and rotenone and the antioxidant activities of *BM* underlie and contribute significantly to its neuroprotective functions. *BM* extract protected *Drosophila melanogaster* (fruitfly) against paraquat and rotenone induced toxicity model of PD through modulation of oxidative stress (Hosamani and Muralidhara, 2009; Hosamani and Muralidhara, 2010). Additionally, *BM* extract has also been reported to protect dopaminergic N27 cells and mice against rotenone and 3-NPA induced toxicity and decreased various markers of i.e. malondialdehyde, ROS levels, protein carbonyl content and hydroperoxides, restored GSH levels (Shinomol *et al.*, 2011 a, b). Furthermore, *BM* treatment also restored the levels of cytosolic antioxidant enzymes, neurotransmitter function, and dopamine levels in Striatum (Shinomol *et al.*, 2011). *BM* extract has been shown to protect the primary cortical neurons aginst Aβ peptide and glutamate-induced neurotoxicity besides reducing the amyloid load in a transgenic mouse model of AD e.g. in PSAPP mice (Hocomb *et al.*, 2006; Limpeanchob *et al.*, 2008). *BM* extract (40 or 160 mg/kg/day) administered at 2 months of age for either 2 or 8 months lowered Aβ1-40 peptide and Aβ1-42 peptide levels in cortex by as much as 60% besides retaining memory and cognition (Hocomb *et al.*, 2006). *BM* extract also inhibited aluminium (Al) induced toxicity in cerebral cortex in rats and *BM* extract (40mg/kg/day) significantly decreased the levels of lipid peroxidation products such as MDA, 4-HNE and protein carbonyl content and elevated the levels of different antioxidant enzymes e.g. SOD, GPx, GST and increased the levels of GSH in cerebral cortex (Jyoti et al., 2007). *BM* extract (40 mg/kg, orally) for 21 days also protected mice against Methyl mercury (MeHg) induced neurotoxicity by preserving the activities of SOD, catalase, GPx besides increasing

the GR activity in cerebellum (Sumathi *et al.*, 2012). Some epidemiological investigations suggested an association between chronic exposure to some metals such as Al, mercury and risk of AD development (Frisardi *et al.*, 2010b; Fujimura *et al.*, 2009; Mutter *et al.*, 2010). Methanolic extract of *BM* also scavenged NO and reduced S-nitroso-N-acetyl-penicillamine (SNAP) induced intracellular oxidants and DNA damage in astrocytes (Russo *et al.*, 2003b). *BM* extract also prevented restraint stress induced changes in the plasma coticosterone and brain monoamines in cerebral cortex and hippocampus in rats (Sheikh *et al.*, 2007). Elevated plasma corticosterone levels and altered monoamines expression has been observed in AD and corticosterone has been shown to enhance kainic acid (KA)-induced toxicity of cortical neurons by inducing mitochondtrial dysfunctions (Du *et al.*, 2009) and increasing Aβ peptide load in a mouse model of AD (Dong and Csernansky, 2009; Kepe *et al.*, 2006; Mohler *et al.*, 2011). In addition, administration of the *BM* extract and *Bacoside A* increased mitochondrial bioenergetic activities and prevented structural and functional impairments of mitochondria along with preservation of mitochondrial dehydrogenases in brain exposed to cigarette smoke and morphine (Sumathy *et al.*, 2002; Anbarasi *et al.*, 2005). Oral administration of *Bacosides* in rats also protected against age related chronic neuroinflammation by significantly decreasing the levels of pro-inflammatory cytokines and iNOS protein expression in middle aged and aged rat brain cortex (Rastogi *et al.*, 2012). Thus, anti-stress, anti-inflammatory, antioxidant and mitochondrial modulatory activities of *BM* may contribute to its neuroprotective functions in various paradigms of toxicity.

BM plant extract and *Bacosides* have also been investigated in several animal models and clinical trials for confirming their cognitive, memory enhancing and nootropic actions.

Bacosides A and *B* have been reported to facilitate learning and memory in normal rats, inhibited the amnesic effects of scopolamine, electroshock and immobilization stress (Dhawan and Singh, 1996). Further, *BM* has been shown to enhance protein kinase activity in the hippocampus may contribute to its cognitive enhancing effects (Singh and Dhawan, 1997). Recently, the standardized extract of *BM* has been found to reverse the cognitive deficits induced by hypobaric hypoxia in rats together with decreasing oxidative stress, plasma corticosterone levels and neuronal degeneration, and increased cytochrome c oxidase activity with a concomitant increase in ATP levels (Hota *et al.*, 2009). *BM* extarct also reversed the scopolamine and benzodiazepines-induced amnesia by significantly improving calmodulin and by partially attenuating activation of MAPK, phosphorylated CREB (pCREB), iNOS, and protein kinase C (PKC) pathways (Saraf *et al.*, 2008; 2010). Overactivation of PKC in prefrontal cortex with aging has been shown to result in working memory impairments and PKC inhibitors may be useful in the treatment of cognitive deficits in the elderly (Birnbaum *et al.*, 2004; Brennan *et al.*, 2009; Hains *et al.*, 2009). Additionally, in normal rats *BM* extract increased the expression of serotonin synthesizing enzyme tryptophan hydroxylase-2 (TPH2) and serotonin transporter (SERT) thereby enhancing learning and memory (Cirrito *et al.*, 2011). Serotonin signaling has also been shown to lower Aβ peptide levels and plaques in a transgenic mouse model of AD (Charles *et al.*, 2011). Various clinical studies in human have been performed to establish the efficacy of *BM* in memory functions and improved memory acquisition and retention in healthy older people has been reported. In four different randomized, double-blind, placebo-controlled trials in healthy older persons (age: > 55 years; n=98), (age: 40-65 y; n=76), chronic administration of *BM* extract (dose 300 mg/ day) significantly improved

memory acquisition, improved speed of visual information processing and memory retention after 12 weeks and 90 days of treatment (Morgan and Stevens, 2010; Roodenrys *et al.*, 2002; Stough *et al.*, 2001, 2008).

The current available drugs (acetylcholinesterase inhibitors) and L-3,4-dihydroxyphenylalanine (L-DOPA) along with the dopamine (DA) agonists for AD and PD respectively, only provide symptomatic relief and do not prevent the progression of these diseases. Oxidative stress, mitochondrial dysfunctions, neuroinflammation and abnormal protein accumulation in the brain are believed to play a crucial role in the development and pathopysiology of AD and PD (Hirsch and Hunot, 2009; Hensley *et al.*, 2010; Lin and Beal, 2006; Yong *et al.*, 2010). Since, multi-factorial etiopathological events have been recognized in AD and PD, they will require pleiotropic interventions to address the varied pathological aspects (Frautschy and Cole, 2010; Geldenhuys *et al.*, 2011). The botanical extracts containing multiple classes of chemical entities with synergic property may hold a better promise for therapeutic benefits and applicability in neuroprotection as compared to single chemical entity. Moreover, under the new Food and Drug Administration (FDA) guidelines standardized botanical extracts can be approved as drugs (Schmidt *et al.*, 2007). Hence, several of triterpenoid saponins possessing pleotropic pharmacological and biological activities that mediate and underlie the neuroprotective and nootropic effects of the *BM* extract may be further useful in the therapeutic management of AD and PD.

VIII- Hypothesis and Research Objectives

Alzheimer's disease (AD) and Parkinsons's disease (PD) are two of the leading neurodegenerative diseases affecting a vast number of elderly people in advanced and developing contries. Although both AD and PD are known to affect specific cell populations, there is strong evidence showing that both the diseases share some common pathogenic mechanisms, including oxidative stress, mitochondrial dysfunctions and neuronal cell death. Importantly, these mechanisms are closely interlinked. Oxidized proteins and lipids are significantly increased in AD and PD patients. In addition to oxidative modification of macromolecules; dysregulation of various redox signaling pathways such as the sirtuins, nuclear factor-kappaB (NF-κB), phosphatidylinositol3-phosphate kinase (PI3-K)-AKT, nuclear erythroid 2-related factor 2 (Nrf2), mitogen activated protein kinase (MAPK) and p66Shc may lead to neuronal dysfunctions and, ultimately, in neuronal death. A detailed understanding of the multiple pathophysiological mechanisms involved in AD and PD progression is a key step towards the development of therapeutic approaches to prevent and treat these neurodegenerative diseases.

Number of *in vivo* and *in vitro* laboratory studies have shown that polyphenolic antioxidants from herbs, fruits and vegetable can protect neurons from oxidative stress-induced cell death by scavenging of free radicals and effects on various neuronal signaling pathways thereby tilting the balance towards cell survival in various animal and cell culture models of AD and PD (Singh *et al.*, 2008). Additionally, another class of phtyochemicals composed of triterpenoid saponins have been shown to modulate the Nrf2, NF-κB and HSPs pathways that may further mitigate oxidative stress, inflammatory cascade and the toxicity of different misfolded proteins involved in the pathophysiology of AD and PD.

Therefore, we proposed that the *Bacopa monniera* (*BM*) extact enriched in triterpenoid saponins *Bacosides* may protect neurons against oxidative stress, preserve mitochondrial functions, and favorably modulate various neuronal redox-signaling pathways leading to cell survival. *BM* extact and *Bacosides* may be useful in the management of neurodegenerative disease like AD and PD. Therefore, we investigated the neuroprotective mechanisms of Indian medicinal plant *BM* in *in vitro* models of AD and PD using various toxins with the following specific objectives:

1. Study whether *Bacopa monniera* (*BM*) can protect against H_2O_2, acrolein, PQ and MPP^+ induced neuronal cell death.

2. Examine the effects of *Bacopa monniera* (*BM*) on the cellular antioxidant status, intracellular reactive oxygen species (ROS) and superoxide levels, and H_2O_2 scavenging activity.

3. To further assess the ability of the *BM* extract to modulate different cell signaling pathways, particularly on the Sirtuins, nuclear factor-kappaB (NF-κB), nuclear erythroid 2-related factor 2 (Nrf2) and phase two antioxidant enzymes, phosphatidylinositol-3-phosphate kinase (PI3-K)-AKT, mitogen activated protein kinases (MAPKs) and p66Shc.

4. Examine the effects of *Bacopa monniera* (*BM*) on the mitochondrial functions against various toxins.

Modulation of hydrogen peroxide and acrolein-induced oxidative stress, mitochondrial dysfunctions and redox regulated pathways by the *Bacopa monniera* extract: potential implication in Alzheimer's disease.

Manjeet Singh[a], Ven Murthy[b], and Charles Ramassamy[a,b*]

a : INRS- Institut Armand Frappier, 531, boul. des Prairies, Laval, Québec, H7V 1B7

b: Department of Medical Biology, Faculty of Medicine, Laval University, Québec, Canada

***Corresponding author**: Prof. Charles Ramassamy

Address: INRS- Institut Armand Frappier

531, boul. des Prairies, Laval, Québec, H7V 1B7

Phone: +001-450-687-5010

Fax: +001-450-687-5510

E-mail: Charles.Ramassamy@iaf.inrs.ca

Keywords: Alzheimer's disease, *Bacopa monniera*, mitochondria, oxidative stress, redox regulation.

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Scientific objectives of the article 1

Alzheimer's disease (AD) is the most common age-related neurodegenerative disorder, affecting millions of people worldwide. Aggregation of Aβ peptide both in soluble and insoluble forms in the brain is likely a key initiation factor in AD. Aβ peptide toxicity is mediated through a variety of downstream pathways including the generation of hydrogen peroxide (H_2O_2), lipid peroxidation products and mitochondrial dysfunctions. Acrolein is one of the most reactive by-products of lipid peroxidation and its levels are significantly higher in vulnerable regions in brains of AD patients. The toxic actions of H_2O_2 in neuronal cells include oxidative modifications of macromolecules such as proteins, lipids, and DNA, as observed in AD. *Bacopa monniera* (*BM*) has a long history of use in India as a memory-enhancing therapy. However, in spite of widespread use of *BM* extract as a brain tonic, the detailed neuroprotective mechanisms against H_2O_2 and acrolein-induced toxicity remains to be investigated. In this article, we demonstrated the neuroprotective effects of the standardized extracts of *BM* against H_2O_2 and acrolein-induced toxicity in human neuroblastoma SK-N-SH cell line and further elucidated the mechanisms underlying this protection.

We showed that a pre-treatment with the *BM* extract protected the SK-N-SH cells H_2O_2 and acrolein-induced toxicity in various cell survival assays, significantly inhibited the generation of intracellular reactive oxygen species (ROS) levels and preserved the mitochondrial membrane potential in SK-N-SH cells. *BM* pre-treatment also prevented the modifications of the activity of several redox-regulated proteins, i.e., NF-κB, Sirt1, ERK1/2, and p66Shc. *BM* extract could have a therapeutic application in the prevention of AD by modulating oxidative stress and mitochondrial dysfunctions, which are involved in the pathophysiology of AD.

Contribution of student

This article has been published in the "Journal of Alzheimer's Disease". All the experimental work, data analyses and the first draft of this article were accomplished by **Manjeet Singh** with substantial inputs from Prof. Charles Ramassamy, my current PhD supervisor. Manjeet Singh submitted the article and both Manjeet Singh and Charles Ramassamy replied to the reviewer's comments.

Résumé de l'article en français

L'Acroléine est l'un des sous-produits de la peroxydation lipidique. Du fait de sa haute réactivité, ce n'est pas seulement un marqueur de la peroxydation lipidique mais il peut aussi être un bon indicateur du stress oxydatif par adduction cellulaire à des groupes nucléophiles. Les niveaux de l'acroléine sont significativement élevés dans certaines régions vulnérables du cerveau des patients atteints de la maladie d'Alzheimer (MA), et dans des cultures primaires d'hippocampe l'acroléine est encore plus toxique que le 4-hydroxyl-nonenal. La toxicité de l'amyloïde-β est médiée à travers la génération du peroxyde d'hydrogène (H_2O_2). L'action du H_2O_2 inclue des modifications oxydatives des protéines, des lipides et de l'ADN tel qu'observées chez les patients atteints de la MA. Le *Bacopa monniera (BM)* à une longue histoire d'utilisation en Inde comme agent améliorant la mémoire. L'objective de notre étude était de déterminer les effets d'extraits de *BM* standardisés contre l'acroléine et l'H_2O_2, et d'élucider les mécanismes sous-jacents à cette protection. Nos résultats montrent qu'un prétraitement avec des extraits de *BM* protège les cellules humaines de neuroblastomes, les SK-N-SH, contre l'H_2O_2 et l'acroléine. Nous avons démontré qu'un prétraitement de *BM* inhibe significativement la génération des espèces réactives intracellulaires et préservé le potentiel membranaire mitochondrial. Le prétraitement de *BM* peut également prévenir les modifications de l'activité de nombreuses protéines redox (NF-κB, Sirt1, ERK1/2, et p66Shc) dans le but de favoriser la survie cellulaire en réponse à un stress oxydatif. Donc, nos résultats démontrent que le *BM* protège les cellules SK-N-SH contre l'H_2O_2 et l'acroléine via différents mécanismes impliqués dans la physiopathologie de la MA, et qui il pourrait avoir des applications thérapeutiques dans la prévention de la MA.

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Modulation of Hydrogen Peroxide and Acrolein-Induced Oxidative Stress, Mitochondrial Dysfunctions and Redox Regulated Pathways by the *Bacopa Monniera* Extract: Potential Implication in Alzheimer's Disease

Manjeet Singh[a], Ven Murthy[b] and Charles Ramassamy[a,b,*]
[a]*INRS-Institut Armand Frappier, Laval, PQ, Canada*
[b]*Department of Medical Biology, Faculty of Medicine, Laval University, PQ, Canada*

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Abstract. Acrolein is one of the by-products of lipid peroxidation. Due to its high reactivity, it is not only a marker of lipid peroxidation but could also be an initiator of oxidative stress by adducting cellular nucleophilic groups. In brains of Alzheimer's disease (AD) patients, levels of acrolein are significantly higher in vulnerable brain region and, on primary hippocampal culture, it is more toxic than 4-hydroxyl-nonenal. The toxicity of the amyloid-β peptide is mediated through the generation of hydrogen peroxide (H_2O_2). The actions of H_2O_2 include oxidative modifications of proteins, lipids, and DNA as observed in AD. *Bacopa monniera* (*BM*) has a long history of use in India as a memory-enhancing therapy. The objective of our study was to investigate the neuroprotective effects of the standardized extracts of *BM* against acrolein and H_2O_2 and to elucidate the mechanisms underlying this protection. Our results show that a pre-treatment with the *BM* extract protected the human neuroblastoma cell line SK-N-SH against H_2O_2 and acrolein. We demonstrated that *BM* pre-treatment significantly inhibited the generation of intracellular reactive oxygen species in addition to preserving the mitochondrial membrane potential. *BM* pre-treatment also prevented the modifications of the activity of several redox regulated proteins, i.e., NF-κB, Sirt1, ERK1/2, and p66Shc, so as to favor cell survival in response to oxidative stress. Thus, our findings demonstrate that *BM* can protect human neuroblastoma cells against H_2O_2 and acrolein through different mechanisms involved in the pathophysiology of AD and could have a therapeutic application in the prevention of AD.

Keywords: Alzheimer's disease, *Bacopa monniera*, mitochondria, oxidative stress, redox regulation

INTRODUCTION

Alzheimer's disease (AD) is the most common age-related neurodegenerative disease affecting million of people worldwide. There is strong evidence supporting the role of mitochondrial dysfunction as well as oxidative stress in the pathophysiology of AD [1,2]. Reac-

*Correspondence to: Prof. Charles Ramassamy, INRS- Institut Armand Frappier, 531, boul. des Prairies, Laval, PQ, H7V 1B7, Canada. Tel.: +1 450 687 5010; Fax: +1 450 687 5510; E-mail: charles.ramassamy@iaf.inrs.ca.

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tive oxygen species (ROS)-induced changes in DNA, proteins, and polyunsaturated fatty acids have been reported in mild cognitive impairment (MCI), in early and late stages of AD patients [3–5], as well as in animal models with selected facets of AD [6]. Oxidation of polyunsaturated fatty acids generates highly electrophilic α,β-unsaturated carbonyl derivatives including acrolein ($CH_2 = CH\text{-}CHO$). In AD brain, levels of acrolein were found to be significantly higher in vulnerable brain regions such as hippocampus, amygdala, and middle temporal gyrus [7,8]. Moreover, the immunoreactivity of acrolein in neurofibrillary tangles and in dystrophic neurites surrounding senile plaques was 50% greater than in controls [9]. Acrolein/guanosine adducts in nuclear DNA were 2-fold higher in hippocampus from AD patients while it is not significantly different for 4-hydroxyl-nonenal (HNE)/guanosine adducts [10,11]. On primary hippocampal cultures, it has been shown that acrolein was toxic in a time- and concentration-dependent manner and its toxicity was higher than HNE at 5 μM concentration [7]. These results suggest that acrolein, due to its high reactivity, is not only a marker of lipid peroxidation but could be an important neurotoxic compound in AD. In addition to being a metabolic by-product of mitochondrial respiration, hydrogen peroxide (H_2O_2) can be formed by the amyloid-β peptide (Aβ) aggregation which would induce oxidative damage, particularly in the presence of metals where H_2O_2 would be converted, via Fenton's reaction, into the highly reactive hydroxyl radical. Metals have been observed at the sites of brain lesions in these diseases [12]. Also, there is mounting evidence indicating that the toxicity of Aβ is mediated through the generation of H_2O_2 [13,14]. In the brain of 3xTg-AD mice model, an increase production of H_2O_2 was found as early as 3 month of age in mitochondria [15]. Therefore, it has been suggested that H_2O_2 could be involved in the degeneration and loss of nerve cells in the brain of AD [16]. Collectively, these studies suggest that oxidative stress and mitochondrial dysfunctions induced by Aβ, H_2O_2, and by-products of lipid peroxidation may play an important role in the AD pathogenesis.

In addition to ROS induced-modifications of cellular macromolecules, there is a dysregulation of some redox regulated transcription factors in AD. Activated nuclear factor kappa B (NF-κB) is elevated in AD brain [17,18] while its inhibition in transgenic mice models of AD was neuroprotective [19,20]. The toxicity of Aβ peptide and H_2O_2 is involved the phosphorylation of the extracellular signal-regulated kinase 1/2 (ERK1/2) [21,22] and p66Shc protein [23], a redox enzyme which generates mitochondrial H_2O_2 [24]. Moreover sustained activation of the ERK1/2 plays an important role in the phosphorylation of the tau protein [25,26], which is a component of neurofibrillary tangles.

Incidence rates of the age-specific AD in rural India are at least three times lower than the age-matched American reference population [27], which could be attributed to genetic, environmental, or dietary causes. *Bacopa monniera* (*BM*) is one of the most important medicinal plants used in the Indian system of traditional medicine for enhancing memory and cognition [28,29]. It has been used as a nootropic agent and brain tonic to restore age-related decline in mental abilities. *BM* extract also protected against Aβ toxicity in primary rat cortical neurons [30]. Interestingly, in an AβPP-PS1 transgenic mice model, *BM* extract reduced Aβ peptide levels by 60% in hippocampus and cortex and improved Y-maze performance [31]. However, the mechanisms underlying its neuroprotective activity remain poorly investigated. Thus, the purpose of the present study was to investigate if a pre-treatment with the standardized extract of *BM* can protect human neuroblastoma cells against the H_2O_2 and acrolein-induced toxicity. Our results show that *BM*-induced neuroprotection is mediated through the suppression of intracellular ROS generation, improving mitochondrial functions, and modulating the expression of some redox regulated proteins, i.e., NF-κB, Sirt1, ERK1/2, and p66Shc, which are dysregulated during aging and in AD. In addition, our results suggest that *bacosides* are likely involved in the neuroprotective effect of *BM*.

MATERIALS AND METHODS

Dulbecco's minimal essential medium (DMEM), fetal bovine serum (FBS), Penicillin/streptomycin, L-Glutamine, H_2O_2, Tox-2 (XTT based), Tox-8 (Resazurin based), 2,4-dinitrophenylhydrazone (DNPH), DL-buthionine-(S,R)-sulfoximine (BSO), acrolein, phenazine methosulfate (PMS), and proteases inhibitor cocktail were obtained from Sigma-Aldrich Inc. The cytotoxicity detection kit, based on lactate dehydrogenase (LDH) was from Roche Diagnostics. Monochlorobimane (MCB) was from Fluka while JC-1 (5, 5', 6, 6'-tetracholro-1, 1', 3, 3'- tetraethylbenzimidazolyl carbocyamine iodide) and 2, 7 dichlorofluoroscein diacetate (DCF-DA) were from Invitrogen. Iodonitrotetrazolium chloride (INT) was from Serva

Electrophoresis Gmbh (Heidelberg, Germany). The nuclear protein extraction kit was obtained from Active Motif (California, USA) and BCA protein estimation kit was from Pierce Biotechnology (Rockford, USA).

Rabbit polyclonal anti-human Sirt1 antibody (eBioscience), rabbit polyclonal anti-NF-κB p65 antibody (Delta Biolabs), mice monoclonal anti-Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) antibody (Millipore), rabbit polyclonal antibody to total and phosphoERK1/2 (Cell Signaling), anti-rat monoclonal antibody to importin α3 (MBL, Japan), rabbit polyclonal antibody to Shc (Abcam), and mouse monoclonal antibody to phospho 66Shc (S36, Abcam) were used in western blot analysis. Mice monoclonal anti-β-actin, mice monoclonal anti-dinitrophenyl (DNP), and mice and rat horseradish peroxidase (HRP) conjugated secondary antibodies were obtained from Sigma Aldrich, Inc. Fluorescence emission with different probes was recorded using the Synergy HT multi-detection microplate reader.

Standardized extracts of Bacopa monniera

Standardized extracts of *BM* were kindly provided by Natural Remedies Private Limited, Bangalore, India. *BM* was certified to contain 11.5% of total *bacosides* (*bacoside A3*, *bacopaside II*, Jujubogenin isomer of *bacopasaponin C*, *bacopasaponin C*, *bacopaside I* and *bacosine*), *apigenin* (2.5%), *Luteolin* (1.2%), and *Sitosterol-D-glucoside* (2.0%) as determined by high performance thin layer chromatography (HPTLC). *BM2* extract contain 40% of *bacosides* with *bacoside A3* (9.2%), *bacopaside II* (8.6%), Jujubogenin isomer of *bacopasaponin C* (7.8%), *bacopasaponin C* (8.7%), *bacopaside I* (7.5%), *bacosine* (0.8%), *apigenin* (2.5%), *luteolin* (1.2%), and *sitosterol-D-glucoside* (2.0%).

Cell culture and treatment

SK-N-SH cells, a human neuroblastoma cell line from American Type Cell Culture (ATCC, Rockville, MD, USA), were maintained in DMEM supplemented with 10% (v/v) FBS, 100 U/ml penicillin, and 100 μg/ml streptomycin. This work was conducted using SK-N-SH cells because they display a neuronal phenotype with multiple neurochemical markers and have been shown to be damaged by Aβ peptide [32].

SK-N-SH cells were plated at a density of 2.0×10^4 cells/well in 96 well plates and incubated at 37°C. After 24 h of plating, cells were starved and treated with different doses of *BM* extracts for 3 h prior to the addition of 200 μM of H_2O_2 or 15 μM of acrolein. These doses were selected on the basis of dose-toxicity response curves and results in almost 50% of cell death after 24 h of treatment.

Cytotoxicity and cell viability assays

XTT assays measure the mitochondrial dehydrogenases activity using the sodium salt of (2, 3-bis [2-Methoxy-4-nitro-5-sulfophenyl]-2H-tetrazolium-5-carboxyanilide inner salt. XTT assay may reflect metabolic alterations and XTT-based test alone would lead to the underestimation of the number of living cells. Therefore, it has been suggested that a valuable approach would be to integrate this assay with another one, allowing for the crossconfirmation of the cytotoxicity and proliferation datasets [33]. XTT assay was thus completed with the Resazurin test. Resazurin is known to act as an intermediate electron acceptor in the electron transport chain between the final reduction of oxygen and cytochrome oxidase by substituting for molecular oxygen as an electron acceptor [34]. The deoxygenated product of resazurin, resorufin, exhibits strong emission at wavelengths greater than 550 nm. Resazurin was able to detect toxicity earlier than XTT [35]. To distinguish between toxicity and proliferation effects of the *BM* extracts, LDH release was measured per the manufacturer's instructions. The LDH assay measures the cellular membrane integrity and is a mean of quantifying dead cells.

For XTT and resazurin assays, the media was completely removed and cells were kept in fresh MEM without phenol red (100 μl/well) containing 20% XTT and 10% resazurin per the manufacturer's instructions. Values obtained from controls, untreated cells were considered as 100% and cell survival was expressed as a percentage of the control value.

Intracellular reactive oxygen species

Intracellular ROS generation was monitored by following the oxidation of 2',7'- dichlorofluorescein-diacetate (DCF-DA), a cell permeable dye which upon hydrolysis by intracellular esterases reacts with H_2O_2 to produce a highly fluorescent compound, 2',7'-dichlorofluorescein (DCF), which is trapped inside the cells. Briefly, after 3 h of treatment, DCF-DA was added to a final concentration of 10 μM for 20 min. Cells were then washed with PBS and treated with 1.0 mM of H_2O_2 and the fluorescence was recorded with λex at 485 nm and λem at 535 nm 1 h after the addition of H_2O_2.

Polarographic measurement of H_2O_2 degradation by BM

The antioxidant capacity of *BM* was analyzed through the H_2O_2 detection with a 100 μM carbon sensor connected to the Free Radical Analyzer Apollo 4000 (WPI, Sarasota, FL). The analysis was conducted in 5.0 ml of PBS buffer with continuous stirring at room temperature. Once the output signal stabilized in PBS, different concentrations of H_2O_2 were added. The output signal was allowed to stabilize subsequent to each addition. Concentrations of H_2O_2 were plotted against the corresponding pA intensity and a standard curve was generated. In the presence of a compound that could degrade H_2O_2, the slope obtained for the standard curve will decrease. All the experiments were performed at least in triplicate on the same day.

Intracellular reduced glutathione (GSH) level

MCB, a cell permeable fluorescent dye, enters cells and forms a highly intracellular fluorescence adduct with GSH which can be estimated. After different treatments, the medium was removed and cells were kept in a MCB solution (100 μM) for 30 min. After washing cells with PBS, the fluorescence was recorded with λex at 360 nm and λem at 480 nm.

Total mitochondrial NADH dehydrogenases activity

Total mitochondrial NADH dehydrogenases activity in intact cells was measured by using NADH-tetrazolium based assay [36]. After 24 h of plating, cells were starved and kept in DMEM without phenol red. Cells were treated with different doses of *BM* for 3 h along with 100 μM each of iodonitrotetrazolium (INT) and PMS. INT is a NADH dehydrogenases substrate and PMS is an intermediate electron carrier used to enhance the sensitivity of the reaction. INT is reduced by mitochondrial dehydrogenases more efficiently as compared to other tetrazolium salts [36]. After 6 h of incubation at 37°C, the absorbance of culture media was measured at 490 nm using a multiwell microplate reader. The activity of non-treated control cells is assumed to be 100%.

Mitochondrial membrane potential (MMP- $\Delta\psi_m$)

MMP was monitored using the cell permeable cationic fluorescent dyes JC-1 and Rhodamine123, which preferentially enters mitochondria due to the highly negative MMP. In mitochondria, JC-1 is under monomeric form (green fluorescence) and in the presence of a high MMP, it forms red aggregates with a large shifts in emission. MMP was taken as the ratio between red and green fluorescence. Briefly, after different treatments, media were removed and cells kept in a JC-1 solution (1.0 μg/ml) for 30 min. After washing with PBS, green and red fluorescence were recorded with λex/λem at 485 nm/535 nm and λex/λem at 530 nm/590 nm, respectively.

The effect of *BM* pre-treatment on H_2O_2-induced changes on MMP was also studied by fluorescence microscopy. SK-N-SH cells were cultured on glass slides in 24-wells plate. Briefly, after different treatments, Rhodamine123 solution (5 μM) was added for 30 min. Cells were then rinsed with PBS and fixed in ice-cold methanol for 10 min. The glass slides were mounted and observed under a fluorescence microscope.

Protein carbonyl measurement

Briefly, after treatment, 20 μg of whole cell extract was derivatized with 10 mM of DNPH 2N-HCL for 15 min at room temperature. Then the levels of DNP-derivatized proteins were detected with the rabbit anti-DNP antibody (1:2000 dilution) followed by the secondary antibody anti-rabbit IgG HRP (1:10000 dilution) both for 1 h in TBS containing 5% skim milk. Detection was realized with Immobilon Western Chemiluminescent HRP Substrate (Millipore, USA) and the bands were visualized and quantified by densitometric analysis using the luminescent imaging system FluorChem (Alpha Innotech).

Effect of BM pre-treatment on the expression of some redox regulated proteins upon oxidative stress by western blotting

After different treatments, cytoplasmic and nuclear proteins were obtained by using a nuclear protein extraction kit containing protease inhibitors. The activation of NF-κB was monitored by the nuclear translocation of the subunit p65 with the anti-p65 antibody (1:300 dilution). p66Shc, pERK1/2, and Sirt1 proteins were analyzed in total proteins extracted by a complete lysis buffer (pH 8.0) containing 50 mM Tris (pH 8.0), 150 mM of NaCl, 0.5% Igepal, 0.1% sodium dodecyl sulfate (SDS), 0.01% Triton X-100, 5 mM EDTA, and a cocktail of proteases inhibitors. These proteins were analyzed using primary antibodies for Sirt1 (1:500),

total and pERK1/2 (1:750), p66Shc (1:500), total Shc (1:500) followed by HRP-conjugated secondary antibodies. β-actin, GAPDH and importin α3 were used as internal protein control. Purity of cytoplasmic and nuclear fractions was checked by analyzing a nuclear specific transcription factor sp3. Detection was realized as described above.

Statistical analysis

All experiments were performed in triplicate from at least three separate experiments and results were expressed as mean ± SEM. Data were statistical analyzed by one way ANOVA followed by a Dunnett multiple means comparison test and the level of significance was considered when p-values was < 0.05.

RESULTS

A pre-treatment with BM protects SK-N-SH cells against H_2O_2 and acrolein -induced toxicity

Figure 1 shows that treatments with the *BM* extract for 3 h before the addition of 200 μM of H_2O_2 protected SK-N-SH cells against H_2O_2-induced toxicity. This protection was assessed 24 h after the addition of H_2O_2 through the survival XTT test and was significant from 50 μg/ml (Fig. 1A). To confirm the cytoprotective effect of BM, we completed the Resazurin test (Fig. 1B). However, higher metabolic activities observed with XTT and Resazurin could be due to a proliferative effect of *BM*. Therefore, LDH release in the medium was measured 24 h after H_2O_2 treatment and a significant protection was observed from 25 μg/ml of *BM* and reached a maximum at 100 μg/ml (Fig. 1C). *BM* alone did not cause any cytotoxicity until the highest tested concentration (100 μg/ml) (data not shown).

Our results showed that acrolein, at 15 μM, induced 50% cell death as assessed by XTT, Resazurin, and LDH tests after 24 h of treatment (Fig. 2A–C). Interestingly, a 3 h pre-treatment with the *BM* extract could protect SK-N-SH cells against the toxicity induced by acrolein from 40 μg/ml of *BM* (Fig. 2A–C).

H_2O_2 decomposes in the media over short time frames (1–2 h) when added as a bolus [37], while acrolein increased the production of superoxide, activated NADPH oxidase activity and reduced GSH level [38] by forming highly reactive conjugates with GSH and proteins which are likely to persist for a significantly longer time [39]. Thus our data showed that *BM* pre-treatment can protect SK-N-SH cells against both transient as well as persistent oxidative stress.

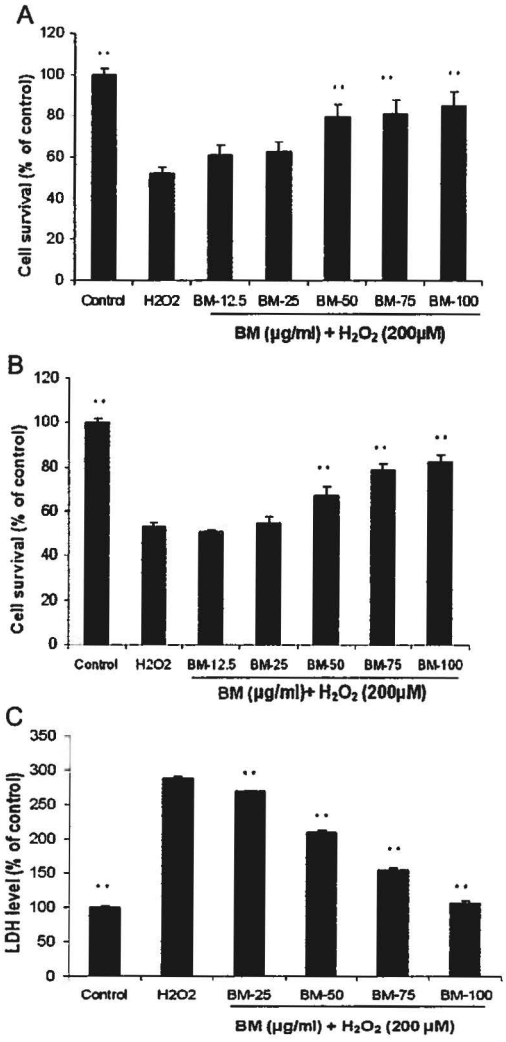

Fig. 1. Effects of a 3 h pre-treatment with *BM* on SK-N-SH cells survival after 24 h of treatment with 200 μM of H_2O_2 as analyzed by (A) XTT, (B) Resazurin, and (C) LDH assays. Results are expressed as percentage of control (taken as 100%). Data are means ± SEM from at least three separate experiments performed in quadruplicate in each group. $^{**}P < 0.01$ *versus* H_2O_2 treated group.

BM extract scavenges H_2O_2 which in turn may decrease intracellular ROS

As shown in Fig. 3A, SK-N-SH cells treated with a high concentration of H_2O_2 (1.0 mM) displayed intense fluorescence after staining with DCF dye, and this fluorescence was significantly decreased when cells were pre-treated with *BM* from 12.5 μg/ml (Fig. 3A). In the

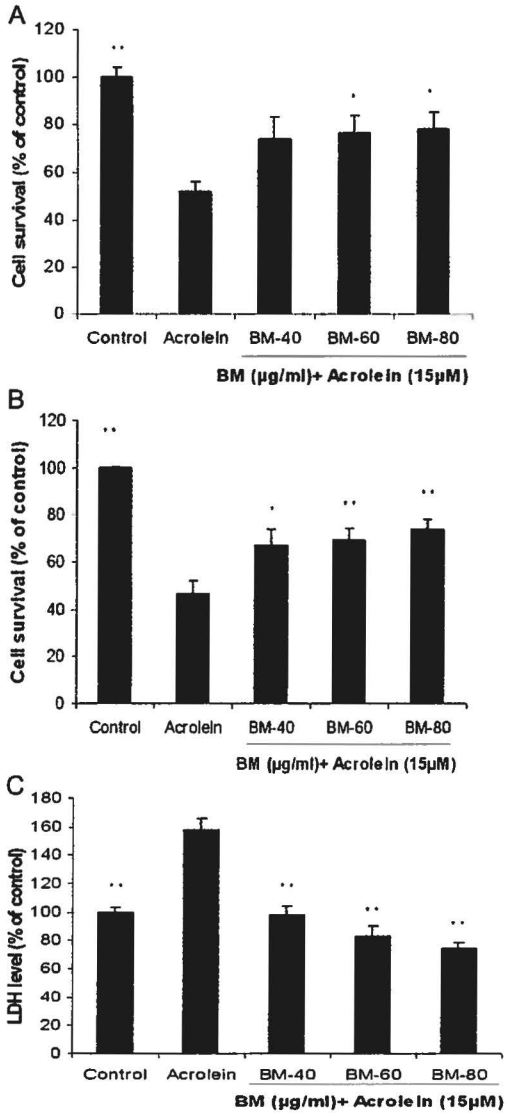

Fig. 2. Effects of a 3 h pre-treatment with *BM* on SK-N-SH cells survival after 24 h of treatment with 15 μM of acrolein as analyzed by (A) XTT, (B) Resazurin, and (C) LDH assays. Results are expressed as percentage of control (taken as 100%). Data are means ± SEM from at least three separate experiments performed in quadruplicate in each group. $^{*}P < 0.05$ and $^{**}P < 0.01$ *versus* acrolein treated group.

presence of the redox-active metal ions such as Fe^{2+}, H_2O_2 decomposes to produce the very reactive and cytotoxic hydroxyl radical (OH). We have next examined whether *BM* pre-treatment can also decrease intracellular ROS in the presence of H_2O_2 and $FeCl_2$. For this, SK-N-SH cells were pre-treated with different doses of *BM* for 1 h followed by a 1 h treatment with 500 μM of H_2O_2 and 50 μM of $FeCl_2$. We found that the DCF fluorescence was significantly decreased when cells were pre-treated with *BM* from 12.5 μg/ml (Fig. 3B).

DCF dye can also react with reactive nitrogen species such as peroxynitrite anions to give intense fluorescence [40]. Since the SK-N-SH cells also produce nitric oxide under normal physiological conditions [41], the ability of *BM* to degrade H_2O_2 was further strengthened through a polarographic method, which uses sensors for measuring H_2O_2 concentrations in real time. As shown in Fig. 3C, the intensity, i.e., picoampere (pA), increased with the addition of graded concentrations (250 μM, 500 μM, 750 μM, 1000 μM, 1250μM) of H_2O_2 in PBS solution. The regression line was obtained by plotting the intensity in pA against the concentrations of H_2O_2 and the slope is 3.099 (Fig. 3C inset). To analyze the ability of the *BM* extract to degrade H_2O_2, different volumes of H_2O_2 were added in a PBS solution containing the *BM* extract. In the presence of the *BM* extract, the slope of the calibrated curve indicates the degradation of H_2O_2 by the *BM* extract. The slopes of the calibrated curve is inversely proportional to the concentration of *BM* being 2.55 and 1.89 with 50 μg/ml and 100 μg/ml of *BM*, respectively (Fig. 3D). These results show the potential of the *BM* extract to degrade H_2O_2 in a concentration dependent manner.

BM pre-treatment maintains mitochondrial membrane potential (MMP)

Oxidative stress is accompanied by a large increase in intracellular calcium levels and has been linked to a decrease in MMP [42], which is widely considered as an indicator of mitochondrial functionality. It was found that mitochondria are one of the main intracellular targets of acrolein [43]. It could induce a dose-dependent increase of ROS and a subsequent decrease in GSH content [44]. We have evaluated the effect of acrolein on MMP, which is considered a critical checkpoint in the cascade of events leading to cell death. Using JC-1 fluorescent dye to determine the MMP in SK-N-SH cells, we found that a treatment with 200 μM and 10 μM of H_2O_2 and acrolein, respectively, for 6 h induced a mitochondrial depolarization which results in the reduction of the intracellular fluorescence (Fig. 4A). So, next we investigated whether *BM* pre-treatment could rescue MMP in SK-N-SH cells. As shown in Fig. 4A, the decrease in MMP after H_2O_2 or acrolein

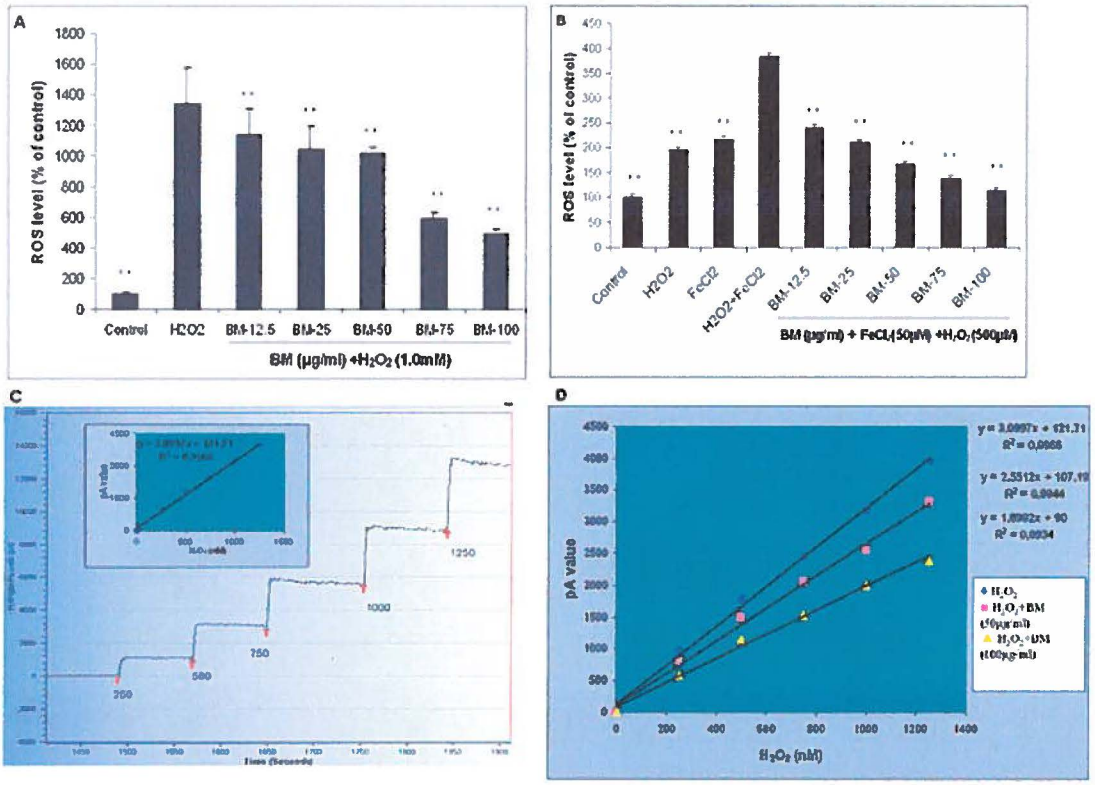

Fig. 3. A) Effect of a 3 h pre-treatment with *BM* on ROS levels in SK-N-SH cells after 1 h of treatment with 1.0 mM of H_2O_2. B) Effect of a 1 h pre-treatment with *BM* on ROS levels in SK-N-SH cells after 1 h of treatment with 50 μM and 500 μM of $FeCl_2$ and H_2O_2, respectively. DCF fluorescence is expressed as arbitrary units as percentage of control (taken as 100%). Data are means ± SEM from at least three separate experiments performed in quadruplicate in each group. $^{**}P < 0.01$ *versus* H_2O_2 or H_2O_2+ $FeCl_2$ treated groups. C) H_2O_2 concentrations as measured by polarographic assay. Once the output signal stabilized in PBS, different concentrations of H_2O_2 were added (indicated by arrow). The output signal was allowed to stabilize subsequent to each addition. Concentrations of H_2O_2 were plotted against the corresponding pA intensity and a standard curve was generated (inset). D) Standard curves obtained with or without *BM extract* as measured by polarographic assay.

treatments was encountered by *BM* pre-treatment from 20 μg/ml. MMP changes induced by a high concentration of H_2O_2 (1.0 mM) for 1 h were also observed by fluorescence microscopy (Fig. 4B). A dose dependent increase in Rhodamine123 fluorescence was observed when cells were pre-treated with *BM* suggesting a role of *BM* in maintaining mitochondrial integrity against oxidative stress.

In addition to the cytochrome *c* oxidase (COX) activity [15], activities of pyruvate dehydrogenase (PDH) and α-ketoglutarate dehydrogenase (KGDH) are also decreased in AD mice models [45,46]. Next we have investigated whether *BM* pre-treatment could activate the mitochondrial dehydrogenases. For this, SK-N-SH cells were treated with *BM* for 6 h along with INT and PMS, and total NADH dehydrogenase activity was measured. A significant increase in total mitochondrial dehydrogenases activity was observed in the presence of *BM*. At the highest dose of *BM*, i.e., 100 μg/ml, total NADH dehydrogenase activity increased by approximately 50% after 6 h of *BM* treatment (Fig. 4C).

BM pre-treatment prevents BSO-induced decrease in GSH levels, H_2O_2, and acrolein-induced oxidized proteins

Figure 5A shows that a pre-treatment with *BM* for 3 h prevented the reduction of GSH induced by the addition of BSO, at 10 mM for 24 h.

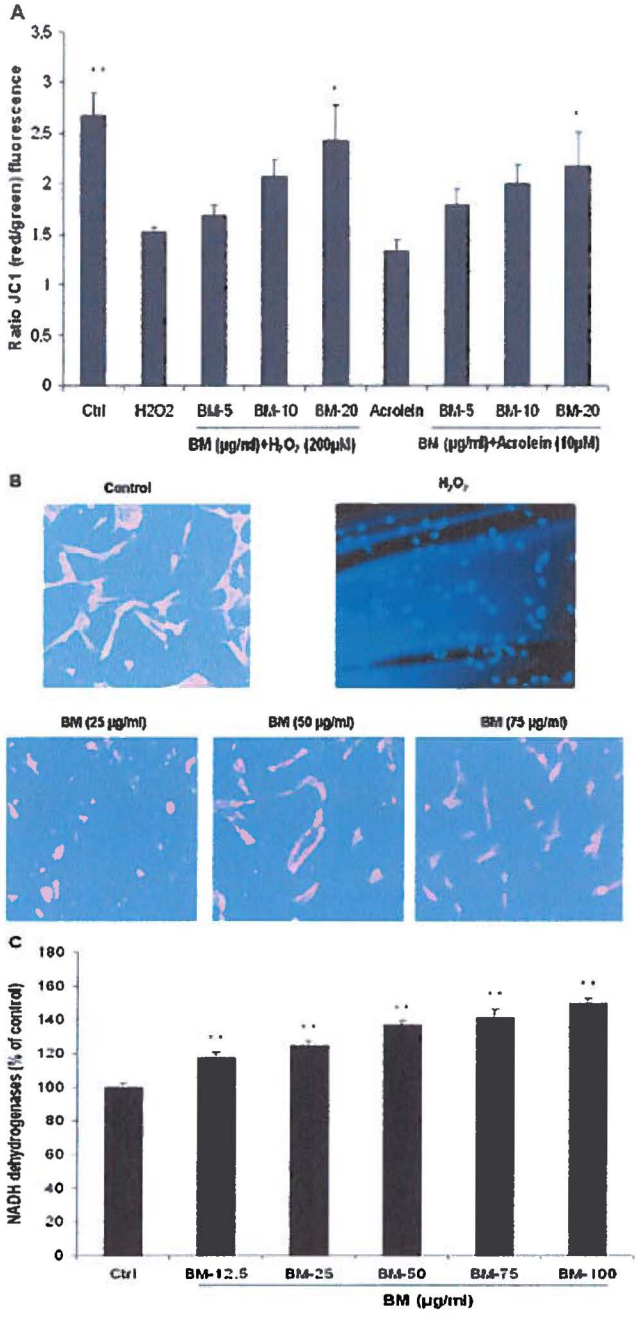

Fig. 4. Effect of a 3 h pre-treatment with *BM* on the mitochondrial membrane potential as detected by (A) the ratio between JC-1 red and green fluorescence after 6 h of treatment with 10 μM of acrolein or 200 μM of H_2O_2; B) with fluorescent dye Rhodamine123 after 1 h treatment with H_2O_2 at 1.0 mM. C) Cellular NADH dehydrogenase activity after 6 h with *BM* alone. Results are expressed as percentage of control (set to 100%). Data are means ± SEM from at least three separate experiments performed in quadruplicate in each group. $^{*}P < 0.05$ and $^{**}P < 0.01$ *versus* H_2O_2 or acrolein (A) or control group (C).

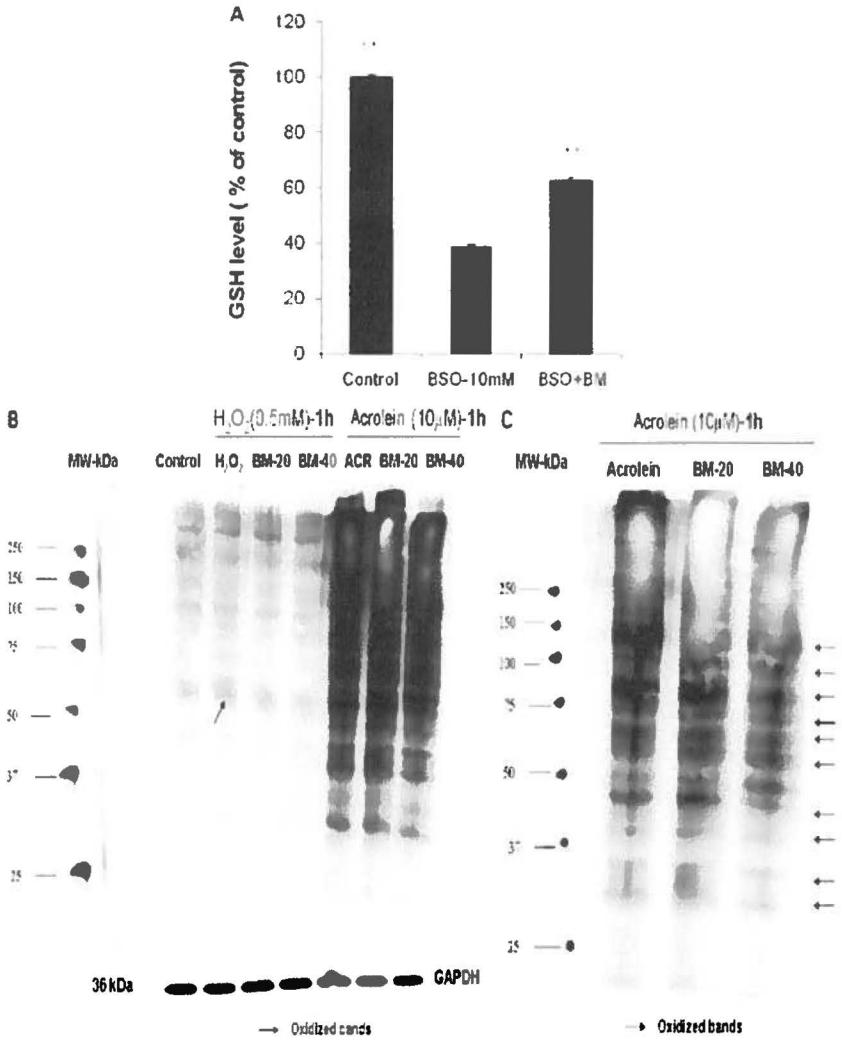

Fig. 5. A) Effect of a 3 h pre-treatment with *BM* on the intracellular level of GSH in SK-N-SH cells as measured by the fluorescent dye MCB after 24 h of treatment with BSO (10 mM). Results are expressed as percentage of control (taken as 100%). Data are means ± SEM of at least three separate experiments performed in triplicate in each group. $^{**}P < 0.01$ *versus* BSO group. B) Effect of a 3 h pre-treatment with *BM* on the levels of oxidized proteins in SK-N-SH cells as measured by Western blot after 1 h of treatment with H_2O_2 (500 μM) or acrolein (10 μM). C) Levels of acrolein induced oxidized proteins after decreasing the contrast of (B).

We investigated whether *BM* pre-treatment could reduce oxidized proteins in SK-N-SH cells. For this, cells were treated for 1 h with H_2O_2 (500 μM) and acrolein (15 μM). At this concentration, H_2O_2 induced an elevation of protein carbonyl and particularly proteins in the range of 50 kDa (Fig. 5B), while acrolein induced carbonylation of proteins between 30-110 kDa (Fig. 5C). A pre-treatment with *BM* from 40 μg/ml decreased the carbonylation of these proteins.

BM pre-treatment modulates the expression of some redox regulated proteins upon oxidative stress

To gain insight into the mechanisms underlying the *BM* induced neuroprotection, we analyzed the effect of *BM* on the expression of some redox regulated proteins. NF-κB is one of the most sensitive transcription factors to oxidative stress. To determine whether *BM* can prevent H_2O_2 and acrolein-induced activation of NF-κB,

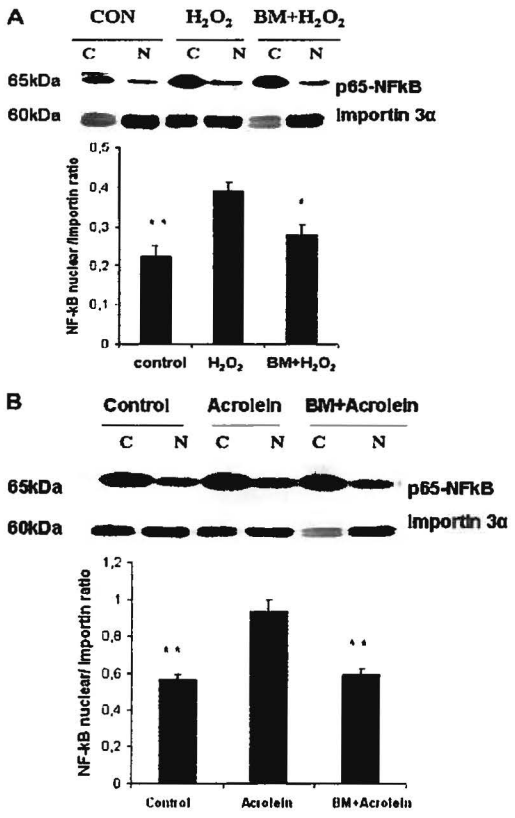

Fig. 6. Effect of *BM* pre-treatment on the NF-κB activation. A) SK-N-SH cells were pre-treated with *BM* (60 μg/ml) for 3 h followed with a treatment with 1.0 mM of H_2O_2 for 45 min. B) Cells were pre-treated with *BM* (20 μg/ml) or 10 μM of acrolein for 1 h. Then the cytoplasmic and nuclear fractions were collected for Western blot analysis. Importin 3α was used as the loading control. Blots represent one of three independent experiments. Bar graphs represent the average of the ratio of the relative density of p65/Importin 3α from at least three independent experiments. $^{*}P < 0.05$ and $^{**}P < 0.01$ *versus* H_2O_2 or acrolein treated groups.

SK-N-SH cells were pre-treated for 3 h with a dose of *BM* (60 μg/ml) followed by H_2O_2 at 1.0 mM for 45 min or with a dose of *BM* (20 μg/ml) followed by 10 μM of acrolein for 1 h. Figure 6A, B showed that non-toxic concentrations of H_2O_2 and acrolein induced a nuclear translocation of p65. Interestingly, this translocation was prevented by *BM* pre-treatment.

Next we measured the effect of *BM* pre-treatment on the expression of pERK1/2, which has recently emerged as a key regulator of tau phosphorylation [25, 26]. Moreover Aβ and H_2O_2 toxicity is also mediated through pERK1/2 [21,22]. We showed that the levels of pERK1/2 were increased at 1 h but were downregulated after 24 h of acrolein treatment at 10 μM. The effects of acrolein on the regulation of pERK1/2 levels were significantly prevented by *BM* pre-treatment at 20 μg/ml while no significant effect of *BM* (60 μg/ml) pre-treatment on H_2O_2 (1.0 mM, 45 min) induced pERK1/2 was observed (Fig. 7A, B).

Phosphorylation and mitochondrial translocation of the redox active protein p66Shc plays an important role in mitochondrial ROS generation and apoptosis [24]. Moreover p66Shc phosphorylation is critical for Aβ-mediated toxicity [23]. We also measured the effects of *BM* on the activation of p66Shc after H_2O_2 and acrolein treatments. For this, cells were pre-treated with *BM* at 60 μg/ml followed by H_2O_2 (1.0 mM) or acrolein at 15 μM, for 30 min. H_2O_2 treatment did not increase the levels of phospho p66Shc, but it was downregulated by at least 50% by *BM* pre-treatment as shown in Fig. 8. On the other hand, acrolein treatment increased the levels of phospho p66Shc and induced phospho p66Shc tetramers formation (Fig. 9A). *BM* pre-treatment decreased the level of phospho p66Shc but did not prevent the formation of tetramers induced by acrolein as shown in Fig. 9A. Tetramers of p66Shc can be reversed by thioredoxins in the presence of GSH [47]. So next we incubated protein homogenates from SK-N-SH cells treated with acrolein or *BM* plus acrolein and GSH at 5 mM for 30 min prior to performing western blot to analyze tetramers reversal. Our results show a significant reversal of phospho p66Shc tetramers in cells treated with *BM* in comparison to acrolein alone (Fig. 9B).

Sirt1 have been shown to protect cells against a variety of stressors. Cellular GSH level also regulates Sirt1 expression [48]. In the presence of a depletion of GSH by BSO, Sirt1 levels decreased when exposed to H_2O_2. This BSO mediated downregulation of Sirt1 was prevented by pre-treating cells with a low dose of *BM* (12.50 μg/ml) (Fig. 10). This low dose of *BM* was chosen as it maintains cellular GSH levels against BSO treatment (Fig. 5A).

Role of bacosides compounds in neuroprotection

The *BM* extract contains triterpenoids saponins, alkaloids, and flavonoids compounds. The triterpenoids saponin *bacosides* have been reported to be active constituents responsible for the biological activity of *BM*. In order to analyze the involvement of *bacosides* in the neuroprotective effect observed with the *BM* extract, we compared the efficacy of the second standardized extract (*BM2*), which contains 40% of *bacosides*. The

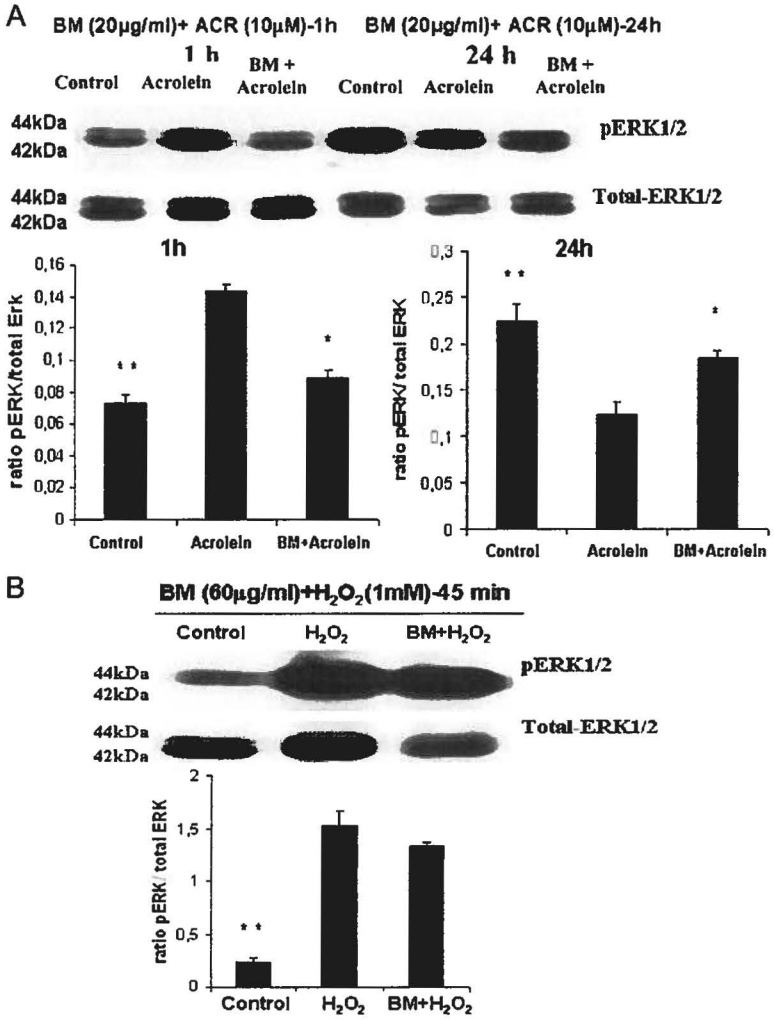

Fig. 7. Effect of *BM* pre-treatment on the pERK1/2 levels. A) SK-N-SH cells were pre-treated with *BM* (20 µg/ml) for 3 h followed by a treatment with acrolein (10 µM) for 1 h and 24 h or (B) with 1.0 mM of H_2O_2 for 45 min. Then total proteins were collected for Western blot analysis. β-actin was used as the loading control. Blots represent one of three independent experiments. Bar graphs represent the average of the ratio of the relative density for phospho/total ERK1/2. Data are means of at least three independent experiments. $^*P < 0.05$ and $^{**}P < 0.01$ *versus* acrolein or H_2O_2 treated groups.

BM2 extract was more effective against H_2O_2-induced toxicity as LDH levels were significantly reduced from 1.0 µg/ml and cell viability was significantly higher from 2.5 µg/ml and reached a maximum at 10 µg/ml (Fig. 11A, B). These results strongly suggest that the neuroprotective effect of the *BM* extract is mediated through *bacosides* compounds. We have then studied the ability of *BM2* to decrease ROS levels. Fig. 11C shows that at 10 µg/ml, *BM2* is as efficient as *BM* indicating that the antioxidant activity of the *BM* or *BM2* extract was not due to *bacosides* compounds but likely due to flavonoids.

DISCUSSION

It is now well recognized that both oxidative stress and mitochondrial dysfunction play an important role

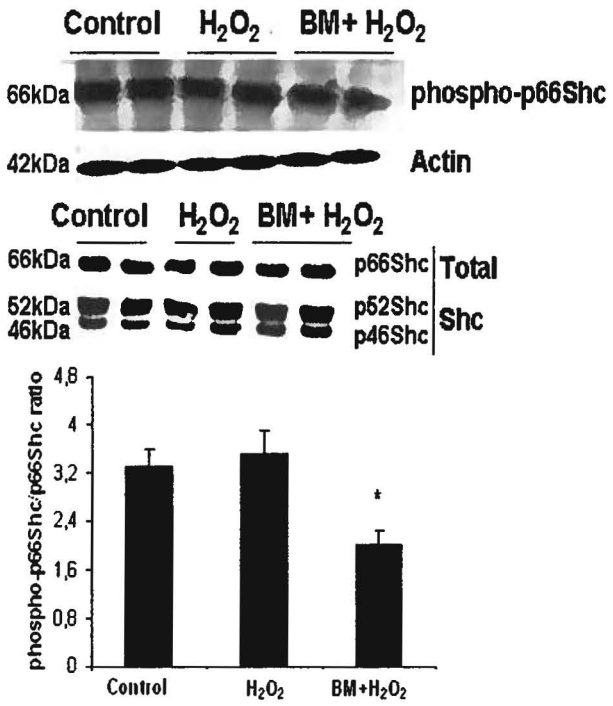

Fig. 8. *BM* pre-treatment down regulates the p66Shc pathway. SK-N-SH cells were pre-treated with *BM* (60 μg/ml) for 3 h followed with a treatment with 1.0 mM of H_2O_2 for 30 min and total proteins were collected for Western blot analysis. β-actin was used as the loading control. Images represent one of three independent experiments. Bar graphs represent the average of the ratio of the relative density for phospho p66Shc/total p66Shc from at least three independent experiments. Data are means ± SEM ($n = 3$). $^*P < 0.05$ *versus* H_2O_2 treated group.

in the pathophysiology of AD [15,45,46,49]. Aβ peptide is known to generate H_2O_2 in the presence of metal ions [13,14] and H_2O_2 in turns promotes Aβ production through c-jun N terminal (JNK)-dependent activation of γ-secretase [50]. The peroxidation of membrane lipids, on the other hand, generates highly electrophilic α,β-unsaturated carbonyl derivatives including acrolein or HNE, and 4-oxononenal [51]. Of α,β-unsaturated aldehydes, acrolein is the most reactive and reacts 110–150 times faster with GSH than HNE or crotonal [39]. Increased levels of acrolein have also been reported in the brain regions of subjects with MCI and patients with early AD [8] and acrolein was found to be neurotoxic to primary hippocampal neuron [7].

BM extract has been used in traditional Indian medicine for almost 3000 years and is classified as a drug used to improve memory, cognition, and as a potent nerve tonic [28,29]. Various clinical studies have been performed to establish the efficacy of *BM* in memory functions [52–54]. *BM* was well tolerated without side effects [29] and the LD_{50} in rats was determined to be as high as 2.7 g/kg when administrated orally [55]. Recently, *BM* extract was found to be able to reduce the Aβ peptide levels by 60% in hippocampus and cortex from an AβPP-PS1 transgenic mice model [31]. Although the *BM* extract and *bacosides* compounds have been investigated extensively for their neuropharmacological activities, their beneficial effect on cellular pathways involved in the physiopathology of AD remains to be established.

We demonstrated that *BM* pre-treatment protected SK-N-SH cells against H_2O_2 and acrolein-induced toxicity and the mitochondrial integrity. Using the fluorescent probe DCF-DA, we showed that *BM* pre-treatment reduced intracellular ROS levels. This property was completed by polarographic assay measuring the degradation of H_2O_2 in real time. *BM* extract may degrade ROS either by direct physical activity or indirectly through the elevation of endogenous antioxidants. We showed that a pre-treatment with the *BM* extract could prevent the depletion of GSH upon BSO treatment and may help in preserving cellular redox potential. These

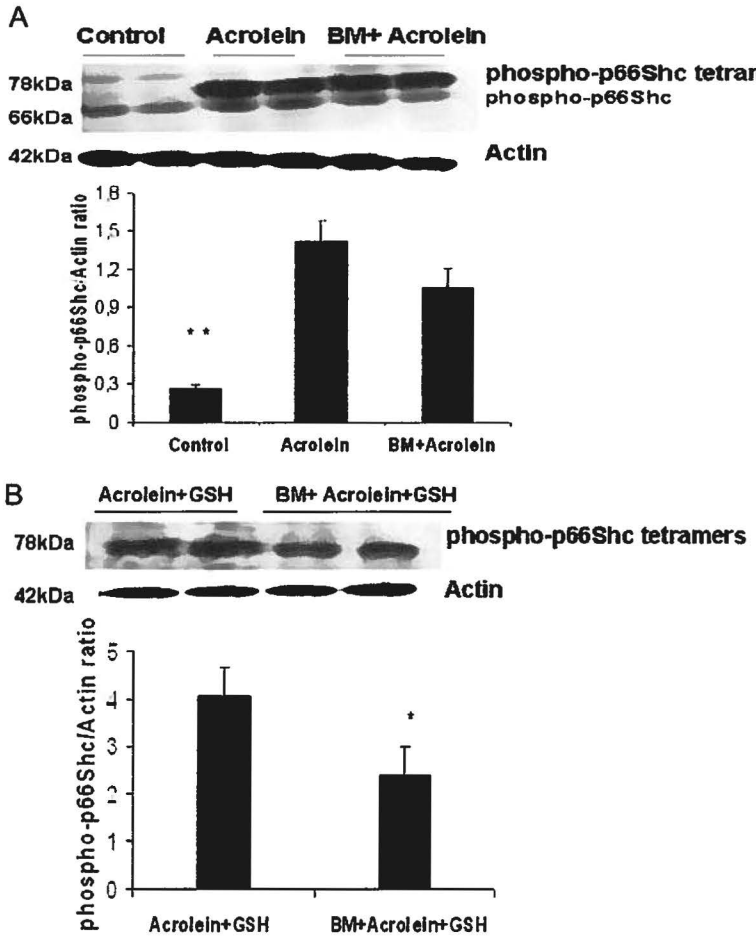

Fig. 9. Effect of *BM* pre-treatment on the p66Shc tetramers formation. SK-N-SH cells were pre-treated with *BM* (60 μg/ml) for 3 h followed with a treatment with 15 μM of acrolein for 30 min and total proteins were collected for Western blotting analysis. β-actin was used as the loading control. Images represent one of three independent experiments. A) Western blotting images and bar graph represents the ratio of the quantification of phospho p66Shc/actin; (B) Western blotting images and bar graph represents the ratio of the quantification phospho p66Shc tetramers/actin. Data are means ± SEM ($n = 3$). * $P < 0.05$ and ** $P < 0.01$ *versus* acrolein or acrolein and GSH treated groups.

results are of interest as several studies have demonstrated a deficit in total GSH levels and an alteration of GSH redox cycling in AD [56]. In contrast, an up-regulation of GSH synthesis protects neurons against Aβ-induced neurotoxicity [57].

Pharmacological studies have identified several triterpenoid saponins as active constituents of the *BM* extract [58–60]. Recently the standardized extract of *BM* has been found to reverse the cognitive deficits induced by hypobaric hypoxia [55]. Mitochondria are one of main intracellular targets of acrolein and H_2O_2. Acrolein has been shown to inhibit mitochondrial respiration, increase ROS production with subsequent decrease in GSH content in purified brain mitochondria [61], and inhibit NADH-linked dehydrogenases [62]. Both Aβ and H_2O_2 can also inactivate some of the mitochondrial enzymes especially KGDH, succinate dehydrogenase, aconitase and COX [63,64]. We further observed that *BM* pre-treatment maintains cellular MMP upon acrolein and H_2O_2 treatments as measured by JC-1 and Rhodamine123 fluorescence probes. These results are of great interest because the basal MMP has been shown to decrease significantly and exclusively in cortical neurons from the triple transgenic

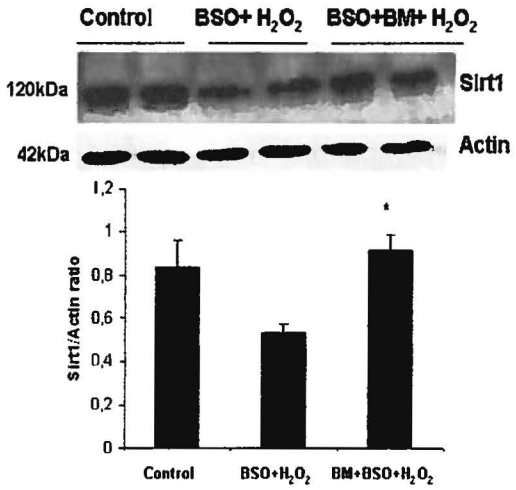

Fig. 10. Effect of *BM* pre-treatment on Sirt1 levels from cells treated with *BM* (12.5 μg/ml) and BSO (5.0 mM) for 24 h followed by H_2O_2 (1.0 mM) treatment for 1 h. Data are means ± SEM with $n = 3$. * $P < 0.05$ *versus* BSO+ H_2O_2 treated group.

AD mice model [46]. Moreover, 3 h of *BM* treatment also significantly increased mitochondrial NADH dehydrogenase activity. Thus, by upregulating various dehydrogenases, *BM* may play an important role in mitochondrial protection against oxidative stress as activities of various mitochondrial dehydrogenases are also reduced in the brains from AD patients

Oxidative damage of several proteins is elevated in MCI and AD brain [3,4,65]. We found that the increase of protein carbonyls in SK-N-SH cells induced by H_2O_2 and acrolein was prevented by *BM* pre-treatment. Interestingly, the molecular weights of oxidized proteins in the presence of acrolein are in the range of those observed in the brain from MCI (carbonic anhydrase II (29.1 kDa), heat shock protein 70 (71.1 kDa), mitogen-activated protein kinase I (41.7 kDa), and syntaxin binding protein (67.9 kDa) or from early AD (phosphoglycerate mutase 1 (28.8 kDa), glial fibrillary acidic protein (49.9 kDa), and fructose bisphospate aldolase C (39.7 kDa) [65]).

The expression of several redox regulated pathways, i.e., NF-κB, pERK1/2, p66Shc, and Sirt1, which play a vital role in cell survival upon oxidative stress, is altered in brains of AD patients. The transcription factor NF-κB is retained in the cytoplasm as a homo- or heterodimer by inhibitory binding molecules called IκBs. Multiple stimuli including inflammation and oxidative stress, trigger the phosphorylation of IκBs, thus releasing NF-κB followed by its nuclear translocation [66]. We found that *BM* pre-treatment prevents the nuclear translocation of the subunit p65 induced by H_2O_2 and acrolein. We and others have previously demonstrated that the inhibition of NF-κB activation was involved in the protective effect against Aβ-induced toxicity [67]. Our results suggest that the prevention of the NF-κB activation by *BM* extract may partially be responsible for increased cell survival in the presence of H_2O_2 and acrolein. These results are of interest because it was found that blocking NF-κB transcriptional activity could inhibit the production of both $A\beta_{1-40}$ and $A\beta_{1-42}$ in Swedish-AβPP-expressing cells [68]. Accordingly, NF-κB is higher in various transgenic animal models of AD [19,20]. Moreover, there is an increase in the nuclear immunoreactivity of NF-κB in the brain from AD patients compared to age-matched control subjects [17,18]. Finally, recent data concerning NF-κB functioning indicates that the development of drugs targeting NF-κB regulation may prove beneficial for memory disorders [69].

Aβ peptide, acrolein, and H_2O_2-induced toxicity is mediated through the phosphorylation of ERK1/2 [21, 22,70]. ERK1/2 has recently emerged as a key regulator of plaque formation and tau hyperphosphorylation [25,26] with increase activity of ERK1/2 in AD brain [71]. We observed that the sustained increase in pERK1/2 levels after 1 h of treatment with acrolein were significantly prevented with *BM* pre-treatment. On the other hand the decrease of the pERK1/2 levels after 24 h of treatment was significantly restored with *BM* pre-treatment. The downregulation of pERK1/2 levels after 24 h of acrolein treatment may be attributed to the activation of Protein phosphatase 2A (PP2A), an ERK1/2 phosphatase, which has been shown to be activated by HNE via caspase3 dependent mechanism [72]. It may be possible that *BM* pre-treatment inhibits PP2A activation by inhibiting activation of caspase 3. These results suggest that *BM* can potentiate the ability of cells to withstand oxidative stress induced by acrolein. By this ability to reduce stress, activation of ERK may be regulated at the basal level.

Moreover ERK1/2 has been shown to be an upstream regulator of NF-κB [73]. This regulation can also explain the concomitant inhibition of ERK1/2 and NF-κB with *BM* pre-treatment.

Sirt-1 is known to deacetylate the subunit p65 predominantly at lysine 310 and inhibit the activity of NF-κB [74]. In the cerebral cortex from AD patients, Sirt-1 levels are significantly reduced [75]. Recently, Sirt-1 activation either by caloric restriction or by resveratrol treatment has been considered as a novel mechanism

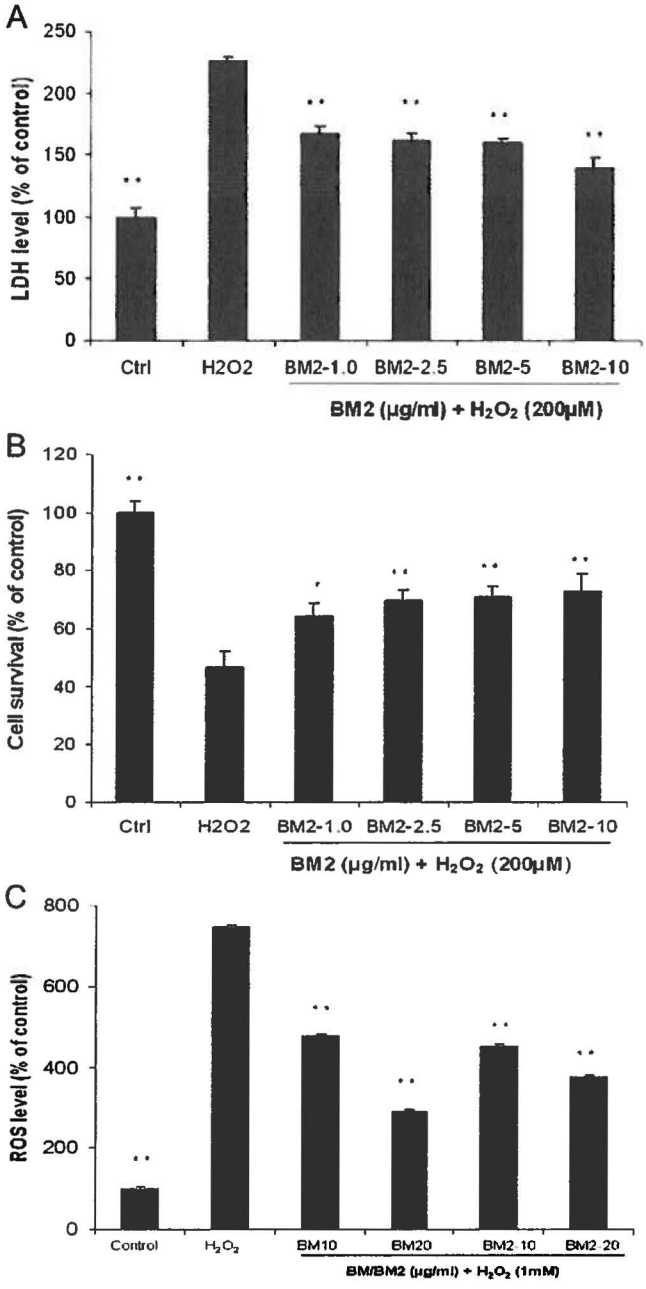

Fig. 11. Effect of a 3 h pre-treatment with *BM2* on SK-N-SH cells survival after 24 h of treatment with 200 μM of H_2O_2 as analyzed by (A) LDH and (B) XTT assays. Results are expressed as percentage of controls (taken as 100%). C) Effect of a co-treatment with *BM* and *BM2* on ROS level in SK-N-SH cells after 1 h of treatment with 1.0 mM of H_2O_2. DCF fluorescence is expressed as arbitrary units with control group considered as 100%. Data are means ± SEM of at least three separate experiments performed in quadruplicate in each group. $^{*}P < 0.05$ and $^{**}P < 0.01$ *versus* H_2O_2 group.

to protect neurons against the Aβ toxicity [76,77]. In our study, Sirt-1 downregulation was prevented by *BM* pre-treatment as observed after 1 h of treatment with H_2O_2 in parallel with the inhibition of NF-κB. These results suggest that the activation of Sirt-1 and the inhibition of NF-κB represent a compensatory mechanism to increase mitochondrial activity and cell survival. Flavonoids present in the *BM* extract could be activators of Sirt-1 as previously described [78].

The mitochondrial adaptor protein p66Shc is emerging as a novel link between mitochondria, oxidative stress, and longevity. Mice lacking p66Shc genes have less ROS generation, live 30% longer, are resistant to oxidative stress, and are also protected from age-related endothelium dysfunctions and diabetes [79,80]. Phosphorylation of p66Shc is critical for Aβ mediated toxicity [23] and under oxidative stress condition, ERK1/2 has been identified as the kinase phosphorylating p66shc on Ser36 [81]. In our study, Ser36 phosphorylation of p66Shc is probably related to the observed early ERK1/2 activation by acrolein. Our results showed that *BM* pre-treatment decreased the phosphorylation of p66Shc induced by acrolein and H_2O_2 indicating that the beneficial effect of *BM* was also mediated through the inhibition of phospho p66Shc. Acrolein treatment upregulated both phospho p66Shc levels and tetramers formation. It has been shown that phospho p66Shc, by oxidizing cytochrome C, generates H_2O_2, which in turn activates the opening of permeability transition pore thereby causing mitochondria rupture and release of various apoptotic factors [24]. Thus by downregulating the levels of phospho p66Shc, *BM* can decrease mitochondrial H_2O_2 generation and prevent the release of various apoptotic factors. Excessive oxidative stress also induces p66Shc tetramers by disulfide bond formation which can be reversed by thioredoxins and GSH systems [47]. In our study, *BM* pre-treatment did not preserve GSH levels against acrolein (data not shown) likely due to the rapid formation of GSH-acrolein adduct. However, *BM* could reverse the phospho-p66Shc tetramers when GSH was exogenously added. Thus, we can speculate that *BM* could reverse the phospho p66Shc tetramers by preserving cellular thioredoxin system. This is the first report of phospho p66Shc downregulation as well as reversal of tetramers formation by a standardized plant extract.

The standardized extract of *BM* contains bacosides and flavonoids. *Bacosides* are the major bioactive triterpenoid saponins isolated and characterized from *BM* [58–60]. Using the *BM2* extract with 40% of bacosides, our results demonstrate the involvement of *bacosides* in the neuroprotective activity of the *BM* extract. The *BM2* extract displayed higher efficacy against H_2O_2-induced toxicity in SK-N-SH cells. This result is in line with the *in vivo* neuroprotective effect the triterpenoid saponins Celastrol in mice treated by MPTP (1-methyl-4-phenyl 1, 2, 3, 6-tetrahydropyridine) or 3-nitropropionic acid [82]. As the *BM* extract, Celastrol decreased the NF-κB and ERK1/2 activation induced by lipopolysaccharide [83]. However, we cannot exclude the effect of flavonoids present in the *BM* extract on the mitochondrial activity since flavonoids present in the standardized extract of *Ginkgo biloba* EGb 761 could modulate the MMP [84], inhibit the activation of NF-κB induced by Aβ [67], and protect neurons against Aβ toxicity [85].

Oxidative stress and mitochondrial dysfunctions do play a role in neuronal loss in AD. H_2O_2 is generated by Aβ and is in turn implicated in Aβ toxicity while acrolein represents a by-product of lipid peroxidation with a neurotoxic effect. Deficits in complex IV, a decrease in MMP- ($\Delta\psi_m$) and ATP level may subsequently result in the opening of permeability transition pore, cytochrome C release, and caspase activation and cell death. We have demonstrated that *BM* pre-treatment protected human SK-N-SH cells against H_2O_2 and acrolein induced neurotoxicity by reducing oxidative stress level and maintaining mitochondrial bioenergetics and functions. The neuroprotective mechanisms of *BM* also involved the prevention of the activation of NF-κB, ERK1/2, and p66Shc pathways as well as preserving Sirt1 levels. Moreover, low doses of *BM* also prevent cellular GSH depletion against BSO treatment. The modulation of these redox active proteins by *BM* pre-treatment may contribute to cell survival mechanisms against oxidative stress. The mechanisms demonstrated in this study could underline the protective effect of the *BM* extract previously observed in a transgenic mice model of AD [31]. Our investigation is the first comprehensive study aimed at the scientific validation of the traditional use of *BM* as a nootropic agent for restoring age-related decline in mental abilities. Further ongoing studies are likely to elucidate additional mechanisms involved in *BM*-induced neuroprotection. In the long term, *BM* may be of therapeutic use in the prevention of AD as well as other age-related neurodegenerative disorders in which oxidative stress and mitochondrial dysfunctions are involved.

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Authors' disclosures available online (http://www.j-alz.com/disclosures/view.php?id=358).

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General discussion of article 1

Strong evidence supports that free radicals associated with senile plaques may mediate or contribute in the pathophysiology of Alzheimer's disease (AD) (McLellan *et al.*, 2003). Fresh Aβ peptide and oligomeric peptide are more efficient at generating reactive oxygen species (ROS) (Tabner *et al.*, 2005), whereas aged (enriched in fibrils) Aβ peptide and Aβ fibrils induced a higher level of lipid peroxidation and nitro-oxidative stress (Ill-Raga *et al.*, 2010; Melo *et al.*, 2009). In a comparative toxicity study between acrolein and ROS, acrolein was found to be more toxic than H_2O_2 and hydroxyl radical (OH•) (Yoshida *et al.*, 2009). Mitochondria are one of main intracellular targets of Aβ peptide, H_2O_2 and acrolein. Using human neuroblastoma SK-N-SH cell, we demonstrated the details of the neuroprotective effects of *Bacopa monniera (BM)* extract against H_2O_2 and acrolein induced-toxicity and further showed that this neuroprotection was mediated by modulating oxidative stress and preserving mitochondrial functions. A complete protection against H_2O_2 and acrolein-induced toxicity was observed in LDH assay by *BM* extract. On the contrary an incomplete protection was observed in other two assays. The reduction of XTT and Resazurin is believed to be mediated by different mitochondrial enzymes and particularly dehydrogenases. Some of these enzymes such as alpha-ketoglutarate dehydrogenase (KGDH), and succinate dehydrogenase (SDH) are susceptible to reversible oxidative inactivation (Hurd *et al.*, 2012; Nulton-Persson and Szweda, 2001; Shi *et al.*, 2011). So, it may be possible that *BM* extract could only partially prevent the oxidative inactivation of KGDH, SDH and acotinase and hence up to 80% of cell survival was observed in Resazurin and XTT assays. Furthermore, we showed that *Bacosides*, the triterpenoid saponins mediated *BM* extract induced neuroprotective effects. The antioxidant

activity of *BM* extract is further important because oxidative stress has been shown to mediate some of the early synaptic degeneration and cognitive decline in AD mouse models (Hartl *et al.*, 2012; Resende *et al.*, 2008). Levels of reduced glutathione (GSH) and vitamin E, have been shown to be decreased and lipid peroxidation products along with oxidized proteins increased before the deposition of senile plaques and neurofibrillary tangles in transgenic mouse models of AD (Ghosh *et al.*, 2012; Zhang *et al.*, 2012).

Several redox-regulated transcription and antioxidant enzymes modulate cellular response to ROS and some of these have been shown to be dysreguled in AD. Activation of transcription factor nuclear factor kappa B (NF-κB) mediates the production of various proinflammatory cytokines, inflammatory proteins and NF-κB inhibition protected neurons against Aβ induced toxicity (Choi *et al.*, 2012; Huang *et al.*, 2012). Drugs targeting NF-κB regulation may prove beneficial for memory disorders. Terpenoids compounds have been used in traditional Indian and Chinese medicines for various ailments and several of these have been shown to possess anti-inflammatory activities by inhibiting the NF-κB activation (Salminen, *et al.*, 2008). Several neuroprotective compounds such as resveratrol have been shown to activate Sirt1 which in turn regulate various antioxidant enzymes such as catalase and manganese superoxide dismutase (MnSOD) (Tanno *et al.*, 2010). Inhibition of extracellular signal-regulated kinase/mitogen-activated protein kinase (ERK/MAPK) and p66Shc signaling pathways also protected neurons against Aβ peptide induced toxicity (España *et al.*, 2010; Wu *et al.*, 2012) whereas, p66Shc is known to be a central regulator of mitochondria-dependent oxidative balance (Pesaresi *et al.*, 2011). Thus, by preserving cellular redox homeostasis and mitochondrial functions *BM* extract may enhance cell survival during oxidative stress.

Standardized extracts of *Bacopa monniera* protect against MPP+ and Paraquat induced- toxicities by modulating mitochondrial activities, proteasomal functions and redox pathways

Manjeet Singh[1], Ven Murthy and Charles Ramassamy[1,2]*

[1]INRS- Institut Annand Frappier

[2]Department of Medical Biology, Faculty ofMedicine

Laval University, Québec, Canada G1K 7P4

*Corresponding author: Prof. Charles Ramassamy

Address: INRS- Institut Annand Frappier

531, boul. des Prairies, Laval, Québec, H7V 1B7

Phone: l-450-687-5010

E-mail: Charles.Ramassamy@iaf.inrs.ca

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Scientific objectives of the article 2

Strong evidence supports the role of free radicals, oxidative stress, mitochondrial and proteasomal dysfunctions in Parkinsons's disease (PD) pathogenesis. The first evidence of mitochondrial complex 1 involvement in the pathophysiology of PD came from drug users who were accidentally exposed to 1-methyl-4-phenyl 1, 2, 3, 6-tetrahydropyridine (MPTP) and developed PD because the metabolite ofMPTP i.e. MPP+ is an inhibitor of the complex 1. Paraquat (PQ), is widely used as a herbicide and several epidemiological studies have identified PQ exposure as a potential risk factor for the onset of PD. In our previous article we demonstrated the modulation of H202 and acrolein-induced oxidative stress and mitochondrial dysfunctions with *BM* extract. Since, the MPP+ and PQ-induced toxicity also involves oxidative stress and mitochondrial dysfunctions, we proposed that with these biological activities of *BM* extract may protect the human neuroblastoma SK-N-SH cell line against the MPP+ and PQ-induced toxicity.

We demonstrated that a pre-treatment with the *BM* extract protected the SK-N-SH cells against MPP+ and PQ-induced toxicity in various cell survival assays, significantly inhibited the generation of intracellular reactive oxygen species (ROS) and superoxide anions, preserved the mitochondrial membrane potential and complexl activity. *BM* pre-treatment also prevented the MPP+ induced GSH depeletion and proteasomal activation in SK-N-SH cells. Additionally, we demonstrated that the *BM* extract alone could activate the transcription factor nuclear factor E2-related factor2 (Nrf2), a master regulator of cellular antioxidant response along with the various phase2 antioxidant enzymes. Thus, by modulating oxidative stress, GSH levels, mitochondrial and proteasomal functions along with the activation of Nrf2 pathway, *BM* extract could have a therapeutic application in the prevention and progression of PD.

Contribution of student

This article has been published in the "Toxicological Sciences". Ail the experimental work, data analyses and the first draft of this article were accomplished by **Manjeet Singh** with appreciable inputs from Prof. Charles Ramassamy, my current PhD supervisor. Charles Ramassamy submitted the article and both Manjeet Singh and Charles Ramassamy replied to the reviewer's comments.

Résumé de l'article en français

La maladie de Parkinson (MP) est l'une des maladies neurodégénératives liées à l'âge les plus courantes et affecte des millions de personnes à travers le monde. Des preuves solides soutiennent le rôle des radicaux libres, du stress oxydatif, des dysfonctionnements mitochondriaux et du protéasome dans la mort neuronale présente dans le cerveau de patients atteints la MP. Les facteurs environnementaux, en particulier, les pesticides représentent une des classes primaires des agents neurotoxiques associés à la MP et plusieurs études épidémiologiques ont identifié l'exposition de l'herbicide, paraquat (PQ) en tant que facteur de risque pour l'apparition de la MP. L'objectif de notre étude était d'étudier les effets neuroprotecteurs des extraits standardisés de *Bacopa monniera (BM)* contre la toxicité induite par le PQ et le MPP+, et d'élucider les mécanismes sous-jacents à cette protection. Nos résultats montrent qu'un traitement préalable avec l'extrait *BM* à partir de *50* J.Lg/ml protège la lignée cellulaire dopaminergique SK-N-SH contre la neurotoxicité induite par le MPP+ ou le PQ dans divers tests de survie cellulaire. Nous avons démontré que le prétraitement avec l'extrait de *BM* empêche la diminution de GSH en plus de préserver le potentiel de membrane mitochondriale et le maintien de l'activité du complexe mitochondrial 1. Le prétraitement avec 10.0 J.Lg/ml de *BM* a également empêché la production intracellulaire d'espèces oxygénées réactives (ROS) ainsi que la diminution des taux de superoxide mitochondriale. Le *BM* active le facteur nucléaire Nrf2 en modulant l'expression de Keapl et en régulant la synthèse du glutathione endogène. L'effet du BM sur la phosphorylation d'Akt renforce son rôle dans l'augmentation de la survie cellulaire. En préservant l'homéostasie redox et la survie cellulaire en situation de stress oxydatif les extraits de *BM* peuvent avoir des utilisations thérapeutiques dans différents maladies

neurodégénératives liées lâge telles que la maladie de Parkinson.

Strong evidence supports the role of free radicals, oxidative stress, toxic protein aggregation, mitochondrial dysfunctions, autophaghic and ubiquitin-proteasome system (UPS) dysfunctions in neuronal death in Parkinson's disease (PD) (Johri and Beal, 2012; Przedborski, 2005). Mitochondrial complex I involvement in the pathophysiology of PD came from drug users who were accidentally exposed to 1-methyl-4-phenyl 1, 2, 3, 6-tetrahydropyridine (MPTP) and developed PD because the metabolite of MPTP i.e. MPP^+ is an inhibitor of the complex I (Langston *et al.*, 1983). Additionally, recent epidemiological studies have positively linked PD with the pesticides paraquat and rotenone, which are known to increase oxidative stress and mitochondrial dysfunctions (Tanner *et al.*, 2011). Therefore, pathways controlling mitochondrial functions and reactive oxygen species (ROS) production by mitochondrial-targeted antioxidants are rapidly emerging as potential therapeutic targets in PD (Beal, 2009).

The antioxidant response element (ARE)-mediated gene activation is coordinated by nuclear factor E2-related factor2 (Nrf2). Nrf2, upon exposure to electrophiles or ROS, activates the expression of some endogenous antioxidant enzymes, modulates the removal of damaged proteins *via* proteasomal degradation or autophagy (Lewis *et al.*, 2010). In human neuroblastoma SK-N-SH cell, we demonstrated the neuroprotective effects of *BM* extract against MPP^+ and paraquat induced-toxicity. Furthermore, we showed that *BM* extract activated AKT, prevented paraquat-induced generation of ROS and superoxide anions, preserved cellular GSH levels and activated the transcription factor Nrf2, a master regulator of cellular antioxidant response along with the various phase2 antioxidant enzymes. Triterpenoid saponins are known to activate the Nrf2/ARE pathways by forming

a Michael adduct with reactive cysteine residues on Keap1 protein (Dinkova-Kostova *et al.*, 2005). Recently decaffeinated coffee and nicotine-free tobacco extracts also conferred neuroprotection in Drosophila models of PD through an Nrf2-dependent mechanism (Trinh *et al.*, 2010). Additionally, AKT activation has been shown to have trophic effect on dopaminergic neurons and prevented dopaminergic axonal loss through suppression of macroautophagy (Cheng *et al.*, 2011; Chung *et al.*, 2011; Ries *et al.*, 2006).

Mitochondrial dysfunctions play a critical role in the pathogenesis of PD. A 25-30 % decrease in the complex I activity along with oxidative damage to various complex I subunits and a reduction in succinate dehydrogenase (SDH) activity has been observed in PD patients (Beal, 2005; Keeney *et al.*, 2006; Parker *et al.*, 1989; Simunovic *et al.*, 2009). MPP^+ has been shown to induce loss in mitochondrial membrane potential (MMP) (Chen *et al.*, 2005) and a rapid increase in mitochondrial fragmentation (Wang *et al.*, 2011). In addition, evidence from PD-related toxins supports that mitochondrial fission; fusion, mitophagy and transport may be involved in the pathogenesis of PD (Arnold *et al.*, 2011; Van Laar and Berman, 2012). *In vitro* and *in vivo* models of PD also suggest that increased ROS production leads to mitochondrial dysfunction in dopaminergic neurons leading to subsequent cell death. In various models of PD, cell death was rescued by over expression of antioxidant genes e.g. glutathione S-transferase (GST), or thioredoxin that reduced ROS levels (Shi *et al.*, 2009; Smeyne *et al.*, 2007). However, recent study also suggests that mitochondrial functions and energy metabolism may be a more important target for the treatment of PD especially in ameliorating the Parkinsonian bradykinesia and synaptic failure (Vincent *et al.*, 2012). Therefore, by preserving mitochondrial functions and decreasing ROS production *BM* extract may have dual protective effects.

Neuroprotective mechanisms of the standardized extracts of *Bacopa monniera* in a paraquat/diquat- mediated acute toxicity

Manjeet Singh[1], Ven Murthy[2] and Charles Ramassamy[1,2]

[1]INRS- Institut Armand Frappier

[2]Department of Medical Biology, Faculty of Medicine
Laval University, Québec, Canada G1K 7P4

***Corresponding author**: Prof. Charles Ramassamy

Address: INRS- Institut Armand Frappier

531, boul. des Prairies, Laval, Québec, H7V 1B7

Phone: 1-450-687-5010

E-mail: Charles.Ramassamy@iaf.inrs.ca

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Scientific objectives of the article

Strong evidence suggests the role of oxidative stress and mitochondrial dysfunctions in the pathogenesis of Parkinsons's disease (PD). Recent epidemiologic and toxicological studies have shown that environmental factors especially, pesticides such as paraquat and rotenone represent one of the primary classes of neurotoxic agents associated with PD. Paraquat (PQ) and diquat (DQ), are bipyridyl compounds widely used as herbicides and undergo redox cycling leading to reactive oxygen species (ROS) formation. In our previous article we demonstrated the modulation of MPP^+ and PQ-induced oxidative stress and mitochondrial dysfunctions with *BM* extract in SK-N-SH cells. Since, both convergent and divergent mechanisms are involved in MPP^+ and PQ/DQ-induced toxicities, we proposed that *BM* extract may protect the rat dopaminergic PC12 cell line against PQ/DQ-induced toxicity.

We demonstrate that a pre-treatment with the *BM* extract protected the PC12 cells against PQ/DQ-induced toxicity in various cell survival assays, significantly inhibited the generation of ROS and superoxide anions and depolarized the mitochondrial membrane potential. *BM* pre-treatment also prevented the down regulation of tyrosine hydroxylase (TH) and antioxidant defense systems such as γ-glutamylcysteine synthetase (γ-GCS) and thioredoxin1 (Trx1) levels besides preventing the activation of AKT and heat shock protein90 (HSP90) proteins. Thus, our findings demonstrated that *BM* can protect dopaminergic cells through modulating cellular redox pathways which are altered in PD and could have a therapeutic application in the prevention of PD.

Contribution of student

This article has been accepted for publication in the "***Neurochemistry International***". All the experimental work, data analyses and article writing were accomplished by **Manjeet Singh** with appreciable inputs from Prof. Charles Ramassamy, my current PhD supervisor.

Résumé de l'article en français

La maladie de Parkinson (MP) est l'une des maladies neurodégénérative liée à l'âge la plus fréquente et touche des millions de personnes à travers le monde. Les preuves suggèrent un rôle du stress oxydatif et des dysfonctionnements mitochondriaux dans la pathogenèse de la MP. Derécentes études épidémiologiques et toxicologiques ont montré que les facteurs environnementau, en particulier les pesticides tels que le paraquat (PQ) et le diquat (DQ) représentent l'une des classes primaires des agents neurotoxiques associés à la MP. L'objectif de notre étude était d'étudier les effets neuroprotecteurs de l'extrait standardisé de *Bacopa monniera* (*BM*) contre la toxicité induite par le PQ/DQ et d'élucider les mécanismes sous-jacents à cette protection. Nos résultats ont montré qu'un prétraitement avec 20.0 µg/ml de l'extrait *BM*, protège les cellules dopaminergiques de la lignée de rat, les PC12, contre la toxicité induite par le PQ/DQ déterminée par différents tests de survie cellulaire. Nous avons démontré que le prétraitement avec 50.0 µg/ml de *BM* empêchait la génération intracellulaire d'espèces réactives de l'oxygène, diminuait les niveaux de superoxide et depolarize le potential mitochondrial. Le prétraitement avec l'extrait de *BM* a également empêché la régulation à la baisse de la tyrosine hydroxylase (TH) et des systèmes de défense antioxydants tels que le niveaux de γ-glutamylcystéine synthétase (γ-GCS) et thioredoxine 1 (Trx1). Ce prétraitement a également empêché l'activation de l'Akt et de la protéiné 90 de choc thermique (HSP90). Ainsi, nos résultats ont démontré que *BM* peut protéger les cellules dopaminergiques par la modulation des voies cellulaires redox qui sont altérées dans la MP et qu'il pourrait ainsi avoir une application thérapeutique dans la prévention de la MP.

Abstract

Parkinson's disease (PD) is one of the most common age related neurodegenerative disease and affects million of people worldwide. Strong evidence suggests a role for oxidative stress and mitochondrial dysfunctions in the pathogenesis of PD. Recent epidemiologic and toxicological studies have shown that environmental factors, especially herbicides such as paraquat and diquat represent one of the primary classes of neurotoxic agents associated with PD. The objective of our study was to investigate the neuroprotective effects of the standardized extract of *Bacopa monniera* (*BM*) against paraquat/diquat-induced toxicity and to elucidate the mechanisms underlying this protection. Our results showed that a pre-treatment with the *BM* extract, from 20.0 μg/ml, protected the rat dopaminergic PC12 cell line against paraquat/diquat-induced toxicity in various cell survival assays. We demonstrated that *BM* pre-treatment, from 5.0 μg/ml, could prevent the generation of intracellular reactive oxygen species (ROS), decreased mitochondrial superoxide levels and depolarized the mitochondria. *BM* pre-treatment also prevented the down- regulation of tyrosine hydroxylase (TH) and antioxidant defense systems such as γ–glutamylcysteine synthetase (γ-GCS) and thioredoxin1 (Trx1) levels. Furthermore, *BM* pre-treatment prevented the activation of Akt and heat shock protein90 (HSP90) proteins. Thus, our findings demonstrated that *BM* can protect PC12 cells through modulating cellular redox pathways which are altered in PD and could have a therapeutic application in the prevention of PD.

Key terms: Parkinson's disease, neuroprotection, oxidative stress, *Bacopa monniera*, redox regulation

1. Introduction

Parkinson's disease (PD) is the second most common age-related neurodegenerative disease affecting million of people worldwide. PD is characterized by the selective loss of dopaminergic neurons in the substantia nigra pars compacta (SNpc) of the brain and deposition of alpha α-synuclein containing lewy's bodies (LBs), leading to the characteristic motor and non motor symptoms (Braak *et al.,* 2003; Lang and Lozano, 1998). Strong evidence supports the role for oxidative stress and mitochondrial dysfunctions in the dopaminergic neuronal death in PD (Henchcliffe and Beal, 2008). Dopamine (DA) is a very reactive metabolite and both its non-enzymatic degradation as well as enzymatic oxidation by the monoamine oxidase (MAO) produce superoxide radicals, reactive quinones, hydrogen peroxide (H_2O_2) and dihydroxyphenylacetic acid (DOPAC) (Graham, 1978; Stokes *et al.,* 1999). Only 5% to 10% of PD cases are genetically related and the role of environmental factors in the etiology of PD has gained attention recently. Among different environmental factors, pesticides represent one of the primary classes of neurotoxic agents associated with PD (Moretto and Colosio, 2011; Tanner *et al.*, 2011). Several studies have documented that mitochondrial dysfunction and oxidative stress in the brain of PD are associated to pesticides exposure. Since the SNpc neurons already functions in a more redox active environment, this may enhance their susceptibility to various toxicants.

Paraquat (PQ) and diquat (DQ), are bipyridyl compounds with a structure similar to 1-methyl-4-phenyl-pyridinium iodide (MPP^+), are widely used as herbicides. Several epidemiological studies have identified PQ exposure as a potential risk factor for the onset of PD (Costello *et al.*, 2009; Hatcher *et al.*, 2008; Tanner *et al.*, 2011). PQ was first produced in 1961 and gained considerable attention because of its extreme toxicity in

human and its systemic exposure has been shown to be able to cross the blood-brain-barrier through the neutral amino acid transporter and to selectively damage the nigrostriatal dopaminergic system in mice causing a 20%–30% selective dopamine neuronal loss in the SNpc (McCormack and Di Monte, 2003). Therefore, PQ is commonly used both in *in vitro* and *in vivo* models of PD (Cannon and Greenamyre, 2010). On the other hand DQ exposure in human produced severe toxic effects on the central nervous system leading to symptoms of PD (Sechi *et al.*, 1992). Although exact mechanisms by which PQ and DQ induce PD in animal models are still unknown, several studies have shown that chronic exposure is associated with the induction of oxidative stress and inflammatory cascade which may be involved in the degeneration of nigral dopaminergic neurons (Bonneh-Barkay *et al.*, 2005; Drechsel and Patel, 2009; Kuter *et al.*, 2010; McCormack *et al.*, 2002). Studies of PQ/DQ neurotoxicity suggest a common mode of toxicity and investigating their interactive properties in different mixing ratios can provide a better insight into the evaluations of their potential toxicity hazard under varying experimental conditions that may mimic human exposures.

Prevalence of PD in Asian Indians is at least three times lower as compared to British Caucasians (Muthane *et al.*, 1998) and unlike the Americans there is no age-related loss of SNpc neurons in Asian Indians, which could be attributed to genetic, environmental or dietary causes (Alladi *et al.*, 2009). *Bacopa monnieri*, Linn. (*Brahmi*, *BM*) has been used in Indian Ayurvedic traditional medicine for almost 3000 years. *BM* is classified as a drug used to improve memory, cognition and as a potent nerve tonic (Howes and Houghton, 2003; Russo and Borrelli, 2005). *BM* has been found to be well tolerated without side effects (Russo and Borrelli, 2005) and the LD_{50} in rats was determined to be as high as 2.7

g/kg when administrated orally (Hota *et al.*, 2009). Recently, we have demonstrated the neuroprotective effects of the standardized extracts of *BM* against H_2O_2 and acrolein, a by-product of lipid peroxidation (Singh, *et al.*, 2010). Pharmacological studies have identified several triterpenoid saponins called as *Bacosides* to be active constituents of the *BM* extract (Chakravarty *et al.*, 2001; Murthy *et al.*, 2006). Although the *BM* extract and *Bacosides* compounds have been investigated extensively for their neuropharmacological activities, their beneficial effects on cellular pathways involved in the physiopathology of PD remain to be established. *BM* pre-treatment had been shown to protect Drosophila against PQ and rotenone-induced neurotoxicity and mortality likely by preventing the oxidative stress and mitochondrial dysfunctions (Hosmani and Muralidhara, 2009; Hosmani and Muralidhara, 2010). In light of these beneficial effects of the *BM* extract on different brain functions, we have thus tested the hypothesis that the *BM* extract can protect dopaminergic neurons against different pesticides. We have recently demonstrated that the *BM* extract can protect the human dopaminergic neuroblastoma cell line SK-N-SH cells against MPP^+-induced toxicity through the preservation of the mitochondrial and proteasomal activities and the reduced glutathione (GSH) levels (Singh *et al.*, 2012). Our preliminary data in SK-N-SH cells showed that the protective effect of the *BM* extract on MPP^+ and PQ was similar. The aim of the present study was to validate the protective effect of the *BM* extract against PQ/DQ mixture on another catecholaminergic cell type and to get further insight into the protective mechanisms of the *BM* extract against PQ/DQ-induced toxicity. In this study, the mechanisms underlying the protective effect of the *BM* extract on PQ/DQ-induced toxicity were initiated with the preservation of ROS and superoxide anions levels.

Our results show that on PC12 cells, *BM* induced-neuroprotection is mediated through the decreasing intracellular ROS and scavenging mitochondrial superoxide anions. *BM* pre-treatment also depolarized the mitochondria and prevented the down-regulation of different redox regulated proteins i.e. γ-glutamylcysteine synthetase (γ-GCS), thioredoxin1 (Trx1), tyrosine hydroxylase (TH) besides preventing the up-regulation of heat shock protein90 (HSP90) and Akt. These results validate the traditional use of *BM* as a nerve tonic agent and suggest its possible therapeutic application in the prevention of PD by modulating oxidative stress and redox signalling pathways.

2. Materials and Methods

2.1. Chemicals and Reagents

Mega cell Dulbecco's modified eagle's medium /Nutrient mixture (DMEM/F12 Ham), Foetal bovine serum (FBS), horse serum, Penicillin/streptomycin, Tox-2 (XTT based), Tox-8 (Resazurin based), dihydroethidium (DHE) and protease inhibitor cocktail were obtained from Sigma-Aldrich Inc. The cytotoxicity detection kit, based on lactate dehydrogenase (LDH) was from Roche Diagnostics. Paraquat (PQ) and Diquat (DQ) mixture containing 1.0 mg/ml each of PQ and DQ was from Ultrascientific, JC-1 (5, 5', 6, 6'- tetracholro- 1, 1', 3, 3'- tetraethylbenzimidazolyl carbocyamine iodide), 2, 7 dichlorofluoroscein diacetate (DCF-DA), and MitoSox red were from Molecular Probes. BCA protein estimation kit was from Pierce Biotechnology (Rockford, USA). Primary antibodies rabbit polyclonal anti-Akt, anti-phospho Akt, anti-Trx1, anti-γ-GCS, and mouse monoclonal anti-TH antibodies were from Abcam. Primary mouse monoclonal anti-HSP90 was from Stressgen. Mouse monoclonal anti-Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) antibody was from Millipore. Rabbit and mouse horseradish peroxidase (HRP)

conjugated secondary antibodies were obtained from Sigma Aldrich Inc. Fluorescence emission obtained with different probes was recorded using the Synergy HT multi-detection microplate reader.

2.2. Standardized extract of *Bacopa monniera (BM)*

Standardized extract of *BM* were kindly provided by Natural Remedies Private Limited, Bangalore, India. *BM* extract was certified to contain 11.5% of total *bacosides* as determined by high performance thin layer chromatography (HPTLC). *BM* extract further contained these bioactive phytocompounds i.e. *Bacoside A3* (1.5%), *Bacopaside II* (1.3%), Jujubogenin isomer of *bacopasaponin C* (1.6%), *Bacopasaponin C* (1.0%), *Bacopaside I* (1.1%), *Bacosine* (0.2%), *Apigenin* (0.1%) and *Luteolin* (0.2%) as determined by high performance liquid chromatography (HPLC). *Bacosides* have been shown to be stable in phosphate buffer solution between pH 6.8 to 9.0 at least for 5 days (Phrompittayarat *et al.*, 2008).

2.3. Cell culture and treatment

PC12 cells, a rat pheochromocytoma cell line from American Type Cell Culture (ATCC, Rockville, MD, USA), were maintained in DMEM/F12Ham containing 6% (v/v) FBS and 3% (v/v) horse serum, 4mM L- glutamine and penicillin/streptomycin at 37°C in a humidified incubator containing 5% CO_2. PC12 cells were plated at a density of 2.0 x 10^4 cells/well in 96 well plates (Corning, NY, USA) coated with 1X collagen and incubated at 37°C. After 24 h of plating, the media was completely removed and cells were kept in DMEM/F12Ham with antibiotics but without serum. Cells were treated with different doses of *BM* extract for 1 h prior to the addition of a mixture containing 80.0 µM of the paraquat (PQ) and 60.0 µM of the diquat (DQ) in the media containing *BM*. This combined dose of

PQ/DQ mixture was selected on the basis of dose toxicity response curves and resulted in almost 50% of cell death after 24 h of treatment.

2.4. Cytotoxicity and cell viability assays

Cytotoxicity and cell survival was measured by using LDH, XTT and Resazurin assays with commercial kits as described previously (Singh *et al.*, 2010). XTT and Resazurin assays may reflect metabolic alterations and these tests alone would lead to the underestimation of the number of living cells. Therefore, a valuable approach would be to integrate these assays with another one, allowing for the cross confirmation of the cytotoxicity and proliferation datasets. To distinguish between toxicity and proliferation effects of the *BM* extracts, LDH assay which measures the cellular membrane integrity and is a mean of quantifying dead cells was also done. The control value was considered as 100% and cell survival was expressed as a percentage of the control value.

2.5. Superoxide anion generation

Superoxide anion levels in mitochondrial and cytosolic compartments were determined by the fluorescent dyes MitoSox red and dihydroethidium (DHE), respectively. This assay is based on the reduction of DHE by superoxide anions to a fluorescent compound ethidium (Bindokas *et al.*, 1996). For this, cells were treated with *BM* for 1 h before the addition of a mixture containing 100.0 µM of PQ and 75.0 µM of DQ for another 1 h. After treatment, the media was removed and cells kept either in a MitoSox red solution (1.0 µM) or DHE solution (5.0 µM) for 30 min. Then, cells were washed with PBS and cellular fluorescence was recorded with λex at 510nm and λem at 580nm.

2.6. Intracellular reactive oxygen species (ROS)

Intracellular ROS generation was monitored by the oxidation of 2', 7'- dichlorofluorescein-diacetate (DCF-DA), a cell permeable dye which upon hydrolysis by intracellular esterases reacts with H_2O_2 to produce a highly fluorescent compound, 2', 7'- dichlorofluorescein (DCF) which is trapped inside the cells. Briefly, after 40 min of treatment with a mixture containing 200.0 μM and 150.0 μM of PQ and DQ respectively, DCF-DA was added to a final concentration of 10.0 μM for 20 min. Cells were then washed with PBS and the fluorescence was recorded with λex at 485nm and λem at 535nm as previously described (Singh *et al.*, 2010).

2.7. Mitochondrial membrane potential (MMP- $\Delta\psi_m$)

MMP was monitored using the cell permeable cationic fluorescent dye JC-1, which preferentially enters mitochondria due to the highly negative MMP as previously described (Singh *et al.*, 2010). Briefly, in healthy cells, the dye JC-1 accumulated in the mitochondrial matrix and become a J-aggregates form, which become fluorescent red. In unhealthy or apoptotic cells, the mitochondrial membrane potential collapses, and the JC-1 cannot accumulate within the mitochondria and in these cells JC-1 remains in the cytoplasm in a green fluorescent monomeric form. For this, cells were treated with *BM* for 1 h before the addition of a mixture of PQ (50.0 μM) and PQ (37.5 μM) for 24 h and 48 h. After treatment, the media was removed and cells were kept in a JC-1 solution (1.0 μg/ml) for 30 min. Then cells were washed with PBS and cellular green fluorescence was recorded with λex at 485nm and λem at 535nm while the red one with λex at 530nm and λem at 590nm using a Synergy HT multi-detection microplate reader. MMP was recorded as the ratio between red and green fluorescence.

2.8. Effect of *BM* treatment on different protein levels

Levels of γ-GCS, HSP90, Trx1, Akt, pAkt and TH were monitored in total proteins by Western blot. For this, cells were pre-treated with *BM* (40.0 µg/ml) for 1 h followed by PQ/DQ mixture treatment for different time intervals. After treatments, total proteins were isolated in a complete lysis buffer containing a cocktail of proteases and phosphate inhibitors as described previously (Singh *et al.*, 2010). Protein concentrations were estimated by a BCA kit. Proteins (25-30µg) were separated on a 10% SDS-PAGE and transferred to the polyvinylidene fluoride (PVDF) membranes. Membranes were blocked with 5% non-fat milk in TBS and then incubated overnight at 4°C with primary antibodies for γ-GCS (1:500), Trx1 (1:300), Akt (1:600), pAkt (1:500), TH (1:500) and HSP90 (1:1000). Then, the membranes were washed 3 times with TBST and incubated with horseradish peroxidase-conjugated secondary antibodies. After final washings the membranes were incubated with enhanced chemilumescent (ECL) western blotting substrates (Millipore, USA) for 5 min and bands were quantified by imaging densitometry using FlourChem HD2 system from Alpha Innotech. GAPDH was used as internal protein control.

2.9. Statistical analysis

All results were confirmed with at least three separate experiments and expressed as mean ±SEM. Data were analyzed for statistical analysis by one way analysis of variance (ANOVA) followed by a Dunnett's multiple means comparison test and the level of significance was considered when p-values <0.05.

3. Results

3.1. BM extract protects PC12 cells against Paraquat/Diquat- induced toxicity, decreases superoxide anion and intracellular ROS generation

Most of PC12 cells are positive for TH and also express dopamine transporters (Alyea and Watson, 2009). Therefore, these cells represent a suitable model to study the protective effect against paraquat/ diquat (PQ/DQ) toxicity. Results on the figure 1 showed that treatments with the *BM* extract for 1 h before the addition of PQ/DQ mixture protected PC12 cells against PQ/DQ-induced toxicity. This protection was assessed 24 hours after the addition of PQ/DQ through the survival LDH test and was significant from 20.0 µg/ml (Fig. 1A). To confirm the cytoprotective effect of *BM*, we completed with two survival assays Resazurin and XTT tests (Fig. 1B, C). A significant protection was also observed from 20.0 µg/ml of *BM* in Resazurin assay (Fig. 1B) while in XTT assay a protective effect was observed only from 40.0 µg/ml of *BM* and reached a maximum at 80.0 µg/ml (Fig. 1C).

Bipyridyl herbicides, such as PQ and DQ are known to undergo redox cycling leading to ROS formation and DQ is more effective at generating ROS as compared to PQ (Drechsel and Patel, 2009; Fussell *et al.*, 2011). PQ/DQ toxicity in mammalian cells is mediated through the production of superoxide anions and mitochondria have been identified as a primary source of PQ/DQ-induced ROS generation in the brain (Castello *et al.*, 2007; Drechsel and Patel, 2009; Wu *et al.*, 2005). So, next we have measured the level of mitochondrial and cytosolic superoxide anions levels by using, respectively, fluorescent dyes MitoSox red and dihydroethidium (DHE). As shown in Fig. 2A and B, there was a significant increase in superoxide anions which was more pronounced in mitochondrial compartment after 1 h of PQ/DQ treatment and this was significantly reduced by *BM* pre-treatment from 20.0 µg/ml. H_2O_2 is a metabolic by-product of mitochondrial respiration and its production is enhanced by mitochondrial complex I inhibitors such as rotenone,

dopaminochrome (a dopamine metabolite) and PQ/DQ. To investigate the antioxidant effect of the *BM* extract, we have measured the intracellular levels of ROS using a cell permeable fluorescent probe DCF-DA. PC12 cells treated with a high concentration of PQ/DQ (200.0 μM/ 150.0 μM) for 1 h displayed intense fluorescence after staining with DCF dye and this fluorescence was significantly lower when PC12 cells were pre-treated with *BM* from 5.0 μg/ml (Fig. 2C). In these experiments, higher doses of PQ/DQ were used to generate substantial levels of intracellular ROS. The ability of *BM* to scavenge superoxide anion may underlie the neuroprotective effects, since both the effects were significant from 20.0 μg/ml.

3.2. BM pre-treatment depolarized the mitochondria in PC12 cells

Mitochondria are a primary source of PQ/DQ-induced ROS in the brain which can induce mitochondrial depolarization. Next, we have investigated whether *BM* pre-treatment could rescue the MMP in PC12 cells. For this, JC-1 fluorescent dye was employed to determine the MMP in PC12 cells. A significant decrease in the JC-1 red/green fluorescence that is indicative of mitochondrial depolarization was observed only with *BM* pre-treatment from 5.0 μg/ml following an exposure with a mixture containing 50.0 μM and of 37.5 μM of PQ and DQ, respectively, for 24 hours (Fig. 3A). However after 48 hours both PQ/DQ mixture as well as *BM* pre-treatment significantly induced mitochondrial depolarization in PC12 cells (Fig. 3B). In these experiments, lower dose of PQ/DQ (50.0 μM/ 37.5 μM) was used to attenuate cell death. Recent studies have demonstrated that mild mitochondrial depolarization is a promising neuroprotective approach to prevent oxidative stress induced mitochondrial calcium overload and subsequent cell death (Garcia-Martinez *et. al.*, 2010).

3.3. Effect of BM pre-treatment on the γ-GCS, Trx1, Akt and HSP90 pathways

The antioxidant activity of *BM* was further strengthened by measuring the effect of *BM* treatment on different redox regulated pathways. We have previously showed that *BM* can activate the Nrf2 activity (Singh *et al.*, 2012). Next, we have studied the level of two Nrf2 regulated proteins i.e. γ-GCS and Trx1. GSH homeostasis is a complex process requiring the contribution of different pathways such as the level of the γ-GCS enzyme, a rate limiting enzyme involved in GSH synthesis (Griffith, 1999) and the Trx1 system. A pre-treatment with *BM* (40.0 μg/ml) for 1 hour significantly prevented the decrease in the γ-GCS level induced by 1 hour treatment with PQ/DQ (80.0 μM/60.0 μM) while no effect on the level of γ-GCS was observed after 24 hours of treatment with PQ/DQ (Fig. 4A). On the contrary, treatments with PQ/DQ (80.0 μM/60.0 μM) for 1 hour in the presence of the *BM* extract had no effect on the expression of Trx1 while it decreased after 24 hours of treatment with PQ/DQ (Fig. 4B). Interestingly, a pre-treatment with *BM* (40.0 μg/ml) significantly prevented the Trx1 down-regulation. Thus, PQ/DQ treatment induced an early decrease in γ-GCS levels while Trx1 levels were down-regulated only after a prolonged period of treatment.

Next, we have analyzed the effect of *BM* pre-treatment on the Akt pathway. Several studies have demonstrated an activation of phosphatidylinositol 3-kinase (PI3-K)/Akt by ROS (Lim and Clément, 2007). Accordingly, our results showed an increase in pAkt while a 1 hour pre-treatment with the *BM* extract prevented the phosphorylation of Akt (Fig. 5).

A significant increase in the levels of HSP90 was observed after 48 hours of treatment with PQ/DQ (Fig. 6) while no effect was observed after 24 hours of treatment. The activation of HSP90 was prevented in the presence of the *BM* extract. These results suggest that *BM* pre-

treatment may modulate different redox regulated and HSPs signalling pathways involved in cell survival.

3.4. BM treatment increases the TH levels

We have next investigated the effect of PQ/DQ and of *BM* on the expression of TH after a short and long period of treatment with PQ/DQ. As shown in Fig. 7, a pre-treatment with *BM* at 40.0 µg/ml significantly prevented the TH down-regulation observed after 1 hour of treatment with PQ/DQ (80.0 µM/60.0 µM). Moreover, an up-regulation in TH level was observed after 48 hours of treatment with the *BM* extract in the presence of PQ/DQ.

4. Discussion

Due to an extensive use, human exposure to multiple pesticides/herbicides occurs frequently. Recently, several epidemiologic studies have identified pesticides/herbicides including PQ/DQ and rotenone, as one of the primary classes of environmental neurotoxic agents associated with PD (Costello *et al.*, 2009; Moretto and Colosio, 2011; Tanner *et al.*, 2011). Many of these chemicals have been studied in isolation, neglecting mixture effects and instead their toxicity should be considered cumulatively for risk assessment to human population. Although, the exact mechanisms by which PQ/DQ induces PD in animal models are still unknown, several studies have shown that they induced mitochondrial dysfunctions and inflammatory cascade involving microglial activation with the production of oxidative stress. PQ cellular uptake is accomplished through the dopamine transporter (DAT) and cellular toxicity is determined by the $PQ(^{2+})/PQ(^{+})$ redox cycling, a process in which it accepts an electron from an appropriate donor and subsequently reduces O_2 to produce superoxide anion radical ($O_2^{\cdot-}$) (Bonneh-Barkay *et al.*, 2005; Drechsel and Patel, 2009; Mangano *et al.*, 2012; Purisai *et al.*, 2007; Rappold *et al.*, 2011; Wu *et al.*, 2005).

Similar to PQ, DQ also undergoes redox cycling leading to ROS formation and DQ is 10-40 times more effective at ROS generation compared to PQ (Bonneh-Barkay *et al.*, 2005; Drechsel and Patel, 2009; Fussell *et al.*, 2011). Dose-dependent axonal degeneration was observed in mouse dorsal root ganglion (DRG) neurons treated with PQ/DQ mixture (Fischer and Glass, 2010). PQ exposure in rats and mice increased mitochondrial H_2O_2 production, depolarized mitochondria, induced mitochondrial oxidative damage, and inactivated the mitochondrial iron sulfur (Fe-S) containing proteins such as aconitase (Cantu *et al.*, 2009, 2011; Chen *et al.*, 2012; Czerniczyniec *et al.*, 2011). Additionally, oxidation and inactivation of several redox regulated proteins in mitochondrial, cytosolic and membrane compartments has been implicated in PQ/DQ-induced toxicity. Oxidation of cytosolic proteins such as Trx1 and DJ-1 along with mitochondrial aconitase, thioredoxin-2 and peroxiredoxin-3 have been implicated in PQ-induced toxicity (Cantu *et al.*, 2009, 2011; Ramachandiran *et al.*, 2007; Roede *et al.*, 2011; Taira *et al.*, 2004; Yang *et al.*, 2010). Therefore, preventing the mitochondrial and cytoplasmic superoxide anions generation, mitigating the elevation of intracellular ROS and maintaining the functional integrity of various proteins and redox regulated pathways by natural antioxidant may mitigate PQ/DQ-induced toxicity and promote cell survival. Although preservation of mitochondrial MMP levels facilitates neuronal survival, however recent studies have demonstrated that mild mitochondrial depolarization also prevented oxidative stress and MPP^+-induced cell death (Garcia-Martinez *et. al.*, 2010; Lim *et al.*, 2012).

Recently, *Bacopa monnieri*, Linn. (*Brahmi*, *BM*) has received more attention because of its various physiological benefits especially related with its ability to improve memory, enhance cognition and as a potent nerve tonic (Howes and Houghton, 2003; Russo and

Borrelli, 2005). Our results showed that *BM* can protect PC12 cells against PQ/DQ-induced toxicity and demonstrated that the *BM* extract was able to depolarize mitochondria and prevent the elevation of ROS and particularly the production of superoxide anions in mitochondria. Recently, the triterpenoid Lanosterol has been shown to induce mild depolarization of mitochondria, to promote autophagy and protect dopaminergic neurons and mice against MPP^+ or 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP)-induced toxicty (Lim *et al.*, 2012). As several triterpenoid saponins or *Bacosides* are considered to be pharmacologically active constituents of the *BM* extract (Chakravarty *et al.*, 2001; Murthy *et al.*, 2006), we have explored if a similar mechanism was involved in the neuroprotective effect of *BM* against PQ.

Our results showed a discrepancy between LDH and XTT assays. There are several reasons for the discrepancy between complete protection in LDH assay and incomplete protection observed in the other two assays. The reduction of XTT and Resazurin is believed to be mediated by different mitochondrial enzymes and particularly dehydrogenases. Some of these enzymes such as alpha-ketoglutarate dehydrogenase (KGDH), succinate dehydrogenase (SDH), and aconitase are susceptible to hydrogen peroxide, superoxide and peroxynitrite anions-induced reversible inactivation (Nulton-Persson and Szweda, 2001; Shi *et al.*, 2011). So, it may be possible that *BM* extract could only partially prevent the oxidative inactivation of KGDH, SDH and acotinase and hence up to 80% of cell survival was observed in Resazurin and XTT assays.

Another strategy to counteract PQ/DQ-induced toxicity would be to enhance the cellular antioxidant defence mechanisms such as Trx1 and γ-GCS levels. Our results showed that *BM* could prevent the decrease of Trx1 and γ-GCS levels in the presence of PQ/DQ and this

effect of *BM* was observed as soon as 1 hour after PQ/DQ treatment. These effects are relevant to the pathophysiology of PD because in the SNpc of PD patients, a substantial decrease in GSH levels correlates with the severity of the disease (Riederer *et al.*, 1989). Our results also demonstrated that γ-GCS and Trx1 levels displayed different pattern of regulation to counteract the effect of PQ/DQ. In contrast to the expression of γ-GCS, the decrease of Trx1 induced by PQ/DQ was observed only after 24 hours of treatment. This finding is consistent with recent studies, which have shown that persistent oxidative stress is required to oxidize Trx1, which in turn facilitates its degradation (Ramachandiran *et al.*, 2007; Roede *et al.*, 2011; Zschauer *et al.*, 2011). For the first time, our results demonstrated that *BM* was also able to prevent the decrease the Trx1 after 24 hours of PQ/DQ treatment. This effect is of great interest because Trx1 can scavenge the intracellular ROS through different cysteine residues and the mitochondrial thioredoxin/peroxiredoxin system represents the main enzymes involved in the regulation of cellular GSH homeostasis and H_2O_2 degradation in cytosolic and mitochondrial compartments, respectively (Drechsel and Patel, 2010; Lillig and Holmgren, 2007; Wang *et al.*, 2010). Trx1 is also involved in the neuroprotective effect against PQ by preventing the activation of the apoptosis signal-regulating kinase 1 (ASK-1) (Hansen *et al.*, 2004; Niso-Santano *et al.*, 2010). Therefore, by preventing the oxidation of these antioxidant enzymes, *BM* pre-treatment can also mitigate PQ/DQ-induced toxicity and promote cell survival.

Akt is another redox regulated protein as it is activated by different oxidants such as H_2O_2, superoxide anions or mitochondrial respiratory complex I inhibitors likely through a transient oxidative inactivation of the phosphatase and tensin homolog (PTEN) and this effect is attenuated by antioxidants (Lim and Clément, 2007; Sharma *et al.*, 2011). Akt

activation is considered as a compensatory mechanism to activate different cell survival pathways against oxidative stress (Aleyasin *et al.*, 2010; Martindale *et al.*, 2002). We have previously demonstrated that the *BM* extract alone activated the Akt pathway (Singh *et al.*, 2012) which may be related with the neurotrophic effects of *Bacosides* as some of structurally related triterpenoids have been shown to induce the phosphorylation of RET (rearranged during transfection), a receptor for glial derived neurotrophic factor (GDNF) and its downstream PI3K-Akt effectors (Mograbi *et al.*, 2001; Tohda and Joyashiki, 2009). In the present study, we showed that PQ/DQ mixture activated the Akt signaling cascades and this activation was prevented due to the preservation of the cellular redox status with the antioxidant activity of *BM.*

For the first time our data demonstrate that HSP90 was activated by PQ/DQ mixture. HSPs are important chaperone proteins involved in the removal of damaged, misfolded, and aggregated proteins. In PD, HSP90 has been linked to many pathogenic events. In the presence of the *BM* extract, the elevation of HSP90 was prevented likely due to the reduction of oxidative stress and thereby lowering damaged proteins. Recently, HSP90 inhibition has been shown to decrease cellular superoxide anion radical production by destabilizing NADPH oxidases, one of enzymes involved in PQT mediated superoxide anion radical production (Chen *et al.*, 2011).

TH is the rate limiting enzyme in the synthesis of dopamine (Dunkley *et al.*, 2004). It also contains a nonheme iron (ferrous) which undergoes oxido-reductive reactions to regulate its functions (Frantom et *al.*, 2006). Furthermore, TH has been reported to be a substrate of the ubiquitin-conjugating enzyme system to promote its proteasomal degradation (Døskeland and Flatmark 2002; Shi and Habecker, 2011). In the presence of PQ/DQ mixture, the

elevation of superoxide anions and H_2O_2 may be responsible for an early decrease in TH level by inducing its proteasomal degradation. *BM* pre-treatment, by decreasing superoxide anions and H_2O_2, prevented the down regulation of TH. Our results are consistent with recent findings demonstrating that low levels of forskolin, a diterpene from Indian Coleus plant, increased TH levels and phosphorylation in a cyclic AMP (cAMP) dependent manner in PC12 within 1 hour of treatment (Park *et al.*, 2012). Moreover, TH transcription in PC12 is also stimulated by cell density and this may have resulted in increased TH level in the presence of the *BM* extract after 48 hours of treatment (Kim *et al.*, 1995). TH overexpression in the presence of the *BM* extract in PC12 cells could further contribute to higher resistance of PC12 cells against PQ/DQ-induced oxidative stress. Indeed, it has been demonstrated that the overexpression of TH protected cells against H_2O_2 and 6-hydroxydopamine (6-OHDA)-induced toxicities and increased the ability of neurons to detoxify peroxide, by increasing glutathione reductase (GR) and glutathione peroxidise (GP_x) activities (Franco *et al.*, 2010). However, we cannot exclude the possibility that the mixture PQ/DQ induced non-dopaminergic cells death because the levels of TH was not decreased after 24 hours of treatment.

Using the rat dopaminergic pheochromocytoma PC12 cells, our results showed that *BM* pre-treatment protected these cells against PQ/DQ-induced toxicity in LDH, XTT and Resazurin assays which are measures of the membrane and mitochondrial integrity. Our results showed that the pre-treatment with *BM* reduced ROS, superoxide anions and depolarized mitochondria in the presence of the PQ/DQ mixture. *BM* extract also preserved the expression of γ-GCS, Trx1 and TH. *BM* can also promote neuronal survival in the presence of PQ/DQ by modulating the Akt and HSP90 proteins. The protective effect of

BM may be attributed to the presence of bioactive compounds which may be responsible for quenching of free radicals or alternatively it may be due to the up-regulation of antioxidant systems or modulation of redox-sensitive pathways (Singh *et al.*, 2012).

Since a multiple hit hypothesis has been proposed in the progress and development of PD (Sulzer, 2007), the botanical extracts containing multiple classes of chemical entities with synergic property may hold a better promise for therapeutic benefits and applicability in neuroprotection as compared to single chemical entity. Our investigation provides novel and important insights into the neuroprotective mechanisms of *BM* in the hope that this will open up new avenues for its research, application and therapeutic uses. In the long term, *BM* may be of therapeutic interest following pesticides intoxication because in addition to the protection against PQ/DQ, we have previously demonstrated that the *BM* extract could also protect against MPP^+ (Singh *et al.*, 2012). *BM* could be used in the prevention and treatment of PD as well as in age-related neurodegenerative disorders in which oxidative stress and mitochondrial dysfunctions are involved.

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Fig. 1A

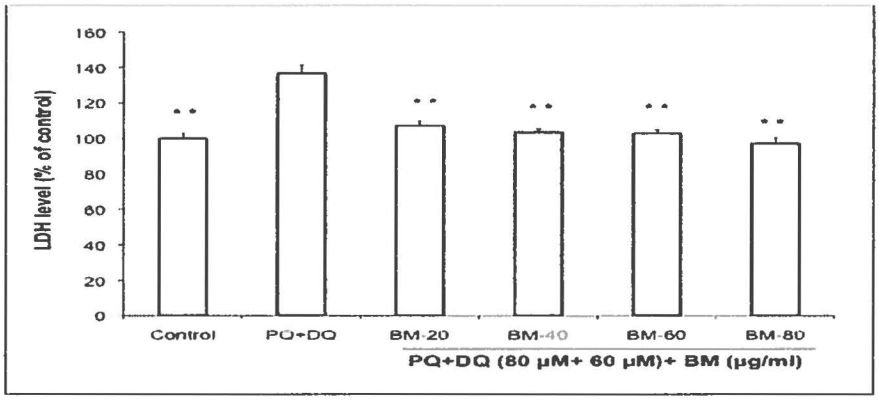

Fig. 1B

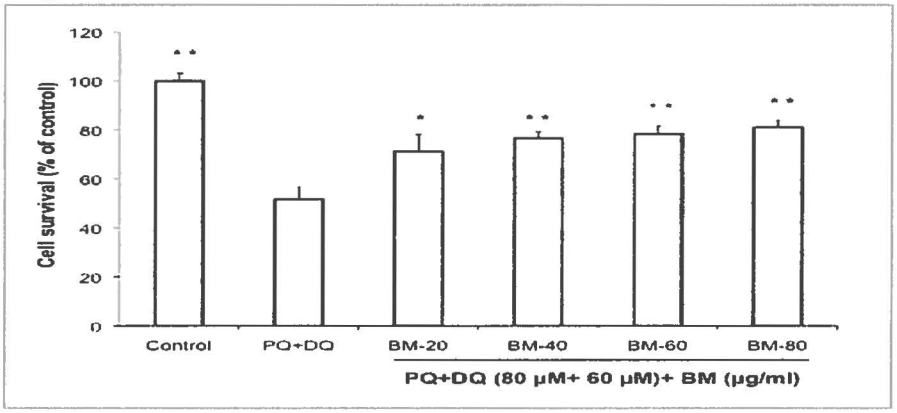

Fig. 1C

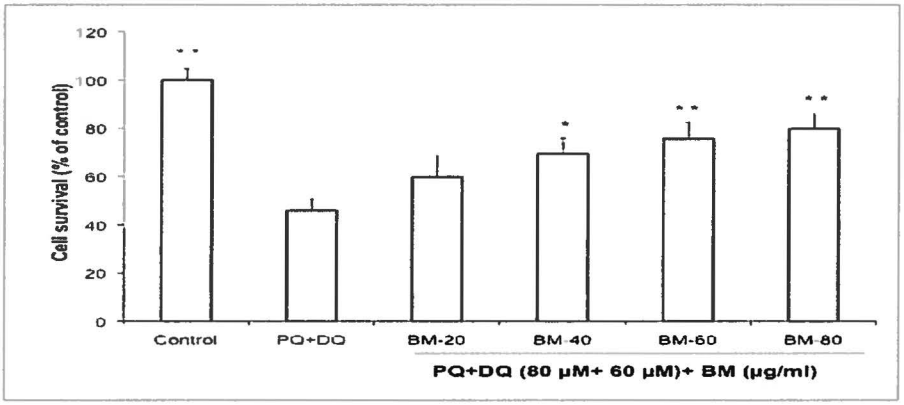

Fig. 1 Effects of *BM* on PC12 cells survival/death in the presence of PQ+DQ.

Cells were treated with *BM* for 1 hour before the addition of 80.0 µM and 60.0 µM of PQ+DQ respectively. Cells death was evaluated 24 hours after PQ+DQ treatment by (**A**) LDH assay and cells survival was analysed by (**B**) Resazurin and (**C**) XTT assays. Results are expressed as percentage of control (taken as 100%). Data are means ± SEM of at least three separate experiments performed in quadruplicate in each group with $*P < 0.05$ and $**P < 0.01$ *versus* PQ+DQ group.

Fig. 2A

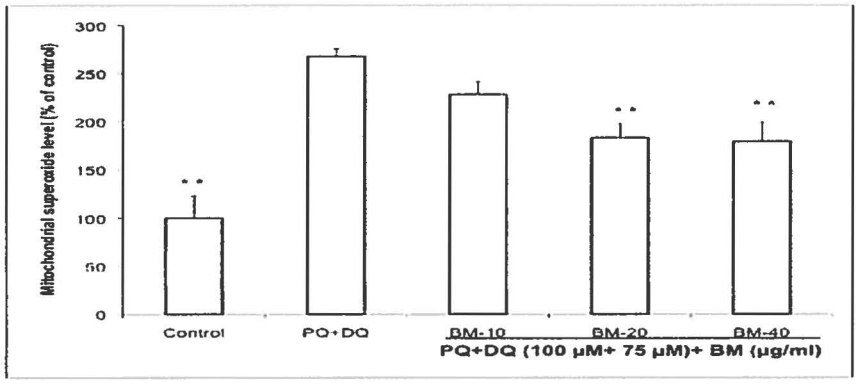

Fig. 2B

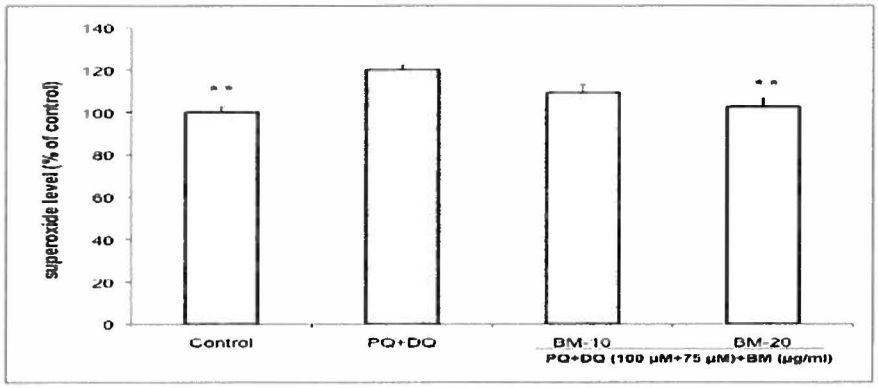

Fig. 2C

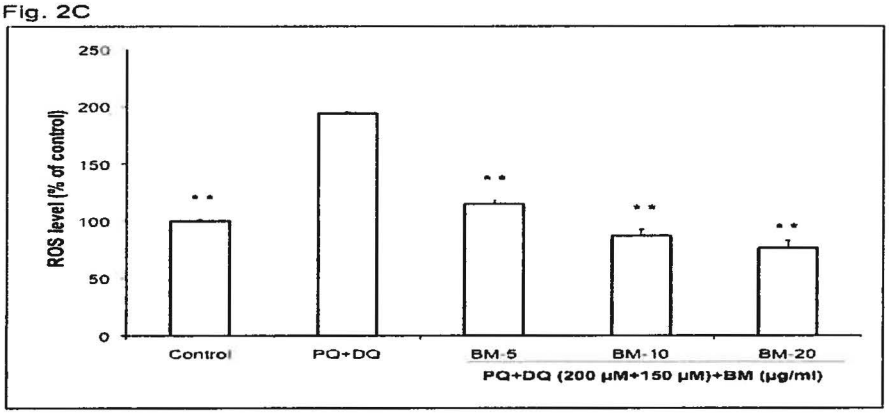

Fig. 2 Effects of *BM* on the levels of ROS, superoxide anions and proteins oxidation in the presence of PQ+DQ. Cells were treated with *BM* for 1 hour before the addition of PQ+DQ. **(A)** Mitochondrial and **(B)** cytoplasmic levels of superoxide anions were monitored by the fluorescent dyes MitoSox red and DHE, respectively, 1 hour after 100.0 µM and 75.0 µM of PQ+DQ treatment respectively. **(C)** Intracellular ROS levels measured by the fluorescent dye DCFDA 1 hour after 200.0 µM and 150.0 µM of PQ+DQ treatment respectively. For 2A, B, C Data are means ± SEM of at least three separate experiments performed in quadruplicate in each group with $^{**}P < 0.01$ *versus* PQ+DQ groups.

Fig. 3A

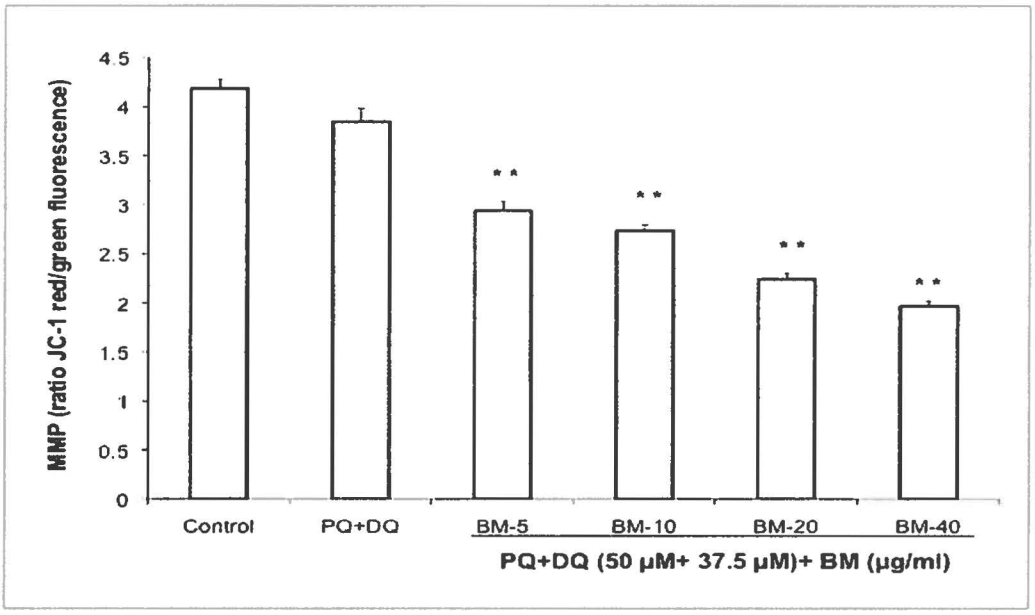

Fig. 3B

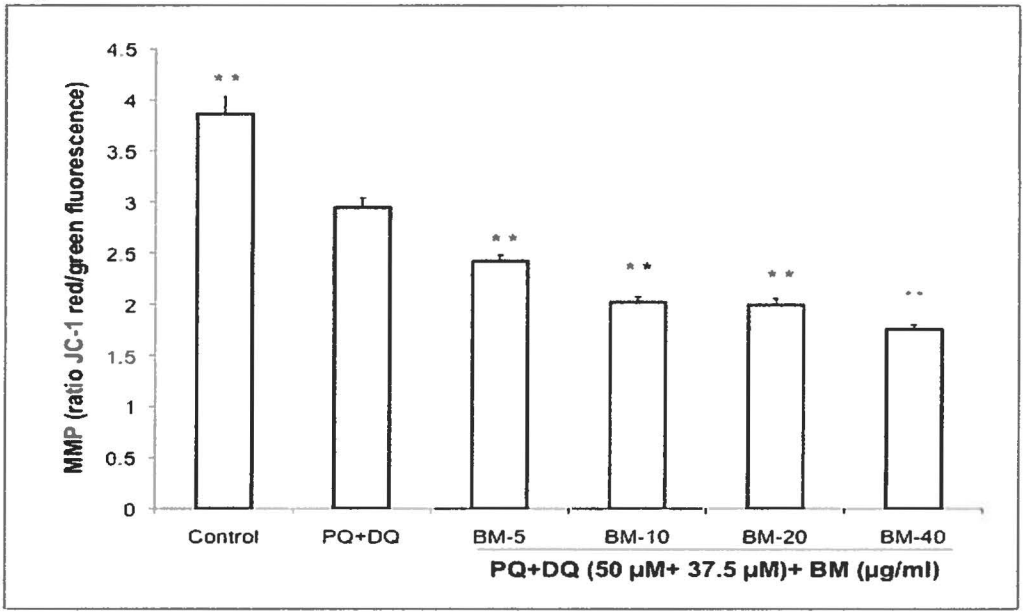

Fig. 3 Effects of *BM* on the mitochondrial membrane potential (MMP) in the presence of PQ+DQ.

PC12 cells were treated for 1 hour with *BM* followed by 50.0 µM and 37.5 of PQ+DQ respectively and then MMP was measured **(A)** 24 hours and **(B)** 48 hours after PQ+DQ treatments by the fluorescent dye JC-1. MMP was expressed as a ratio between JC-1 red and green fluorescence. Data are means ± SEM of at least three separate experiments performed in quadruplicate in each group with $^{**}P < 0.01$ *versus* PQ+DQ group.

Fig. 4A

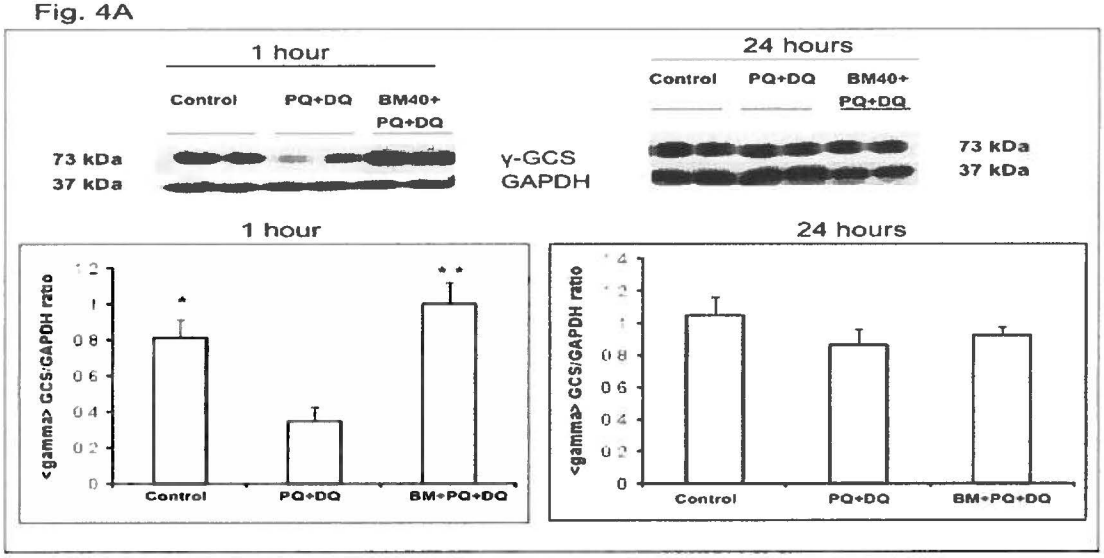

Fig. 4B

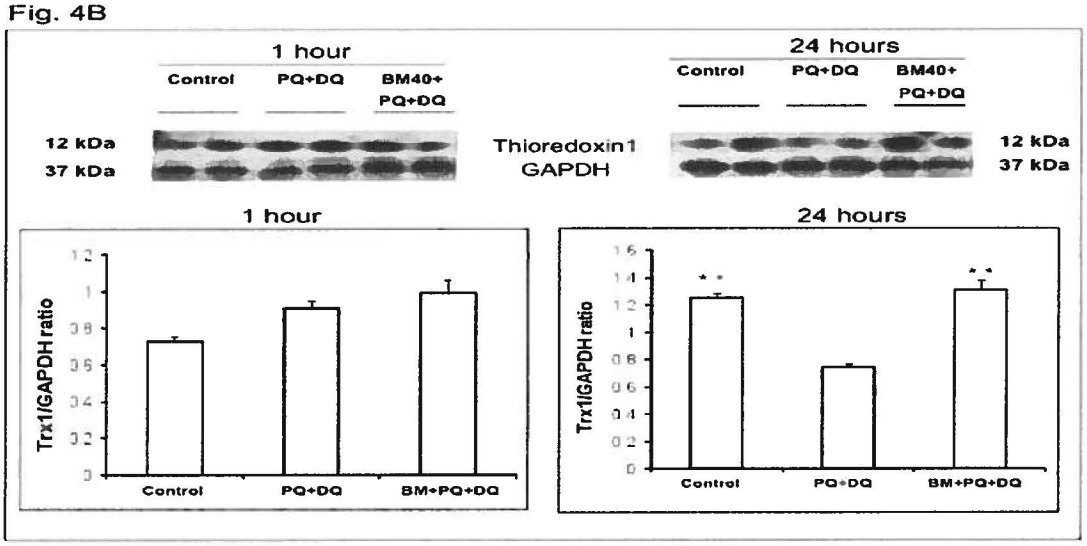

Fig. 4 Effect of *BM* on the levels of γ-GCS and Trx1 in the presence of PQ+DQ in PC12 cells.

PC12 cells were treated for 1 hour with *BM* (40 μg/ml) followed by 80.0 μM and 60.0 μM of PQ+DQ respectively for 1 hour and 24 hours. Total proteins were isolated for γ-GCS and Trx1 proteins analysis by Western blot and GAPDH was used as the loading control. Blots represent one of three independent experiments. (**A**) Bar graphs represent quantitative results of ratio between total γ–glutamylcysteine synthetase (γ-GCS) and GAPDH and (**B**) the ratio between total thioredoxin1 (Trx1) and GAPDH. Data are means ± SEM of at least three separate experiments performed in triplicate in each group. $*P < 0.05$ and $**P < 0.01$ *versus* PQ+DQ group.

Fig. 5

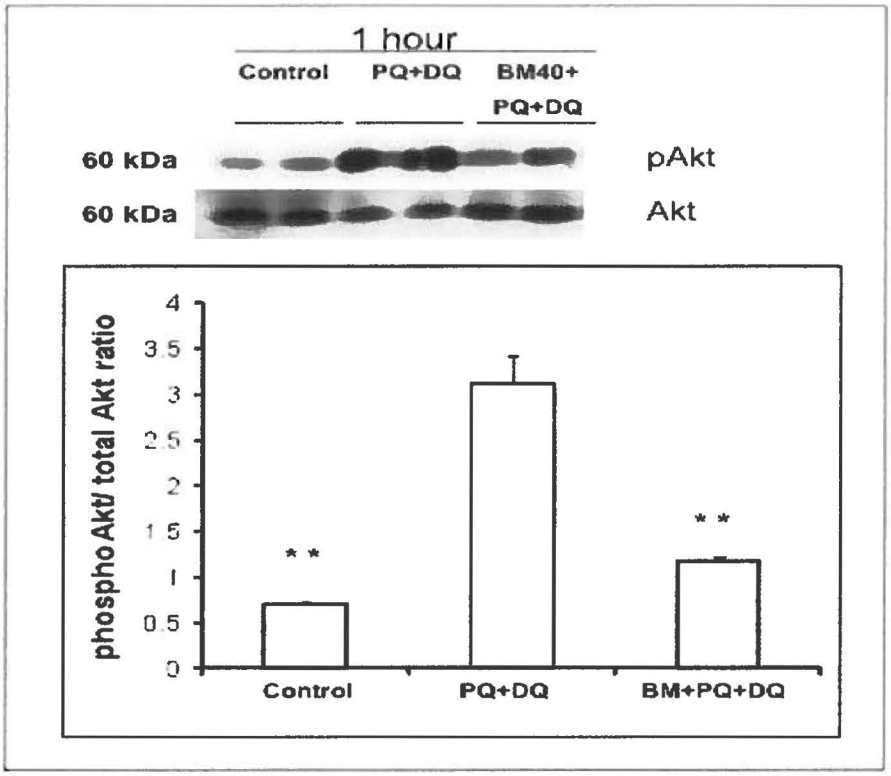

Fig. 5 Effects of *BM* on the activation of Akt in the presence of PQ+DQ.

PC12 cells were treated for 1 hour with *BM* (40 μg/ml) followed by 80.0 μM and 60.0 μM of PQ+DQ respectively for 1 hour. Levels of phopho Akt were measured by Western blot and total Akt was used as the loading control. Blots represent one of three independent experiments. Bar graph represents quantitative results of ratio between phopho Akt and total Akt. Data are means ± SEM of at least three separate experiments performed in triplicate in each group. $^{**}P < 0.01$ *versus* PQ+DQ group.

Fig. 6

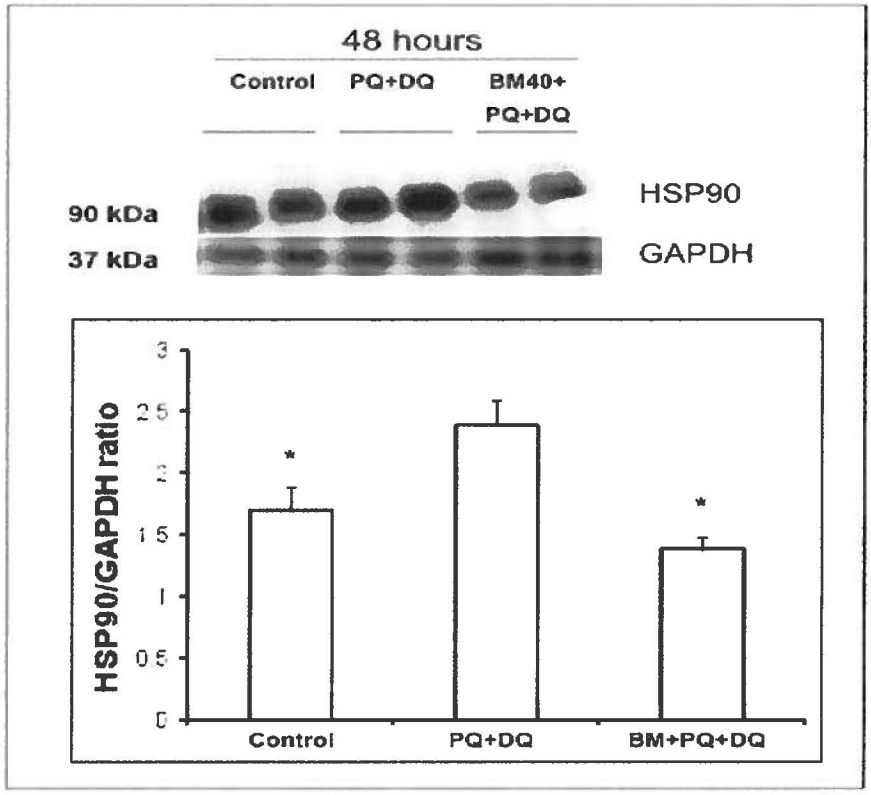

Fig. 6 Effect of *BM* on the levels of HSP90 in PC12 cells in the presence of PQ+DQ.
PC12 cells were treated for 1 hour with *BM* (40 μg/ml) followed by 80.0 μM and 60.0 μM of PQ+DQ respectively for 48 hours. Total proteins were collected for HSP90 Western blot analysis with GAPDH being used for loading control. Blots represent one of three independent experiments. Western blotting images HSP90 with GAPDH after 48 hours of PQ+DQ treatment. Bar graphs represent quantitative results of the ratio of HSP90/GAPDH. Data are means of at least three independent experiments (n= 3). *$P < 0.05$ *versus* PQ+DQ group.

Fig. 7

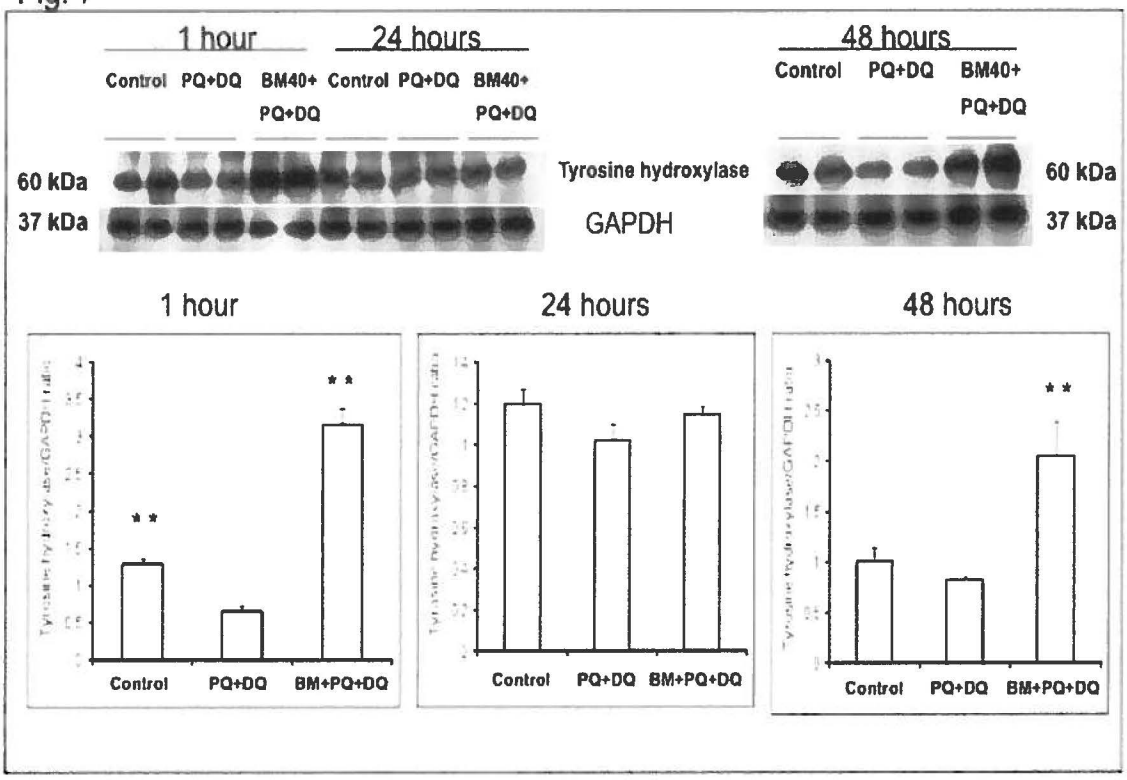

Fig. 7 Effects of *BM* on the levels of TH in the presence of PQ+DQ. PC12 cells were treated for 1 hour with *BM* (40 μg/ml) followed by 80.0 μM and 60.0 μM of PQ+DQ respectively for 1, 24 and 48 hours. Levels of TH protein were measured by Western blot and GAPDH was used as the loading control. Bar graphs represent quantitative results of ratio between total TH and GAPDH. Data are means ± SEM of at least three separate experiments performed in triplicate in each group. $^{**}P < 0.01$ *versus* PQ+DQ group.

Chronic exposure to the pesticides, such as paraquat and rotenone, has been suggested as a risk factor for Parkinson's disease (PD) (Tanner *et al.*, 2011). Paraquat (PQ) is the second most widely used herbicide in the world and was identified as a neurotoxicant based on the structural similarities to MPP^+ (1-methyl-4-phenylpyridine). Unlike MPP^+ it is not an efficient inhibitor of mitochondrial complex-1 (IC20 of 7 mM) (Richardson *et al.*, 2005). PQ specifically oxidized the cytosolic form of thioredoxin and conversely, MPP^+ oxidized the mitochondrial form of thioredoxin (Ramachandiran *et al.*, 2007). PQ is a redox cycler, which in turn leads to reactive oxygen species (ROS) formation and superoxide anions generation (Drechsel and Patel, 2009). Diquat (DQ) is another herbicide that generates superoxide anions through redox cycling; however, DQ is more effective at generating ROS than PQ (Fussell *et al.*, 2011). Therefore, antioxidants controlling ROS production and degradation may protect against PQ and DQ-induced toxicity.

Since PQ induced apoptosis in rat dopaminergic PC12 cells but not in human neuroblastoma SH-SY5Y cells (Klintworth *et al.*, 2007), we demonstrated the neuroprotective effects of *BM* extract against PQ/DQ-induced toxicity in PC12 cells. We showed that *BM* extract prevented PQ/DQ-induced generation of ROS and superoxide anions, and modulated the expression of various redox-regulated proteins. The antioxidant activity of *BM* extract may be involved in the neuroprotection as oxidative stress has been shown to induce cell death in neuroblastoma cells through the induction of autophagy, apoptosis and necrosis (Castino *et al.*, 2010, 2011; Zhang *et al.*, 2009).

Several redox-regulated antioxidant proteins that modulate cellular responses to ROS were dysreguled in PC12 cells after PQ/DQ treatment. Tyrosine hydroxylase (TH) is a rate-

limiting enzyme in dopamine (DA) biosynthesis. PQ/DQ treatment significantly reduced TH levels in PC12 cells and this effect may be either mediated by direct oxidation of TH by ROS or indirectly through the PQ-induced accumulation of alpha (α)-synuclein that may represses TH levels (Wills *et al.*, 2012; Zhu *et al.*, 2012). Improving the striatal TH expression using drugs or gene therapy has received considerable interest as a therapeutic strategy in PD (Feve, 2012; Zhu *et al.*, 2012). PQ/DQ treatment also significantly reduced thioredoxin1 (Trx1) levels in PC12 cells and this effect was reversed by *BM* extract. Trx1 is crucial for the activity of different transcription factors e.g. nuclear factor E2-related factor2 (Nrf2) and nuclear factor kappa B (NF-κB) (Hansen *et al.*, 2004; Lillig and Holmgren, 2007). *BM* extract also prevented the down-regulation of γ-glutamylcysteine synthetase (γ-GCS), a rate-limiting enzyme involved in the synthesis of reduced glutathione (GSH), the most abundant nonprotein thiol that has been shown to protect cells against exogenous and endogenous toxins, including ROS, lipid peroxidation products and electrophiles (Dickinson and Forman, 2002). Perhaps even more interesting was the finding of a down-regulation of phospho-Akt, which is generally considered as pro-survival, with *BM* extract. However, activation of the phosphoinositide-3kinase (PI3K)-Akt-mammalian target of rapamycin (mTOR) (PI3K-Akt-mTOR) signaling pathway has been shown to promote necrotic cell death *via* suppression of autophagy (Wu *et al.*, 2009). For the first time our data demonstrate that HSP90 was activated by PQ/DQ mixture. Recently, receptor interacting protein 1 (RIP1), a protein modulating apoptosis and necroptosis, has been identified as a HSP90-associated kinase and HSP90 inhibitors reduced RIP1 level (Chen *et al.*, 2012). Thus, by further modulating various redox-signaling pathways *BM* extract may enhance the neuronal survival during PQ/DQ-induced oxidative stress.

Chapter 5. Discussion

Alzheimer's disease (AD) and Parkinson's disease (PD) are two of the most common age related neurodegenerative disorders affecting millions of people worldwide. One of the most important hallmarks of these diseases is the accumulation and aggregation of misfolded pathogenic amyloid proteins, which are highly organized cross-β-sheet-rich protein or peptide aggregates (Jucker and Walker, 2011; Ross and Poirier, 2004). However, it is still unkown whether the amyloid deposits are the cause or a consequence of ongoing neurodegenerative changes or the end products of cellular protective and adaptive mechanisms (Treusch *et al.*, 2009). Pathologically AD is characterized by formation of intracellular neurofibrillary tangles (NFTs) composed of tau protein and extracellular deposition of amyloid-beta (Aβ) plaques within the hippocampus and neocortex accompanied by loss of neurons and synapses (Duyckaerts *et al.*, 2009; Holtzman *et al.*, 2011; Mattson, 2004). Accordingly the '*amyloid cascade hypothesis*' posits that misfolding and aggregation of Aβ is a fundamental event in the pathogenesis of AD (Hardy and Selkoe, 2002). PD on the other hand is characterized by a profound and selective loss of nigrostriatal dopaminergic neurons leading to primarily motor symptoms (tremor, rigidity, bradykinesia, and postural instability) and non motor symptoms. The main pathological features of PD are selective loss of dopaminergic neurons in the Substantia Nigra pars compacta (SNpc) and the presence of intra-cytoplasmic, eosinophilic proteinaceous inclusions called Lewy bodies (LBs) and dystrophic Lewy neuritis in surviving neurons (Spillantini *et al.*, 1997,1998). Although AD and PD are two distinct clinicopathological entities, several studies have now demonstared that the clinical features and the pathology of sporadic AD and PD may overlap (McKeith 2000, 2006; Lippa *et al.*, 2007). Infact,

approximately 25% of all patients with AD develop Parkinsonism and 50% of AD cases exhibit LBs, and about 50% of all cases of PD develop AD-type dementia after 65 years of age (Hansen *et al.*, 1990). Neurons are lost in aging brain, however both genetic and environmental factors in the later stages of life may intensify cholinergic and dopaminergic neuronal cell death resulting in neurodegenerative diseases such as AD and PD (Bishop *et al.*, 2010; Collier *et al.*, 2011). Genetic components inspite of contributing to < 10% of familial variants of AD and PD have provided invaluable insights in understanding the different mechanisms involved in neurodegeneration (Ashe and Zahs, 2010; Bertram *et al.*, 2010; Martin *et al.*, 2010, 2011). Both the diseases are characterized by an elevation in oxidative stress markers, abnormal toxic protein aggregation, mitochondrial dysfunctions, neuroinflammation, autophaghic and ubiquitin-proteasome system (UPS) dysfunctions which may lead to the selective loss of neurons in different areas of brain (Ang *et al.*, 2010; Cookson and Bandmann, 2010; Crews and Masliah, 2010; Glass *et al.*, 2010; Hegde and Upadhya, 2011; Jucker and Walker, 2011; Lin and Beal, 2006).

I- Environmental and dietary factors in neurodegenerative diseases

Epidemiological studies have further provided some important insights and valuable clues in the prevention and pathogenesis of AD and PD by identifying environmental and dietary factors associated with the increased or decreased risk of AD and PD. Among different environmental factors, pesticides especially that can cause mitochondrial dysfunctions and oxidative stress represent one of the primary classes of neurotoxic agents associated with PD (Moretto and Colosio, 2011; Tanner *et al.*, 2011). Recently, several studies have shown that higher adherence to a Mediterranean diet (MediD) could be associated with slower cognitive decline, reduced risk of AD, and decreased mortality in AD patients (Frisardi, *et*

al., 2010a). Similarly, coffee users also exhibit a significantly reduced risk of developing PD as compared to the general population (Hancock *et al.*, 2007; Morelli *et al.*, 2010). Additionally, in some countries like India where several medicinal plants are used as nootropic agents and brain tonics to restore age-related decline in mental abilities, the incidence rates of the age-specific AD in rural Indians are at least three times lower than those of an age-matched American reference population (Chandra, *et al.*, 2001). Moreover, prevalence of PD in Asian Indians is also at least three times lower than British Caucasians (Muthane *et al.*, 1998) and unlike the Americans there is no age-related loss of SNpc neurons in Asian Indians (Alladi *et al.*, 2009). These studies suggest that MediD, coffee extract or medicinal plants may contain some neuroprotective agents. Some of the biologically active components of MediD, coffee extract and medicinal plants belong to the family of polyphenols or terpenes. Polyphenol antioxidants have received worldwide attention because a number of these have shown neuroprotective effects in both *in vitro* and *in vivo* models of AD and PD by modulating protein aggregation, oxidative stress, mitochondrial functions, inflammatory cascade and cellular redox mechanisms (Ebrahimi and Schluesener 2012; Singh *et al.*, 2008; Williams and Spencer, 2012). On the other hand, very few studies are available on the beneficial effects of terpenes, which are natural components of the human diet and have drawn worldwide attention because of their cholesterol-lowering properties (Moreau, *et al.*, 2002). In the USA, the triterpenoid intake is 30 mg/kg/day, whereas in Mediterranean countries their intake may reach up to 400 mg/kg/day based upon a diet rich in olive oil, fruits and vegetables (Saleem, 2009). It is possible that some of the beneficial effects of the MediD, coffee extract and medicinal plants in AD and PD may be mediated by these terpene compounds. These findings from

epidemiological studies may be further useful in determining the dietary or environmental interventions needed for promoting healthy brain aging.

II- Oxidative stress and maintainence of cellular redox homeostasis

Although there are several intracellular sites of reactive oxygen species (ROS production), mitochondria are the single largest source of ROS and around 1-2% of oxygen consumption is diverted into mitochondrial ROS (Balaban *et al.*, 2005). The initial ROS formed within mitochondria is superoxide anion ($O_2^{\cdot -}$) (Collins *et al.*, 2012; Finkel, 2011). $O_2^{\cdot -}$ generation is an indicator of functional status because its production is altered by many cellular factors including the membrane potential, the reduction state of electron carriers and post-translational modification or damage to the respiratory chains (Collins *et al.*, 2012). However, $O_2^{\cdot -}$ is mostly converted to hydrogen peroxide (H_2O_2) by copper/zinc superoxide dismutase (Cu-Zn-SOD/SOD-1) and manganese superoxide dismutase (MnSOD/SOD2). In addition, in the cell, H_2O_2 can react with other molecules such as redox-active metals involving iron and copper leading to the formation of hydroxyl radicals ($OH^{\cdot}$) through the Fenton reaction. Cells are endowed with glutathione peroxidase enzymes, which work in conjunction with peroxiredoxin-thioredoxin system to remove H_2O_2 within mitochondria. However, when ROS and reactive nitrogen species (RNS) accumulate beyond a homeostatic set point they induce net oxidation of cellular macromolecules leading to cell death or apoptosis, also referred to as type programmed cell death (PCD) and autophagy which is characterized as type II PCD (Circu and Aw, 2010). Elevated ROS and RNS levels can also result in damage to phospholipids and polyunsaturated free fatty acids (PUFAs), which are both highly prevalent in the brain and very susceptible to oxidative damage resulting in a large number of compounds such as

malondialdehyde (MDA), 4-hydroxy-nonenal (4-HNE), 4-oxo-2-nonenal (4-ONE), trans-4-hydroxy-2-hexenal (HHE), acrolein and nitro-fatty acids (Bochkov *et al.*, 2010). Lipid peroxidation products participate in multiple signaling events including antioxidant response pathways, heat shock response, stress-responsive mitogen activated protein kinases (MAPKs), and nuclear factor kappa B (NF-κB) signaling pathways (Higdon *et al.*, 2012; Schopfer *et al.*, 2011). In AD and PD, lipid peroxidation products has been shown to be increased in the post-mortem brain tissue compared to age-matched controls, as shown by increase in the levels of MDA, 4-HNE protein adducts, 4-ONE, acrolein, HHE, isoprostanes, and isofurans (Bradley *et al.*, 2010, 2012; Dexter *et al.*, 1989; Shamoto-Nagai *et al.*, 2007; Yoritaka *et al.*, 1996). In addition to lipids, proteins are the most frequent and one of the prime targets for oxidative damage and elevations in the levels of oxidized proteins as well as in protein hydrophobicity in brain increased with age (Dasuri *et al.*, 2010a,b; Smith *et al.*, 1991). Cells maintain a healthy proteome through the lysosomal system and UPS, which are responsible for the proteolytic removal of damaged proteins (Davies, 2001; Ciechanover, 2005). Severe or sustained oxidative stress impaired the function of the UPS and decreased intracellular proteolysis (Davies, 2001; Shang and Taylor, 2011). Thus, modulation of ROS and RNS levels, preventing lipid peroxidation and protein oxidation through natural antioxidants is an important strategy for neuroprotection against oxidative stress (Biasutto *et al.*, 2011; Linseman, 2009).

The maintenance of intracellular redox homeostasis is dependent on a complex web of cellular redox circuit that consists of a battery of small molecules and antioxidant enzymes, which are tightly regulated by various redox-regulated transcriptional factors. Nuclear factor E2-regulated factor2 (Nrf2) is one of the most important sensitive transcription

factors to ROS and master regulator of cellular antioxidant response. Under normal physiological conditions, it is retained in their inactive forms in cytoplasm by binding to its inhibitory protein, Kelch-like ECH-associated protein (Keap1) (Jaiswal, 2004; Kaspar *et al.*, 2009). An increase in the Nrf2 level in the nucleus is required for activation of the antioxidant responsive element (ARE) genes (Zhang and Gordon, 2004). Nrf2 is emerging as a guardian of healthspan and gatekeeper of species longevity. Activation of Nrf2-ARE pathway induces multiple avenues of cytoprotection by activating the transcription of more than 200 genes that are crucial in the metabolism of drugs and toxins, protection against oxidative stress and inflammation, including the production of some endogenous antioxidant enzymes such as glutathione S- transferase (GST), glutathione reductase (GR), glutathione peroxidises (GPx) and of γ-Glutamycysteine ligase or synthetase (γ-GCL/ g-GCS), a rate limiting enzyme involved in reduced glutathione (GSH) synthesis. The Nrf2-ARE pathway also plays an integral role in stability of proteins and in the removal of damaged proteins *via* proteasomal degradation or autophagy (Lewis *et al.*, 2010). A decline in the transcriptional activity of Nrf2 with aging, which subsequently lead to the age-related loss of GSH synthesis has been observed in mice and rats (Duan *et al.*, 2009; Suh *et al.*, 2004). Another endogenous Nrf2 activator gene p62/SQSTM1 (sequestosome1) also undergoes oxidative damage in its promoter region during aging (Du *et al.*, 2009; Jain *et al.*, 2010). P62/SQSTM1 is not only an important machinery for removal of toxic protein aggregates through autophay but it also regulates the activation of Nrf2 through inactivation of Keap1 (Komatasu *et al.*, 2010). Thus, both p62 and Nrf2 complement each other functions in response to oxidative stress by creating a positive feedback mechanism. Hence, sustained activation of Nrf2-ARE and p62 pathways is emerging as a novel

pharmacological target for neuroprotective therapy that in turn will preserve the cellular redox homeostasis with aging and mitigate the high concentrations of ROS and RNS that can be important mediators of damage to nucleic acids, lipids and proteins contributing to age-associated neurodegenerative diseases such as AD and PD (Cuadrado *et al.*, 2009; Salminen *et al.*, 2012).

III- Antioxidant effects of *BM* extract

Interestingly, using a cell permeable fluorescent dye dichlorofluorescein-diacetate (DCF-DA), which upon hydrolysis by intracellular esterases reacts with H_2O_2 to produce a highly fluorescent compound, 2', 7'- dichlorofluorescein (DCF) (Kalyanaraman *et al.*, 2012), we observed that *BM* extract possessed potent anti-oxidant activity against various paradigms of oxidative stress i.e. H_2O_2, paraquat (PQ) and iron (Singh *et al.*, 2010, 2012). Furthermore, H_2O_2 degradation was also measured using cell-free system i.e. an Apollo 4000 Free Radical Analyzer (Drechsel and Patel, 2010). Additionally, the antioxidant activity of *BM* extract was also screened by using another two fluorescent dyes MitoSox red and dihydroethidium (DHE), which specifically measure $O_2^{\bullet -}$ levels in mitochondrial and cytosolic compartments, respectively (Bindokas *et al.*, 1996; Kirkland *et al.*, 2007). We observed that *BM* extract also possessed $O_2^{\bullet -}$ scavenging activity (Singh *et al.*, 2012). Furthermore, using western blot approach, we observed that *BM* extract prevented protein oxidation induced by H_2O_2 and acrolein treatments (Singh *et al.*, 2010, 2012). Furthermore, we measured the intracellular GSH levels using another cell permeable fluorescent dye monochlorobimane (MCB), which reacts with GSH in the presence of GSTs to produce a highly fluorescent bimane-GSH adduct (Shrieve *et al.*, 1988) and showed that *BM* extract

prevented L-buthionine-[S,R]-sulfoximine (BSO) and 1-methyl-4-phenyl-pyridinium iodide (MPP^+) induced intracellular GSH depletion (Singh *et al.*, 2010, 2012).

These findings are consistent with recent *in vitro* work where methanolic extract of *BM* scavenged nitric oxide (NO) and intracellular ROS in astrocytes (Russo *et al.*, 2003a). Additionally, *Bacosides* quenched superoxide anions and hydroxyl radicals (Pawar *et al.*, 2001; Russo *et. al.*, 2003b). The overall antioxidant effects of *BM* extract remain important as excessive generation of $O_2^{\cdot -}$, intracellular ROS, NO and H_2O_2 have been shown to induce neuronal cell death through apoptotic and autophagic pathways (Chen *et al.*, 2009; Castino *et al.*, 2011; Franklin, 2011; Higgins *et al.*, 2011; Lee *et al.*, 2012). The catalase like effect/activity of *BM* extract is further interesting as oxidative stress, probably mediated by the H_2O_2, generated during the Aβ peptide interactions with metals and metabolism of dopamine (DA), is an essential and crucial step in the neuronal cell death in AD and PD (Graham, 1978; Rival *et al.*, 2009). Moreover, recent studies have shown that Aβ peptide deactivated catalase, resulting in increased levels of intracellular oxidative stress (Habib *et al.*, 2010). Mitochondria-targeted catalase or treatment with a catalase mimetic, EUK-207 decreased Aβ peptide deposits, oxidative DNA damage and lipid peroxidation in amygdala and hippocampus in a transgenic mouse model of AD (Clausen *et al.*, 2012; Mao *et al.*, 2012). Similarly, superoxide dismutase/catalase mimetics also protected primary dopaminergic mesensephalic cultures as well as adult mice against PQ and 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) induced toxicity (Peng *et al.*, 2005; 2009; Perier *et al.*, 2010). The superoxide dismutase/catalase mimetic activities of *BM* extract further strengthen its application as a mitochondrial-targeted antioxidant and

especially as an effective therapeutic approach to ameliorate the oxidative stress in the pathophyiology of AD and PD.

IV- *BM* extract and lipid peroxidation

BM extract also showed potent neuroprotective activity against acrolein induced toxicity in various cell survival assays and prevented acrolein-induced dysregulation of redox-regulated proteins such as NF-κB, extracellular regulated protein kinases (ERK1/2) and p66Shc (Singh *et al.*, 2010). This is further interesting because in a comparative toxicity study acrolein was found to be more toxic than H_2O_2 and hydroxyl radical ($OH^{\bullet}$) (Yoshida *et al.*, 2009). Furthermore, amongst different α, β-unsaturated aldehydes, acrolein reacts 110–150 times faster with GSH than HNE or crotonal and this rapid cellular GSH depletion may underlie acrolein-induced oxidative damage and neurotoxicity (Hamann and Shi, 2009). Although the overall neuroprotective mechanisms of *BM* extract against acrolein-induced toxicity remains unclear, the ability of *BM* extract to up-regulate GSTs may partially mediate this effect as the detoxification of acrolein involves multiple pathways, which include reduction, oxidation, and conjugation with GSH that is catalyzed by GSTs. Additionally, *BM* extract prevented acrolein-induced protein oxidation (Singh *et al.*, 2010). Furthermore, we specifically followed the oxidation of p66Shc, which has been shown to play an important role in mitochondrial metabolism, ROS generation and apoptosis thereby emerging as a novel mitochondrial redox enzyme or sensor (Gertz and Steegborn, 2010; Giorgio *et al.*, 2005; Nemoto *et al.*, 2006). In our studies acrolein treatment induced p66Shc tetramers in SK-N-SH cells and *BM* treatment could reverse the phospho-p66Shc tetramers formation when GSH was exogenously added (Singh *et al.*, 2010). These findings are consistent with previous reports regarding p66Shc tetramers generation in presence of

oxidative stress that were further reversed in the presence of reducing agents (Gertz *et al.*, 2008). These studies suggest that by decreasing protein oxidation and protecting against lipid peroxidation product acrolein, *BM* extract may ameliorate the pathophysiology of AD and PD.

V- *BM* extract activated the Nrf2, a master regulator of cellular antioxidant response

Finally, we also observed that *BM* extract activated the Nrf2 pathway and enhanced the activation of various phase-2 antioxidant enzymes such as GPx, GR, GST, Trx1 besides increasing the levels of γ-GCS/γ-GCL, a rate limiting enzyme involved in the GSH synthesis (Griffith, 1999; Singh *et al.*, 2012). The effects of *BM* extract on Nrf2 pathway and various phase-2 antioxidant enzymes such as GPx, GR, GST and antioxidant proteins γ-GCS, Trx1 and GSH levels are of special interest as Nrf2-ARE pathway is differentially activated in AD and PD and an adaptive response to oxidative stress may be a key survival mechanism for neurons affected by AD and PD. For instance, in SNpc of PD, it has been shown that neuromelanin-containing neurons exhibited strong nuclear Nrf2 localization whereas as in AD, Nrf2 was retained in the cytoplasm (Ramsey *et al.,* 2007). Consequently, in the frontal cortex from the AD brains the levels of different antioxidants such as GSH, GPx, GST, GR, and Trx1 have been shown to be significantly declined (Ramsey *et al.,* 2007, Ansari and Scheff, 2010). On the contarary the expression of Nrf2 regulated proteins GST and heme oxygenase-1 (HO-1) increased in the SNpc neurons containing LBs from the brain tissue from PD patients (Power and Blumbergs, 2009; Schipper *et al.,* 1998; Shi *et al.*, 2009). However, their activation may not be sufficient enough to protect DA neurons against ongoing pathophysiological insults and exogenous agents may be needed to boost the activity of Nrf2-ARE pathway to impart neuroprotection. Our results showed that *BM*

can activate the Nrf2 pathway through the degradation of Keap1 (Singh *et al.*, 2012). In recent studies decaffeinated coffee and nicotine-free tobacco extracts conferred neuroprotection in *Drosophila* models of AD and PD through an Nrf2-dependent mechanism by mitigating ROS generation and oxidative damage by modulating the expression of phase-2 antioxidant enzymes and GSH levels (Trinh *et al.*, 2010). Furthermore, *BM* extract also preserved the GSH levels against various insults and induced GSH synthesis and turnover with the elevation of the expression of γ-GCL; which is a heterodimer consisting of a 73-kDa heavy or catalytic subunit (γ-GCLC) and a 28-kDa light modulatory subunit (γ-GCLM) and both the subunits contain an ARE sequence in their promoter regions (Chan *et al.*, 2001; Suh *et al.*, 2004). These results are relevant for the *BM* extract induced-neuroprotection because various toxins such as Aβ, H_2O_2, acrolein, BSO, PQ, MPP^+ and DA are known to deplete cellular GSH through different mechanisms (Drechsel *et al.*, 2007; Graham, 1978; Schildknecht *et al.*, 2009). Moreover, In the SNpc of PD, the depletion of GSH is one of the earliest biochemical changes and the degree of GSH depletion correlates with disease severity (Jenner, 1998; Riederer *et al.*, 1989). Additionally, several studies have demonstrated a deficit in total GSH levels in transgenic mouse models of AD before the deposition of amyloid plaques and up-regulation of GSH synthesis protected neurons against Aβ-induced oxidative stress and neurotoxicity (Zampagni *et al.*, 2012; Zhang *et al.*, 2012). Given these considerations, different neuroprotective therapeutic strategies based on the activation of Nrf2 along with preserving cellular GSH levels are also being explored for targeting the oxidative stress in AD and PD (Cuadrado *et al.*, 2009).

Furthermore, *BM* extract also increased the activities of phase-2 antioxidant enzymes GST, GPx, GR and Trx1 (Singh *et al.*, 2012). The family of GPx enzymes is responsible for the enzymatic degradation of H_2O_2, organic hydroperoxides, peroxynitrite and control the activities of cyclooxygenase (COX) and lipoxygenase (LOX) enzymes (Arthur, 2000; Conrad *et al.*, 2007). Over-expression of GPX-1 protected dopaminergic cells and mice against 6-hydroxydopamine (6-OHDA)- and PQ-induced toxicity whereas GPX4 over-expression protected cortical neurons from H_2O_2- and Aβ peptide-induced toxicity (Cheng *et al.*, 1998; Gardaneh *et al.*, 2011; Ran *et al.*, 2006; Ridet *et al.*, 2006). On the other hand, GR regulated intracellular GSH recycling from glutathione disulfide (GSSG, or oxidized glutathione) using nicotinamide adenine dinucleotide phosphate (NADPH) as an effective tool for cell survival under oxidative stress (Dickinson and Forman, 2002; Harvey *et al.*, 2009). Moreover, brain mitochondrial H_2O_2 removal is primarily dependent on the GPx and GR systems (Zoccarato *et al.*, 2004). GST is a family of 21 structurally diverse enzymes in humans, including both soluble and membrane-bound proteins, involved in the regulation of oxidative stress by conjugating and detoxifying electrophiles such as 4-HNE and acrolein with reduced GSH, and studies have shown that GST polymorphism play a role in the determination of onset of AD (Blair, 2006; Pinhel *et al.*, 2008). Recent studies have also shown that GST-Pi and GST-Omega played an important role in the progression of PD (Kim *et al.*, 2012; Shi *et al.*, 2009; Smeyne *et al.*, 2007). Trx1 is another Nrf2-ARE regulated protein that sscavenges intracellular ROS through different cysteine residues in the mitochondria and cytosol compartments (Hansen *et al.*, 2004; Lillig and Holmgren, 2007). Additionally, thioredoxin activated various cell survival signaling pathways by up-regulating brain-derived neurotrophic factor (BDNF) and glial cell line-derived

neurotrophic factor (GDNF) (Kong *et al.*, 2010). *BM* extract thus by modulating the key components of Nrf2-ARE pathway including the GPx, GR, GST, and Trx1 my further mitigate intracellular ROS levels and formation of lipid hydroperoxides, thereby further enhancing cell survival in the presence of various toxic insults.

VI- *BM* extract prevented the activation of the NF-κB

There is sufficient evidence indicating that NF-κB contributes to neurodegenerative processes and controls the expression of several inflammatory genes related to AD and PD pathology, including that of cyclooxygenase-2 (COX-2) and iNOS (Gao *et al.*, 2008; Hayden and Ghosh, 2008; Saijo *et al.*, 2009). In fact, several studies indicate NF-κB inhibition may provide therapeutic benefits in the treatment of chronic neuroinflammatory conditions as well as neuroprotection in AD and PD. Using western blot approach, we observed that *BM* extract prevented NF-κB activation induced by H_2O_2 and acrolein treatments (Singh *et al.*, 2010). These results are important as activated NF-κB has been detected in degenerating neurons, glial cells and dopamineric cells in the brain of AD and PD patients respectively (Hunot et al., 1997; Kaltschmidt *et al.*, 1997), and selective inhibition of NF-κB protected dopaminergic neurons from the MPTP- and 6-OHDA-induced toxicity (Ghosh *et al.*, 2007, 2009; Lee *et al.*, 2011). Besides, NF-κB inhibitors also protected neuronal cells against Aβ peptide induced toxicity and preserved cognition and memory in a transgenic mice model of AD (Longpré *et al.*, 2006; Echeverria *et al.*, 2009; Sung *et al.*, 2004). Different mechanisms may be involved in the overall effect of *BM* extract on NF-κB activation. Interestingly, some triterpenoid can also interact and oxidize Cys-179 of IKKβ and Cys-38 of p65 protein thereby inhibiting NF-κB signaling (Salminen *et al.*, 2008). Secondly, Sirt1 is another redox-regulated protein capable of inhibiting the

NF-κB signalling in microglia and astrocytes thereby protecting AD neurons against Aβ peptide toxicity (Chen *et al.*, 2005; Longpré *et al.*, 2006). Moreover, recent studies have also identified Nrf2 as one of the mediators of NF-κB-dependent inflammatory gene expression, microglial dynamics, neuroinflammation, and neuronal death in mice models of PD (Lastres-Becker *et al.*, 2012; Rojo *et al.*, 2010). Thus by preventing the NF-κB activation *BM* extract may modulate the chronic neuroinflammatory conditions involved in the pathophysiology AD and PD.

VII- *BM* extract preserved mitochondrial functions

In addition to being an important source of ROS generation, mitochondria are also an important target of ROS in AD and PD (Lin and Beal, 2006). Mitochondrial dysfunctions including reduced activity of cytochrome C oxidase (COX, Complex IV of mitochondria electron transport chain, ETC) and Complex I have been observed in AD and PD, respectively, in brain tissue, fibroblasts, and platelets (Cardoso *et al.*, 2004; Schapira *et al.*, 1989). In addition to complex I and complex IV activities, a decrease in protein level and activity of various mitochondrial dehydrogenases such as pyruvate dehydrogenase (PDH), succinate dehydrogenase (SDH) and α-ketoglutarate dehydrogenase (KGDH) has been reported in AD and PD patients (Hauptmann *et al.*, 2009; Rhein *et al.*, 2009; Simunovic *et al.*, 2009). Both Aβ and H_2O_2 has been shown to inactivate some of the mitochondrial enzymes especially KGDH, SDH, aconitase and COX (Casley *et al.*, 2002; Nulton-Persson and Szweda, 2001; Shi *et al.*, 2011). Moreover, accidental discovery of MPTP whose toxic metabolite MPP^+, through selective uptake in dopaminergic neurons, caused Parkinsonism in designer-drug abusers due to mitochondrial dysfunctions especially by inhibiting complex I further cemented the role of mitochondrial dysfunctions in PD (Langston *et al.*,

1983). Additionally, recent epidemiological studies have positively linked PD with the pesticides PQ and rotenone, which are known to increase oxidative stress and mitochondrial dysfunctions (Tanner *et al.*, 2011). PQ exposure in rats and mice increased mitochondrial H_2O_2 production, depolarized mitochondria, induced mitochondrial oxidative damage, and inactivated the mitochondrial iron sulfur (Fe-S) containing proteins such as aconitase (Cantu *et al.*, 2009, 2011; Chen *et al.*, 2012; Czerniczyniec *et al.*, 2011). Therefore, pathways controlling mitochondrial functions, dynamics and ROS production by mitochondrial-targeted antioxidants are rapidly emerging as potential therapeutic targets in AD and PD (Beal, 2009; Dumont *et al.*, 2010; Manczak *et al.*, 2010).

In our study mitochondrial membraine potential (MMP) was assessed using cell permeable fluorescent dyes 5, 5', 6, 6'- tetracholro- 1, 1', 3, 3'- tetraethylbenzimidazolyl carbocyamine iodide (JC-1) and rhodamine123, which preferentially accumulate in mitochondria. In contrast to rhodamine 123, JC-1 is more consistent and sensitive in its response to mitochondrial depolarization (Salvioli *et al.*, 1997). JC-1 exists in its monomeric form, but in the presence of a high MMP, aggregates are formed that are associated with a shift in fluorescence (from green to red). In our studies, we observed that *BM* extract preserved MMP levels against various toxins i.e. acrolein, H_2O_2, PQ and MPP^+ induced toxicity (Singh *et al.*, 2010, 2012). Additionally, the ability of *BM* extract on mitochondrial dehydrogenase activity was also screened by using the sodium salt of (2, 3-bis [2-Methoxy-4-nitro-5-sulfophenyl]-2H-tetrazolium-5-carboxyanilide inner salt (XTT) and Resazurin (also known as Alamar Blue) cell survival assays (Abu-Amero and Bosley, 2005; Roehm *et al.*, 1991). Using XTT and Resazurin assays we have reported that *BM* extract also protected cells against acrolein, H_2O_2, PQ and MPP^+ induced toxicity (Singh *et*

al., 2010, 2012). XTT and Resazurin assays required long incubation times hence, total mitochondrial NADH dehydrogenase activity in intact cells was also measured by using iodonitrotetrazolium (INT) and phenazine methosulfate (PMS) (Luo *et al.*, 2006). We observed that *BM* extract alone stimulated mitochondrial NADH dehydrogenases activity besides preventing the deficit induced by MPP^+-treatment (Singh *et al.*, 2010; 2012). Furthermore, using semi-purified mitochondrial fractions from SK-N-SH cells, we observed that *BM* extract prevented MPP^+-induced mitochondrial complex1 inhibition (Singh *et al.*, 2012). The effects of *BM* extract on mitochondrial functions are of special interest as deficits in mitochondrial bioenergetic and redox status i.e. loss of adenosine triphosphate (ATP) and MMP, decrease in nicotinamide adenine dinucleotide (reduced form; NADH) levels, decreased mitochondrial respiration, GSH depletion and increased mitochondrial ROS have been reported at 3 months of age and precede the development of AD pathology and synaptic deficits in triple transgenic (3xTg-AD) mouse model of AD (Ghosh *et al.*, 2012; Lee *et al.*, 2012; Yao *et al.*, 2009). Furthermore, partial deficiency of mitochondrial complex I made DA neurons and mice more vulnerable to the neurotoxins MPTP and rotenone (Choi *et al.*, 2011; Sterky *et al.*, 2012).

These results are consistence with the property of *BM* extract and *Bacoside* to maintain the structural and functional integrity of the various mitochondrial enzymes against various toxins including 3-nitropropionic acid (3-NPA), PQT and rotenone (Anbarasi *et al.*, 2005; Hosmani and Muralidhara, 2009; Hosmani and Muralidhara, 2010; Shinomol *et al.*, 2011; Sumathy *et al.*, 2002). Irrespective of the mechanisms involved, the overall effect of *BM* extract on Nrf2-ARE pathway and mitochondrial functions remains important, as there is interplay between oxidative stress and mitochondrial dysfunctions. Cellular GSH levels

have been shown to regulate mitochondrial ROS generation and GSH depletion is associated with inhibition of mitochondrial complexI, fast axonal mitochondrial transport and mitochondrial fragmentation (Chinta and Anderson, 2006; Kim-Han *et al.*, 2011; Shen *et al.*, 2005; Wang *et al.*, 2011). Additionally, the activation of transcription factor Nrf2 has been shown to be crucial and effective in blocking the neurotoxicity resulting from mitochondrial dysfunctions caused by complexI and complexII inhibitors MPTP and 3-NP, malonate respectively (Calkins *et al.* 2005; Chen *et al.* 2009; Cuadrado *et al.* 2009; Lee *et al.*, 2003). Moreover, *BM* extract also activated deacetylase Sirt1, and recent studies have implicated the transcriptional coactivator peroxisome proliferator-activated receptor gamma (PPARγ) coactivator-1 alpha (PGC-1α), which is considered a master regulator of mitochondrial biogenesis and metabolism, as a downstream target of Sirt1-induced neuroprotection through modulation of various mitochondrial functions (Albani *et al.*, 2009; Mudò *et al.*, 2012; Wareski *et al.*, 2009). On the other hand effects of *BM* extract on phospho p66Shc and phospho ERK1/2 status are further interesting as oxidative stress, probably generated by p66Shc is essential for mitochondrial dysfunctions during various pathophysiological conditions (Orsini *et al.*, 2004; Pesaresi *et al.*, 2011). Moreover, recent studies have shown that decreased mitochondrial biogenesis and enhanced autophagy is mediated through pERK1/2 during chronic MPP^+ toxicity (Zhu *et al.*, 2012). Therefore, pathways controlling mitochondrial functions are rapidly emerging as potential therapeutic targets in neuroprotection and these findings strengthen the application of *BM* extract as an effective therapeutic approach to modulate mitochondrial dysfunctions in AD and PD. The probable neuroprotective mechanisms of *BM* extract against various neurotoxins are summarized below.

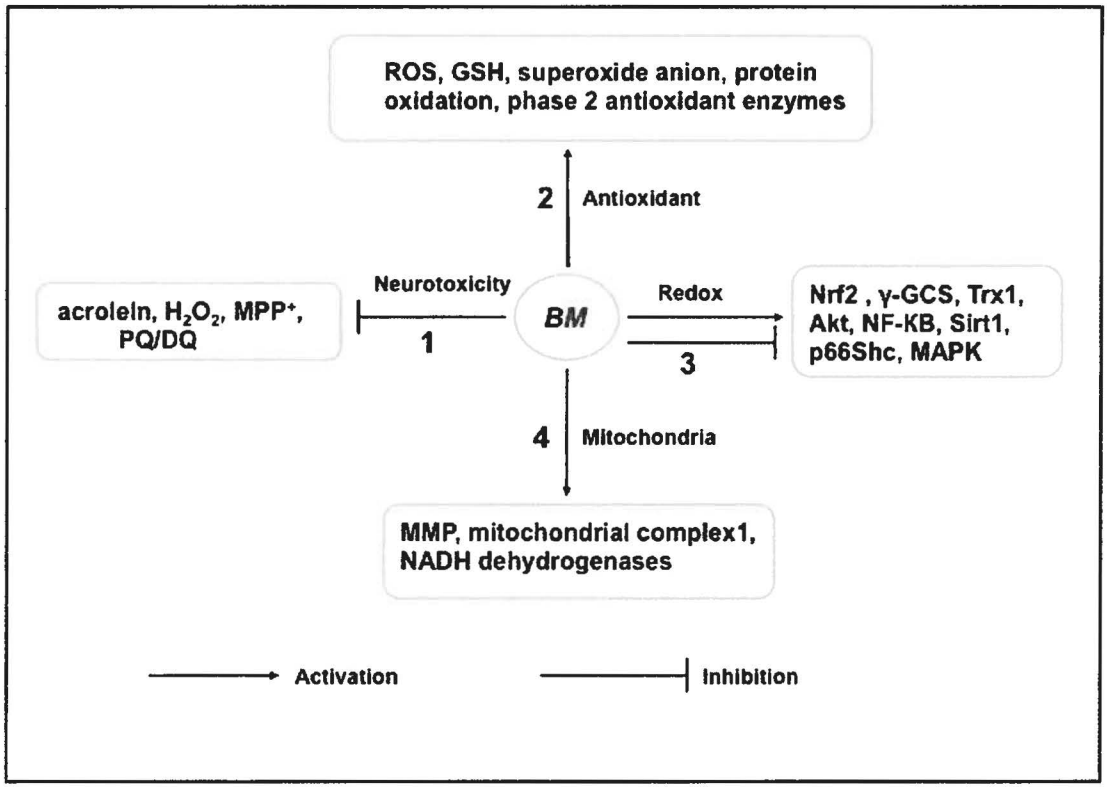

Fig.1 Summary of possible neuroprotective mechanisms induced by *BM* against (1) acrolein, H_2O_2, MPP^+ and PQ/DQ-induced toxicities. (2, 3, 4) Our results demonstrated that *BM* could protect against acrolein, H_2O_2, MPP^+ and PQ/DQ-induced toxicities through different mechanisms (2) by direct scavenging of ROS, superoxide anion, preventing GSH depletion and protein oxidation and inducing phase 2 antioxidant enzymes, (3) by maintaining cellular redox homeostasis with the activation of the Nrf2 pathway along with modulation of Akt, NF-κB, Sirt1, p66Shc and MAPK, (C) by preserving mitochondrial functions such as MMP, mitochondrial NADH dehydrogenase, and complex I activities.

VIII- Modulation of redox-signaling pathways by natural and synthetic Triterpenoids

Triterpenoids constitute a novel class of compounds known to modulate different redox-

signaling pathways (Liby *et al.*, 2007). The major chemical constituents isolated and characterized from *BM* are triterpenoid saponins. Several pharmacological (Singh and Dhawan 1982; Singh *et al.* 1988) and clinical studies (Stough *et al.*, 2001, 2008) have identified several triterpenoid saponins as active constituents of the *BM* extract (Chakravarty *et al.*, 2001; Murthy *et al.*, 2006). More than 20,000 triterpenoids exist in nature (Phillips *et al.*, 2006), including in a large variety of vegetarian foods and medicinal herbs, and are widely used in Indian and Chinese systems of traditional medicines (Liu, 1995). In fact, triterpenoids of oleanolic acid (OA) and ursolic acid (UA) have emerged as attractive drug scaffolds because they are relatively non-toxic, cytoprotective, hypoglycemic, anti-inflammatory, anti-hyperlipidemic, and anti-tumorigenic (Liu, 1995). Novel derivatives of OA, such as 2-cyano-3, 12-dioxooleana-1,9(11)-dien-28-oic acid (CDDO) and related compounds with improved pharmacological potency, have also been synthesized (Liby *et al.*, 2007). CDDO and related compounds are at least 200,000 times more potent than the parent compounds especially with respect to the antioxidant and anti-inflammatory activities (Dinkova-Kostova *et al.*, 2005; Liby *et al.*, 2007; Sporn *et al.*, 2011). Recently some natural and synthetic triterpenoid saponins such as Celastrol, Ginsenoside Rg1, Ginsenoside Rb1, Glycyrrhizin from Indian and Chinese medicinal plants along with a synthetic tritrepenoid 2-cyano-3, 12-dioxooleana-1, 9-dien-28-oic acid methyl ester (CDDO-Me) have also shown neuroprotection aginst 6-hydroxydopamine (6-OHDA) and 1-methy 4-phenylpyridinium (MPP^+) induced toxicities in mouse models of PD (Cleren *et al.*, 2005, Gao *et al.*, 2009, Hwang and Jeong, 2010; Kao *et al.*, 2009; Yang *et al.*, 2009; Yim *et al.*, 2007). One of the most important neuroprotective mechanisms of Celastrol and CDDO is their ability to decrease ROS formation through activation of Nrf2-

dependent ARE genes by forming a Michael adduct with reactive cysteine residues on Keap1 protein (Dinkova-Kostova *et al.*, 2005; Trott *et al.*, 2008, Yang *et al.*, 2009). CDDO-MethyAmide also significantly improved spatial memory, reduced plaque burden, prevented microgliosis and reduced oxidative stress in transgenic mouse model of AD (Dumont *et al.*, 2009). Additionally, CDDO-ethylamide and CDDO-trifluoroethylamide (CDDO-TFEA) also reduced oxidative stress, improved motor impairment and increased longevity in mouse models amyotrophic lateral sclerosis (ALS) and Huntington's disease (HD) through activation of the Nrf2-ARE pathway (Neymotin *et al.*, 2011; Stack *et al.*, 2010). More importantly, bardoxolone methyl (CDDO-Me; RTA 402), an oral antioxidant, inflammation modulator and an Nrf2 inducer, is in a pivotal phase III clinical study in patients with type 2 diabetes mellitus (Pergola *et al.*, 2011). In addition to antioxidant activity, many of these triterpenoids have been shown to modulate inflammatory processes through inhibition of TNF-α and NF-κB pathways through several mechanisms including oxidation of Cys-179 of IKKβ and Cys-38 of p65 protein (Salminen *et al.*, 2008; Yadav *et al.*, 2010). The synthetic oleanane triterpenoid CDDO-Me has been shown to inhibit the NF-κB pathway by direct inhibition of IKappaKinase beta (IKKbeta) on Cys-179 (Ahmad *et al.*, 2006). Moreover, the triterpenoid ginsenoside Rg1 also rescued Aβ-mediated mitochondrial dysfunctions by preserving MMP and ATP levels, along with improvements in cytochrome c oxidase activity in primary cortical neurons (Huang *et al.*, 2012). Celastrol also showed potent anti-lipoperoxidative effects in mitochondria and it was about 15 times more effective than alpha-tocopherol (Sassa *et al.*, 1990). Hence, different triterpenoid saponins along with *Bacosides* from *BM* extract represent a new class of electrophilic metabolites capable of significantly enhancing innate stress response, by activating a

battery of stress-induced proteins *via* the Nrf2 pathway. Additionally, by modulating the several other redox regulated pathways, mitochondrial functions and neuroinflammatory cascade they may further enhance the neuronal capacity to overcome variety of stresses encountered in the pathophysiology of AD and PD.

IX- Standardized plant extracts in neuroprotection in AD and PD

Current studies have implicated an association between multiple risk factors including aging, genetic predisposition, and environmental exposure in the onset and development of AD and PD (Collier *et al.,* 2011; Gao and Hong, 2011; Horowitz and Greenamyre, 2010). Most of the current therapies available for AD and PD, provide only symptomatic relief and hence there is a great and urgent need to broaden our search for novel neuroprotective therapeutic agents. One such novel and invaluable approach will be to target the common linking pathways between aging and neurodegeneration, such as oxidative stress, mitochondrial dysfunctions and redox dysregulation that seem to play an important role in neuronal cell death (Collier *et al.,* 2011; Farooqui and Farooqui, 2009; Henchcliffe and Beal, 2008; Lin and Beal, 2006). Since a multiple hit hypothesis has been proposed in the progress and development of AD and PD, it is highly unlikely that monotherapies based on single drugs will provide significant measurable amelioration in the disease progression or neuroprotection in AD and PD (Frautschy and Cole, 2010; Geldenhuys *et al.,* 2011; Sulzer, 2007). Rather, a rationale and judicious combination of compounds simultaneously interfering and modulating multiple signaling mechanisms involved in the AD and PD pathophyiology seems to be one of the most promising therapeutic approaches. The botanical extracts containing multiple classes of chemical entities with synergic property may hold a better promise for therapeutic benefits and applicability in neuroprotection as

compared to single chemical entity. Moreover, under new Food and Drug Administration (FDA) guidelines standardized botanical extracts can be approved as drugs and an extract of green tea i.e. Polyphenon E; became the first approved botanical drug in the market (Schmidt *et al.*, 2007) and over 200 investigational new drug (IND) applications covering botanical drugs are still pending for approval. However, clinical trials of natural antioxidants and plant extracts in AD have not produced promising results (Mecocci and Polidori, 2012). In a randomized clinical trial, curcumin (1-4 g/day for 6 months) failed to improve cognitive performance in mild-to-moderate AD patients and did not have any beneficial effects on pro-inflammatory biomarkers, serum Aβ peptide levels and isoprostanes (Baum *et al.*, 2008). Moreover, in another randomized, double-blind, placebo-controlled clinical Ginkgo Evaluation of Memory (GEM) study in elderly persons aged >75 years, the use of *Ginkgo biloba* (*GB*), 120 mg twice daily, did not result in less cognitive decline in older adults with normal cognition or with mild cognitive impairment (Snitz *et al.*, 2009). This lack of effect could be attributable to the low bioavailability of oral curcumin or *GB* notably in the brain, or their inability to accumulate in different cellular compartments and modulate ROS, RNS and redox signaling pathways. Secondly, their neuroprotective activity in various *in vitro* and *in vivo* models mostly reflect their ability to prevent the degenerative changes, but in clinical trials in patients suffering from irreversible and extensive neuronal loss they are used for therapeutic purpose and hence it would be unwise to extrapolate these results from animal studies. Inspite of these shortcomings pharmacological agents that have the ability to modulate the oxidative stress, mitochondrial functions and redox signaling pathways such as Nrf2/ARE, NF-κB, sirtuins along with the MAPKs, hold a great promise for therapeutic intervention in neurodegenerative disease.

Chapter 6. Conclusions and Perspectives

Traditional medicines have been used by mankind through the ages and have contributed in the prevention and treatment of several chronic diseases. More recently, herbs from traditional Chinese and Indian medicine systems, such as *Ginkgo biloba*, ginseng (*Panax ginseng*), *Withania somnifera* and *Bacopa monniera* have received increased attention because of their reputed beneficial effects on cognitive processes during aging. Incidence and prevalence of the age-specific neurodegenerative diseases such as Alzheimer's disease (AD) and Parkinson's disease (PD) is less in Asian Indians as compared to other ethnic groups. Moreover, diets, especially along the Mediterranean coast containing fruit, vegetables and herbs, are correlated with healthiness and recently several studies have shown that higher adherence to a Mediterranean diet (MediD) could be associated with slower cognitive decline and reduced risk of AD. It may be possible that MediD and Indian medicinal plants contain some neuroprotective compounds.

AD and PD are two of the most common age-related neurodegenerative disorders affecting millions of people worldwide. Both diseases are characterized by abnormal toxic protein accumulation, oxidative stress and mitochondrial dysfunctions. Most of the current therapies available for AD and PD provide only symptomatic relief. Hence, there is a great and urgent need to broaden our search for novel neuroprotective disease-modifying therapeutic agents that can prevent or delay the onset of these diseases, or reverse the degenerative processes in brain. In our current project we investigated the neuroprotective mechanisms of *Bacopa monniera* (*BM*), one of the most important medicinal plants used in the Indian system of traditional medicine as a nootropic agent and brain tonic to restore age-related decline in cognitive abilities. We demonstrated *BM* extract induced

neuroprotective effects against various toxins in different neuronal cell lines, and showed that the neuroprotection is mediated by modulating oxidative stress and preserving mitochondrial functions. Additionally, we showed that the *BM* extract could activate the transcription factor nuclear factor E2-related factor2 (Nrf2), a master regulator of cellular antioxidant response along with the various phase2 antioxidant enzymes. Besides, *BM* extract could also modulate the expression of several other redox-signaing proteins e.g. mitogen activated protein kinases (MAPKs), nuclear factor kappa B (NF-κB), sirtuin1 and p66Shc. Modulation of these proteins may further enhance cell survival during various stress paradigms. Finally, we reported that *Bacosides*, the triterpenoid saponins compounds, were involved in the neuroprotection against various toxins. Our data provides a deeper insight into the molecular mechanisms underlying the neuroprotective effects of *BM* extract against multiple neurotoxins and this should further open up the possibility of its application in targeting the diverse pathophysiological mechanisms involved in AD and PD.

Inspite of widespread use of *BM* extract as a brain tonic, the detailed neuroprotective mechanisms in an animal model of AD and PD remains to be investigated. Several clinical trials in human have documented the memory enhancing effects of *BM* extract. However, no studies are available on the pharmacokinetics profile of *BM* extract and *Bacosides* compounds. Further studies are warranted to determine the *BM* extract and *Bacosides* compounds levels in plasma and brain along with their effects on the aberrant redox signaling, inflammation, oxidative and nitrosative damage, mitochondrial dysfunctions, neuronal loss and abnormal protein aggregation in appropiate animal models of AD and PD. Pentacyclic triterpenes are secondary plant metabolites and, particularly, the lupane,

oleanane and ursane triterpenes display various pharmacological effects while being devoid of prominent toxicity. Polyphenols and pentacyclic triterpenoid saponins are the key ingredients in traditional Indian and Chinese medicine. Mediterranean spices and fruits, besides other nutraceuticals, contain pentacyclic triterpenes and some of the triterpene rich foodstuffs are consumed in large amounts in Mediterranean countries. Triterpenes have been shown to possess potent antioxidant, anti-inflammatory activity in addition to modulation of heat shock proteins (HSPs) and neurotrophic effects. Mounting evidence shows that the etiopathology of AD and PD is extremely complex and multifactorial, and therefore unlikely to be mitigated by any drug acting on a single pathway or target. Therefore, novel drug entities with the ability to address multiple targets that are involved in the complex pathophysiology of AD and PD seem to offer better therapeutic benefits as compared to single-targeted drugs. Hence, these triterpenes may present promising lead compounds for the development of newer multi-targeting bioactive agents for the prevention and treatment of various neurodegenerative diseases. Under these conditions the correlation of a triterpene enriched nutrition and the beneficial effects of consuming MediD, Indian and Chinese medicinal plants in the context of age-related neurodegenerative disorders further needs detailed epidemiological and laboratory investigations.

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Annex 1: Other Significant Contributions

Cet article a dû être retiré en raison de restrictions liées au droit d'auteur.

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Review

Role of By-Products of Lipid Oxidation in Alzheimer's Disease Brain: A Focus on Acrolein

Manjeet Singh, Dang Thanh Nam, Madeleine Arseneault and Charles Ramassamy*
INRS-Institut Armand-Frappier, Laval, PQ, Canada

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Abstract. Abundant data consistently support the idea that oxidative stress occurs and is a constant feature of Alzheimer's disease (AD). Some recent evidence indicated that phenomenon is an early event and might be implicated in the pathogenesis of this disease. Lipid peroxidation leads to the formation of a number of aldehydes by-products, including malondialdehyde (MDA), 4-hydroxy-2-nonenal (HNE), and acrolein. The most abundant aldehydes are HNE and MDA while acrolein is the most reactive. Increased levels of specific HNE-histidine and glutathione-HNE Michael adducts in AD brain has been reported. Proteomic analysis demonstrated a large number of protein-bound HNE in AD brain. F2-isoprostanes (F2-IsoPs) levels and neuroprostanes were also significantly increased in mild cognitive impairment (MCI) patients and in late-stage AD. In brain from patients with AD, acrolein has been found to be elevated in hippocampus and temporal cortex where oxidative stress is high. Due to its high reactivity, acrolein is not only a marker of lipid peroxidation but also an initiator of oxidative stress by adducting cellular nucleophilic groups found on proteins, lipids, and nucleic acids. Interestingly, data indicates that lipid peroxidation occurs in the brain of MCI and also in preclinical AD patients suggesting that oxidative damage may play an early role in the pathogenesis of AD. In this review, we will summarize some mechanisms implicated in the toxicity of by-products of lipid peroxidation such as IsoPs, HNE, and acrolein and their implication in AD.

Keywords: Arachidonic acid, docosahexaenoic acid, 4-hydroxy-nonenal, isoprostanes, neuroprostanes, oxidative stress, polyamines

INTRODUCTION

The brain is subject to oxidative damage because it contains high levels of polyunsaturated fatty acids (PUFA) and high levels of redox transition metal ions in addition to its high oxygen consumption. In contrast, levels of antioxidants are relatively low. Among different pathogenic hypothesis, the oxidative stress hypothesis in Alzheimer's disease (AD) is attractive because it associates several others such as the trace element hypothesis (iron, copper) [1,2], the mitochondrial hypothesis [3,4], and the amyloid-β peptide (Aβ) hypothesis [5,6]. In recent years, numerous studies have strongly suggested that free radical-mediated oxidative damage plays an early role in the pathogenesis of AD (see review by [7,8]). In MCI and early AD, multiple articles have documented increased lipid peroxidation, protein carbonylation, glycol-oxidation, and DNA and RNA oxidation with a widespread oxidative damage throughout multiple brain regions [9]. Interestingly, levels of oxidative markers increased in a disease-dependent manner and correlated with the Mini-Mental Status Examination scores [10].

*Correspondence to: Charles Ramassamy, INRS-Institut Armand-Frappier, 531, boul. des Prairies, H7V 1B7 Laval, PQ, Canada. E-mail: charles.ramassamy@iaf.inrs.ca.

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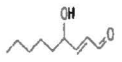
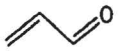
PUFA and their metabolites contribute to some crucial physiological roles including membrane structure and fluidity, cell signaling, energy production, and regulation of genes expression. PUFA, especially arachidonic (AA) and docosahexenoic acid (DHA) which builds the brain phospholipids, are vulnerable to reactive oxygen species, due to their double bonds. The presence of a double bond in the fatty acid weakens the C–H bonds on the carbon atom nearby the double bond and thus facilitates H° abstraction from a methylene group ($-CH_2-$). This reaction generates an unpaired electron on the carbon ($-{}^{\circ}CH-$). An important aspect of lipid peroxidation is its self-propagating process, and this will proceed until substrate is consumed or termination occurs. In this way, lipid peroxidation is fundamentally different from other forms of free radical injury in that it is a self-sustaining process capable of extensive tissue damage. There are two outcomes to lipid peroxidation: structural damage to membranes and generation of oxidized products, some of which are chemically reactive and can covalently modify macromolecules and others may be considered as second toxic messengers which disseminate and augment initial free radical events. Peroxidation of lipids can disturb the assembly of the membrane, causing changes in fluidity and permeability, alterations of ion transport, and inhibition of metabolic processes. Lipid peroxidation releases high amounts of different by-products such as isoprostanes (IsoPs), neuroprotanes, malondialdehyde (MDA), bioactive α,β-unsaturated aldehydes including 4-hydroxy-2-*trans*-nonenal (HNE), and acrolein. There has been much focus on possible roles of lipid peroxidation-derived aldehydes in contributing to neuronal dysfunction in neurodegenerative diseases associated with oxidative stress. Numerous studies have documented increased levels of reactive products of lipid peroxidation in diseased regions of AD brain [11–19]. Also, proteins modified by lipid peroxidation products were present in diseased regions of the brain but not in regions uninvolved in AD. Within diseased regions of the brain, most studies have observed immunoreactivity of by-products of lipid peroxidation with neuronal cytoplasm and neurofibrillary tangles [17,20,21]. In addition, these reactive products of lipid peroxidation have been proposed to participate in pathologic post-translational modifications of the paired helical filaments formed by tau and Aβ peptide. *In vivo* studies showed that increased lipid peroxidation leads to upregulation of BACE1 expression, which may lead to increased $A\beta_{1-42}$ production [22]. Lipid peroxidation products and $A\beta_{1-42}$ have been shown to induce JNK pathways, leading to neuronal apoptosis [23]. There is growing evidence that oxidative stress and by-products of lipid peroxidation can produce neurotoxicity by causing nerve terminal dysfunction and synaptic loss. These observations are of great importance because lipid peroxidation is not a consequence of AD amyloidosis [24,25] and strengthen the notion that brain oxidative stress and lipid peroxidation are potential therapeutic target early in the course of AD. The goal of this review was to describe some mechanisms of cytotoxicity of by-products of lipid peroxidation in brain and their potential implication in AD with a focus on acrolein.

ISOPROSTANES IN ALZHEIMER'S DISEASE BRAIN

The first class of IsoPs discovered were the F2-IsoPs, so named because they contain F-type prostane rings analogous to prostaglandin F2α. F2-IsoPs are produced exclusively from free radical damage to AA (Table 1), a fatty acid that is evenly distributed throughout white matter and gray matter [26,27]. IsoPs derived *in situ* from peroxidation of esterified AA in membrane phospholipids independently of the cyclooxygenase activity [26]. The possible different pathways for the formation of F2-IsoPs during the oxidation of AA lead to four series of regioisomers (5-, 8-, 12- and 15- series) which can comprise eight racemic diasteromers depending on the carbon atom to which the side chain hydroxyl is attached [26,28] (Fig. 1). In contrast to cyclooxygenase-derived prostaglandins, nonenzymatic generation of IsoPs favors the formation of compounds in which the stereochemistry of the side chains is oriented *cis* in relation to the prostane ring. The 5- and 15-series regioisomers are formed in significantly greater amounts than the 8- and 12-series regioisomers [29] because the arachidonyl hydroperoxides that give rise to the 8- and 12-series regioisomers readily undergo further oxidation while 5- and 15-series regioisomers cannot and thus accumulate at higher concentrations in tissues and fluids [30]. IsoPs analogues may be by-products of peroxidation from other PUFAs, such as eicosapentaenoic acid (C20:5, n-3) and DHA (C22:6, n-3) that generate F3-IsoPs and F4-IsoPs, respectively (Table 1). The latter compounds are also termed neuroprostanes, due to the high levels of their precursor in brain [27,31] as DHA account for 40% of the PUFA [32]. Although DHA is present in high concentrations in neurons, these cells are incapable of

Table 1
Compounds generated during lipid peroxidation of membrane phospholipids in brain

Fatty acids	Hydroxy-alkenals	Isoprostanes	Neuroprostanes	Acrolein
C18:2 *n*-6 Linoleic acid CH_3-CH_2-CH_2-CH_2-CH_2-CH=CH-CH_2-CH=CH-CH_2-$(CH_2)_6$-COOH	HNE 			
C20:4 *n*-6 Arachidonic acid CH_3-CH_2-CH_2-CH_2-CH_2-CH=CH-CH_2-CH=CH-CH_2-CH=CH-CH_2-CH=CH-$(CH_2)_3$-COOH 	HNE	F2 IsoPs (64 isomers)		
C22:6 *n*-3 Docosahexaenoic acid CH_3-CH_2-CH=CH-CH_2-CH=CH-CH_2-CH=CH-CH_2-CH=CH-CH_2-CH=CH-CH_2-CH=CH$(CH_2)_2$COOH 			F4-IsoPs (F4-neuroprostanes)	
C20:5, *n*-3 Eicosapentaenoic acid 		F3-IsoPs		

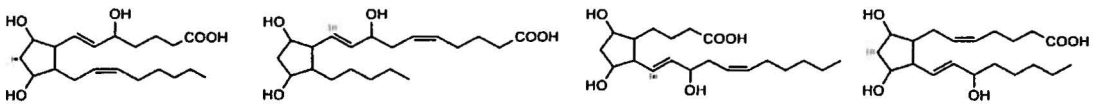


Fig. 1. Four series of regioisomers (5, 8, 12, 15 series) of F2-Isopss.

elongating and desaturating essential fatty acids to form DHA. This latter is synthesized primarily by astrocytes after which it is secreted and taken up by neurons [33]. For these reasons it was proposed that neuroprostanes may be more sensitive and specific for oxidative damage to neurons in brain. Eight subfamilies of neuroprostanes could be formed from DHA owing to free radical attack at positions C6, C9, C12, C15, and C18 [31] (Fig. 2). Once produced, IsoPs are hydrolyzed probably by phospholipases and released from phospholipids. Some IsoPs are highly reactive electrophiles that readily form Michael adducts with cellular thiols, including those found on cysteine residues in proteins and glutathione (GSH) [34]. These cyclopentenone IsoPs could be rapidly metabolized *in vivo* by the glutathione S-transferase enzymes (GSTs) to water-soluble modified GSH conjugates [35]. However, GSTs activities were found to be reduced in AD [36,37].

F2-IsoPs and neuroprostanes levels have been shown to be elevated in frontal, temporal, parietal, occipital lobes, and hippocampus from AD patients as compared to controls with neuroprostanes levels being greater than F2-IsoPs in these regions [9,31,38,39] (see also Table 2). Another class of chemically reactive lipid peroxidation products has been identified: γ-ketoaldehyde isoketals derived from AA and neuroketals derived from DHA and neuroprostanes [40]. During the *in vitro* co-oxidation of equal amount of DHA and AA by iron/ascorbic acid, the amounts of neuroketals formed are lower than the amounts of F4-neuroprostanes but higher than isoketals [40,41]. However, their presence in AD brain remains to be determined. These data indicate greater free radical damage in DHA- than AA-containing compartments in diseased regions of AD brain. These by-products of lipid peroxidation, F2-

Table 2
Levels of F2 and F4-IsoPs in different regions of the brain from control and AD patients

	F4-IsoPs (pg/mg tissue) (data are estimated from bar graph) from [31]		F4-IsoPs (ng/g tissue) (data are estimated from bar graph) from [9]		
	Controls	AD	Controls	MCI	Late AD
Hippocampus			39.5 ± 13.15	92.05 ± 13.15	289.3 ± 52.6*
Frontal lobe			131.5 ± 13.15	236.7 ± 78.9	236.7 ± 65.75
Parietal lobe	75 ± 50	37.5 ± 18.75	144.65 ± 13.15	381.35 ± 65.75*	289.3 ± 39.45*
Temporal	12.5 ± 1.25	50 ± 18.75*			
Occipital lobe	12.5 ± 1.25	100 ± 62.5*	52.6 ± 13.15	210.4 ± 19.72*	210.4 ± 65.75*
	F2-IsoPs (pg/g tissue) [39]		F2-IsoPs (ng/g tissue) (data are estimated from bar graph) from [9]		
	Controls	AD	Controls	MCI	Late AD
Hippocampus			1.632 ± 0.136	2.448 ± 0.544	4.08 ± 0.544*
Frontal lobe	200 (81–260)	410 (240–880)*	1.496 ± 0.136	2.312 ± 0.408*	2.856 ± 0.816*
Inferior Parietal lobe			2.04 ± 0.136	3.4 ± 0.408*	2.85 ± 0.408*
Temporal	205 (110–300)	445 (250–685)*			
Occipital lobe			1.496 ± 0.136	2.584 ± 0.136*	2.312 ± 0.136*

*Significant difference between AD, MCI *versus* control groups.
NS: No significant difference between AD and control groups.

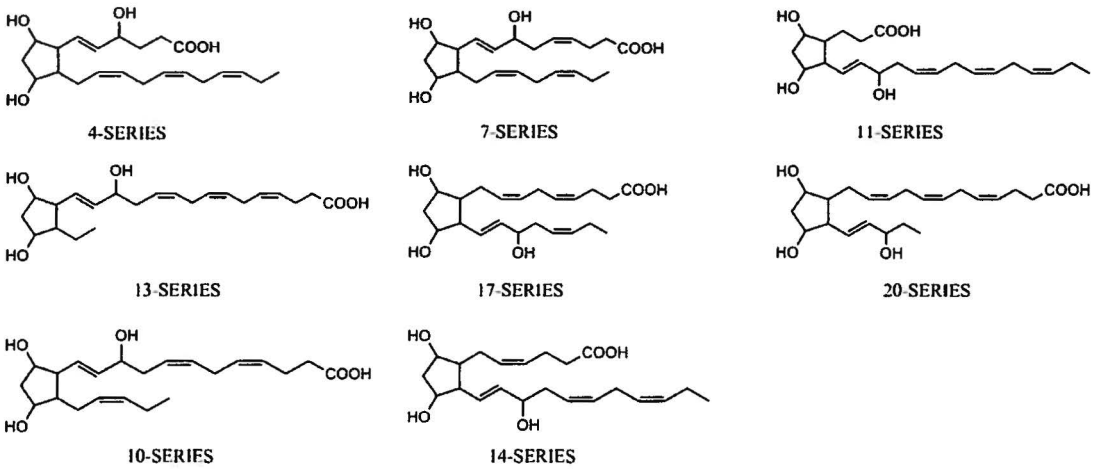


Fig. 2. Structures of different neuroprostanes.

IsoPs and neuroprostanes, were also significantly increased in MCI patients and in late-stage AD compared with controls subjects in different regions of the brain but there was no significant difference between late-stage AD compared with MCI patients [9]. These results suggest that oxidative damage is not a late effect of the neurodegenerative process and could be at least one of the factors involved in the pathogenesis of AD. Molecular modeling of IsoPs containing phospholipids shows that they are remarkably distorted, nonflexible molecules. Therefore, the presence of IsoPs can compromise membrane integrity, fluidity, and neuronal functions and network as observed in AD.

HNE IN ALZHEIMER'S DISEASE BRAIN

HNE results from the lipid peroxidation of n-6 PUFA (linoleic acid and AA) (Table 1) and is thought to be one of the most reactive and significant mediator of free-radical damage [42]. It is difficult to measure the exact quantity of HNE *in vivo* since they are rapidly consumed by reacting with cellular components such as GSH, proteins, and nucleic acid. HNE reacts with lysine, histidine, and cysteine residues in proteins to form Michael-adducts and Schiff base products (Nε-lysine). The order for reactivity of protein residues towards HNE was found to be Cys >> His > Lys. Thiol was demonstrated to be the most reactive nucleophile

towards the lipid aldehyde [42]. However, the thiol-conjugate may be the least stable adduct and His adduct being the most stable [44].

HNE has received considerable attention as a potential effector of oxidative damage and Aβ peptide-mediated neurotoxicity in AD with a significant increase of free HNE in AD brain (see review by [45]). Using 1,3-cyclohexanedione derivatization, HPLC, and a fluorescence detection method, Markesbery and Lovell found that free HNE was significantly higher in amygdala and in hippocampus from AD subjects as compared to age-matched controls [20] (Table 3). Interestingly, this increase was observed in a disease progression-related manner in superior and middle temporal gyrus (SMTG) from MCI and early AD patients [46]. In hippocampus from AD, the distribution of HNE differs among CA sectors with higher intracellular accumulation of HNE-histidine Michael adduct in pyramidal cells most notably at the CA2, CA3, and CA4 regions. These results show that pyramidal neurons in the CA1 sector are more resistant to lipid peroxidation and the CA2, CA3, and CA4 sectors are prone to undergo lipid peroxidation [47]. Recently, Bradley and colleagues [25] showed that free HNE was not significantly different in hippocampus, middle temporal gyrus, and cerebellum from preclinical AD compared to control subjects. In contrast, protein bound HNE were significantly increased in hippocampus from preclinical AD. Surprisingly, protein bound HNE decreased in the cerebellum from preclinical AD (Table 3). Preclinical AD represents cognitively normal individuals who show pronounced AD pathology at autopsy with intermediate or high-likelihood criteria for the histopathological diagnosis of AD by NIA-RI criteria [48]. These individuals would have eventually developed clinical AD if they had lived longer.

In compartments with high GSH levels such as the cytosol, HNE is likely to conjugate to GSH. The chemical reaction of HNE with GSH results in a mixture of diastereomers. At least three isomers of HNE-GSH could be detected and quantified in human brain. Increased levels of the HNE-GSH Michael adduct were found in hippocampus, entorhinal cortex, frontal and temporal cortex, and in cerebellum from AD patients [49]. In normal cells, HNE, HNE-GSH, and related aldehydes are metabolized by the multidrug resistant protein 1 (MRP-1), by the GSTs and by the aldo-keto oxidoreductases enzymes. The HNE-GSH conjugate is effluxed from neurons by MRP-1. The MRP-1 and GST proteins may act in synergy to confer cellular protection. However, in AD brain, GST and MRP-1 were found to be HNE-modified and likely dysfunctional [37], which might account for the loss of GST activity in AD [36,37], and contribute to the increased levels of HNE and accumulation of HNE-protein adducts. HNE could be also detoxified by aldo-keto oxidoreductases enzymes. Among the 4 major aldo-keto oxidoreductases: aldehyde dehydrogenase (ALDH), aldehyde reductase, aldose reductase and alcohol dehydrogenase (ADH), only ALDH and aldose reductase were expressed in cerebral cortex, hippocampus, basal ganglia, and midbrain while these 4 enzymes were present in cerebellum. In cerebrum and hippocampus, aldose reductase was localized in pyramidal neurons while mitochondrial class 2 ALDH (ALDH2) was localized in glia cells and in senile plaques. In temporal cortex from patients with AD, the activity of ALDH was significantly increased compared to age-matched controls [50]. These results suggest that in brain regions involved in AD, neuronal and glial cells utilize different mechanisms to detoxify HNE.

The tissue distribution of HNE-protein adducts varies with apolipoprotein E genotype (*APOE*) [17,21]. Another study has observed modified proteins in or adjacent to neuritic plaques [32], a distribution that is similar to aged genetically modified mice expressing a mutant human amyloid-β protein precursor gene [33]. Early AD had significantly elevated levels of HNE modified protein in the inferior parietal lobule (IPL) compared to age-matched controls. By 2D gel electrophoresis, six proteins were found to be excessively bound to HNE in early AD-IPL, compared to controls. These identified proteins include manganese superoxide dismutase (MnSOD), alpha enolase (α-enolase), dihydropyriminidase-related protein 2 (DRP-2), malate dehydrogenase, triosephosphate isomerase, and F1 AT-Pase, alpha subunit [11,13]. Many of these proteins also are reported to be oxidatively modified in brain from MCI and AD [51]. These results suggest that that impairment of target proteins through the production of HNE adducts leads to protein dysfunction and eventually neuronal death, thus contributing to the biological events that may lead MCI patients to progress to AD.

HNE may contribute to the vulnerability of DNA to oxidation in AD brain. Because of their abundant lysine residues, histones may be a target for HNE modification. The binding of HNE to histones affects the conformation of the histones and therefore their ability to bind to DNA. Interestingly, acetylated histones appear to be more susceptible to HNE modifications than control histones [52]. HNE could also react with nucleosides. *In vitro* reaction of HNE with four different nu-

Table 3
Levels of free HNE, HNE-modified DNA and protein bound HNE in different regions of the brain from preclinical, MCI, early or AD and control groups

	Free HNE (nmol/mg protein) [20]		Free HNE nmol/mg protein) [46]			Free HNE (pmol/mg protein) [24]		HNE-modified DNA (HNE-dG adducts/10^9 normal nucleosides) [53]		Protein bound HNE (% of controls) [25]	
Brain structure	Controls	AD	Controls	MCI	Early AD	Controls	Preclinical AD	Controls	AD	Controls	Preclinical AD
Hippocampus	0.256 ± 0.056	0.543 ± 0.123*	0.4 ± 0.1	1.5 ± 0.3*,^{NS}	1.45 ± 0.5*		NS	464 ± 316	556 ± 379^{NS}	100 ± 16.3	194.2 ± 12.9*
Amygdala	0.193 ± 0.062	0.486 ± 0.096*									
Inferior parietal lobule		NS						546 ± 520	238 ± 72^{NS}		
Superior/Middle Temporal gyrus		NS	0.8 ± 0.1	2.45 ± 0.3 *,^{NS}	2.9 ± 0.3*		NS				NS
Cerebellum		NS	0.8 ± 0.1	1.5 ± 0.2*,^{NS}	NS		NS			100 ± 9.9	70.9 ± 3.9*

*: Significant difference between preclinical AD, MCI, early AD or AD *versus* control groups.
NS: No significant differences between preclinical, early AD, AD and control groups or MCI *versus* early AD groups.

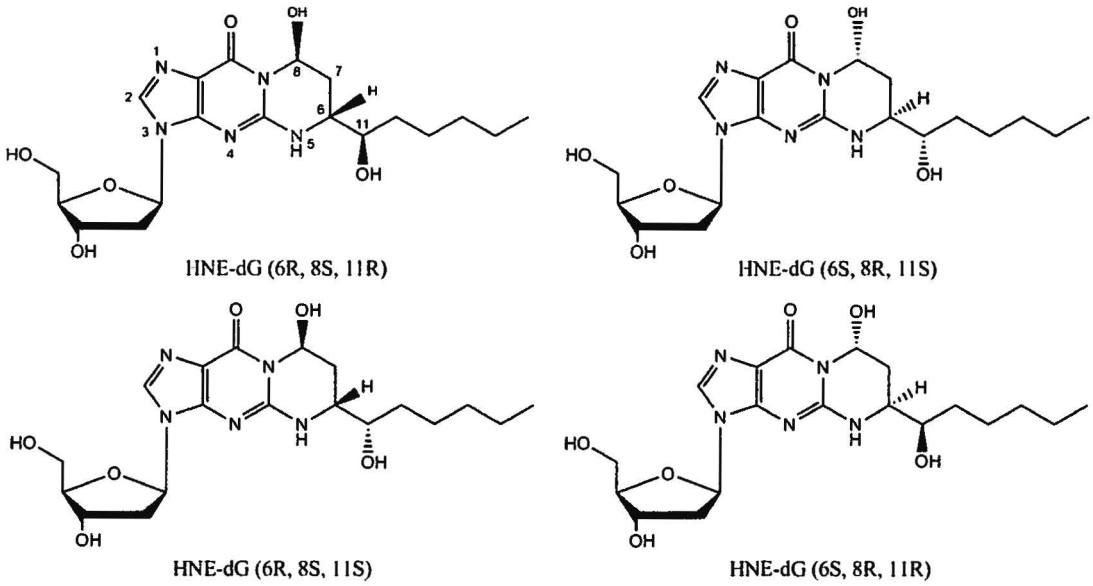


Fig. 3. Stereoisomeric HNE-dG adducts from the reaction of 2'-Deoxyguanosine with HNE, modified from [42].

cleosides (dA/dT/dG/dC) showed that the deoxyguanosine was the most reactive nucleoside. HNE has an enal functionality (a bis-electrophile) and can react with deoxyguanosine through an initial Michael addition of the exocyclic amino group followed by ring closure of N-1 onto the aldehyde group to generate the HNE-derived cyclic 1,*N*2-propanodeoxyguanosine adducts (HNE-dG adducts). Four diastereomeric adducts (6*R*, 8*S*, 11*R*), (6*S*, 8*R*, 11*S*), (6*R*, 8*S*, 11*S*), and (6*S*, 8*R*, 11*R*) are possible (Fig. 3). However, using a sensitive and specific capillary liquid chromatography nanoelectrospray isotope dilution tandem mass spectrometry method, Liu and colleagues [53] have quantified endogenous HNE-dG levels in brain tissue. In the hippocampal and inferior parietal lobule region, the levels of the HNE-dG adducts were not significantly different between AD and control subjects (Table 3). Thus, HNE-dG adduct levels are not altered in the AD

subjects relative to controls.

HNE is not only a potent electrophile reacting with a variety of nucleophilic sites in DNA and protein and generating various types of adducts, but may as well act as a stress signaling molecule. HNE can accumulate at concentrations of 10 μM to 5 mM in response to oxidative insults [42] and invokes a wide range of biological activities, including the selective suppression of the basal and inducible NF-κB activity [54]. The transcription factor NF-κB can prevent neuronal death in experimental models of neurodegenerative disorders by inducing the expression of anti-apoptotic proteins including Bcl-2 and manganese superoxide dismutase. HNE can disrupt ions homeostasis such as Ca^{2+}, impair Na^+/K^+-ATPase activity, disrupt the microtubule structure, and activate the caspase-3 and the stress signaling pathways leading to cell death [55,56]. HNE causes an impairment of the glucose transport in cultured rat hippocampal neurons and an alteration of the glutamate transport in rat neocortical synaptosomes [57]. HNE is also associated with the apoptotic type of neural cell death. For instance, HNE significantly binds p53 in AD IPL, but not in MCI IPL [58, 59]. This difference may suggest a direct involvement of lipid peroxidation product in p53 pathway in an advanced stage of neurodegeneration, but not in an earlier phase. p53 is a multifunctional protein whose main role is to maintain genomic integrity and might contribute to apoptosis by direct signaling to mitochondria [59]. HNE is capable of inducing apoptosis in PC12 cells and cultured rat hippocampal neurons, suggesting that this aldehyde is a mediator of oxidative stress-induced neuronal apoptosis [60]. Therefore, uncontrolled and/or excessive production of HNE could interfere with normal cellular signaling and lead to development of pathological conditions such as observed in AD.

ACROLEIN IN ALZHEIMER'S DISEASE BRAIN

In addition to the disruption of the structural integrity of cellular membranes and the fragmentation of PUFAs, lipid peroxidation generates highly electrophilic α, β-unsaturated carbonyl derivatives including acrolein. Current evidence suggests that *in vivo* acrolein is formed in the metal-catalyzed oxidation of PUFAs including AA [61] (Table 1). Acrolein is an α, β-unsaturated aldehyde with two functional groups that can participate in chemical reactions: the aldehyde group and the carbon-carbon double bond. The molecular formula of acrolein is CH_2=CH-CHO. Acrolein is the strongest electrophile among the unsaturated aldehydes and therefore displays strong reactivity with nucleophilic compounds [42]. Indeed, there is abundant *in vitro* evidence that acrolein can form adducts with amino acids residues such as lysine, histidine and cysteine [42,62]. Lysine and histidine are relatively less nucleophilic than cysteine and are therefore, unlikely to be immediate targets for acrolein. The reaction of acrolein with amino groups (RNH_2) is much slower than those with the thiol group. As a strong electrophile molecule, acrolein can react about 110–150 times faster with the thiol group than 4-HNE. Therefore, it can deplete cellular GSH levels by forming the acrolein-GSH adduct S-(2-aldehydo-ethyl) glutathione. With cysteine the 1:1 adduct is only an intermediate product which rapidly reacts with a second molecule of cysteine to form the thiazolidine derivative. This is the only product in reactions of acrolein with cysteine [63]. The thiol-acrolein adducts are considerably more stable than those formed from all other α, β-unsaturated aldehydes as the dissociation constant of acrolein adducts are about 10 to 10000 times lower than those with other aldehydes [42,63–65].

Among histidine, cysteine and lysine, the latter generated the most stable product and the predominant adducts [61] such as the β-substituted propanals (R-NH-CH_2-CH_2-CHO) or the Schiff's base cross-links (R-NH-CH_2-CH_2-CH=N-R). The Nε-(3-formyl-3,4-dehydropiperidino) lysine (FDP-lysine) is the major adduct formed in reaction of acrolein with protein. This reaction requires the attachment of two acrolein molecules to one lysine side chain [61]. The mechanism for the formation of FDP-lysine has been proposed as follows: acrolein undergoes nucleophilic addition of the lysine amino group at the double bond (C-3) to form a secondary amine derivative with retention of the aldehyde group. This intermediate reacts with another acrolein molecule via a Michael addition and generates an imine derivative. After aldol condensation followed by dehydration, the reaction is completed to generate the FDP-lysine derivative. However, due to the instabilities of these products, methods to detect them in proteins have not yet been established. Protein-bound acrolein such as FDP-lysine may be involved in the pathogenesis of numerous diseases as they were detected in plaque deposits in atherosclerosis [61] and in brain from AD patients [15].

In AD brain, levels of acrolein were found to be significantly higher in several brain regions such as

Table 4
Levels of acrolein and protein bound acrolein in different regions of the brain from control, preclinical AD, MCI, early AD and AD groups

	Acrolein (nmol/mg protein) [76]		Acrolein (nmol/mg protein) [46]			Acrolein (nmol/mg protein) [25]		Protein bound acrolein [25] (% of controls)	
Brain structure	Controls	AD	Controls	MCI	Early AD	Controls	Preclinical AD	Controls	Preclinical AD
Hippocampus	0.7 ± 0.1	5 ± 1.6*	1.2 ± 0.3	NS	2.7 ± 0.2*	1.9	4.9*		NS
Amygdala	0.3 ± 0.05	2.5 ± 0.9*							
Inferior parietal lobule		NS							
Middle Temporal gyrus			0.4 ± 0.1	1.1 ± 0.1*,NS	1.3 ± 0.2*		NS		NS
Cerebellum			0.7 ± 0.1	NS	1.3 ± 0.1*	5.1	1.1*		NS

*: Significant difference between preclinical AD, MCI, early AD, AD *versus* control groups.
NS: no significant difference between preclinical, MCI or AD *versus* control groups or MCI *versus* early groups.

hippocampus, amygdala, middle temporal gyrus, and cerebellum (Table 4). Williams and collaborators [46] have shown that the levels of acrolein were significantly higher in hippocampus and cerebellum from early AD as compared to MCI and control subjects but no difference was observed between control and MCI. However, in the middle temporal gyrus from early onset form of AD and from MCI, the levels of acrolein were significantly higher compared to controls [46]. Recently, acrolein levels were significantly higher in hippocampus and lower in cerebellum from preclinical AD compared to normal subjects [25]. These results indicated that lipid peroxidation and formation of acrolein are present very early in the disease in multiple brain regions.

Acrolein can attack, modify, and inactivate proteins to form protein-bound carbonyls, leading to altered cellular metabolism. Immunohistochemical analysis revealed that the staining of protein-bound acrolein was mainly localized in neurons and rarely seen in glial cells in contrast to protein-bound crotoaldehyde that was mainly seen in glial cells and rarely in neurons [66]. In AD, acrolein has been found to be associated with proteins that were detected in neurofibrillary tangles and dystrophic neuritis surrounding senile plaques [15].

Among the four reactive carbonyl compounds acrolein, MDA, glyoxal, and methylglyoxal, acrolein and methylglyoxal were the most reactive compounds followed by glyoxal and MDA in terms of formation of tau dimers and higher molecular weight oligomers. All tau isoforms were completely converted to high molecular weight aggregates by 10 mM acrolein [67]. Moreover, the triple tau mutant S396D/T403E/S404D reacts faster and forms more thioflavine T aggregates in the reaction with acrolein as compared with wild-type tau. In neuronal culture models, acrolein has been shown to be able to modulate tau phosphorylation at the site recognized by the antibody PHF1, a different site than that modified by protein kinase C [68]. This effect of acrolein is likely due to the activation of both glycogen synthase kinase 3 (GSK-3) and p38 stress-activated protein kinase. GSK-3 is responsible for most of tau phosphorylation at proline-directed sites with the epitope recognized by the antibody PHF1 [69]. These results suggest that acrolein could modulate tau phosphorylation through different pathways and accelerate tangle formation *in vivo*. A better knowledge of the reaction of these reactive carbonyl compounds could be interesting to delay or inhibit tangle formation and neuronal dysfunction in AD and in other tauopathies.

In addition to reactions with proteins, acrolein can also react with a variety of nucleophilics sites in DNA. It has been shown that acrolein can react with the N2 group of guanine in DNA leading to the formation of a cyclic adduct, gamma-hydroxy-1,N2-propano-2'-deoxyguanosine (gamma-HOPdG) [70]. Among the exocyclic adducts, acrolein-deoxyguanosine was the major adduct detected in DNA extracted from rodent and human tissues [71,72]. Different adducts of acrolein with deoxyguanosine are illustrated in Fig. 4 [73]. Exocyclic adducts are formed through a pair of regioisomeric Michael additions with initial bond formation occurring at either the N^2 or N-1 position of deoxyguanosine, followed by ring closure to form exocyclic hydroxyl-isomeric adducts, the *R* and *S* isomers of 3*H*-8-hydroxy-3-($^{\beta}$-D-2'-deoxyribofuranosyl)-5,6,7,8-tetrahydropyrido[3,2-*a*]purin-9-one (8-OH-PdG) and the *R* and *S* isomers of 3*H*-6-

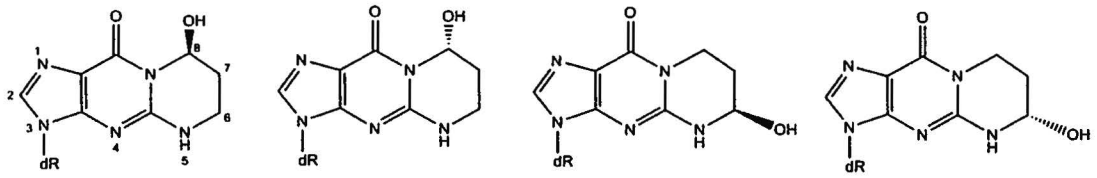


Fig. 4. Different adducts of acrolein with deoxyguanosine, modified from [43].

hydroxy-3-(β-D-2'-deoxyribofuranosyl)-5,6,7,8-tetrahydropyrido [3,2-a] purin-9-one (6-OH-PdG) [74]. The 8-OH-PdG adduct dominates over the 6-OH-PdG. The 8-OH-PdG adduct is biologically important in the formation of the ring-opened, acyclic N^2 oxopropyls in duplex DNA, which are capable of forming DNA-DNA, DNA-peptide, and DNA-protein cross-links [75]. In late onset AD, a 2-fold increase in levels of acrolein/guanosine adducts in nDNA were isolated from the hippocampus of AD as compared to age-matched controls [73]. These adducts are biologically relevant in that they may promote DNA-DNA and DNA-protein cross-linking [75]. In addition, the presence of these bulky exocyclic adducts may alter transcription factor binding and thereby limit the transcription of essential proteins. It can also cause chromosomal aberrations, sister chromatid exchanges, or point mutations. In contrast, levels of the HNE/guanosine adduct in nDNA from parietal lobe and hippocampus from late onset AD subjects were not significantly different to normal control subjects [53]. These results suggest that, in AD, acrolein could induce more toxic reactions compared to HNE.

ACROLEIN IS MORE TOXIC THAN HNE

As a potent alkylating agent, acrolein may react with the matrix tissue or the cell surface proteins, causing alteration of structure and function of matrix proteins. For instance, on primary hippocampal neurons, acrolein is neurotoxic in a time-and concentration-dependent manner and is more toxic than HNE at 5 μM concentration. Treatment with 0.5 μM of acrolein led to 25% decrease in neuronal survival after 6 h and all cells were dead with 10 μM of acrolein (correspond to 0.44 μmol/mg protein) whereas 24 h of treatment with 2.5 μM of acrolein led to nearly complete neuronal death [76]. For comparison to levels of acrolein observed in AD brain tissue, the concentrations of acrolein used in this study (0.44 μmol/mg protein) are higher than those measured in hippocampus (5 ± 1.6 nmole/mg protein) and amygdala (2.5 ± 0.9 nmole/mg protein) from AD brain [76]. However, we cannot exclude the possibility that exposure to lower concentrations of acrolein for much longer periods of time, such as years or decades (as likely occurs in AD brain) could induce cell death. The level of toxicity also depends on neuronal culture and on cell type. On cortical neuron cultures, treatment with 10 μM of acrolein for 6 h led to an approximately 20% decrease in neuronal survival and all cells were dead at a concentration of 25 μM [77]. As compared to HNE, at the concentration of 5 microM, acrolein induced 20 to 30% higher toxicity with greater decrease in cell viability up to 12 h. At 12 h all neurons treated with acrolein were dead while neurons treated with 5 microM HNE required 24 h for complete neuron loss [76].

ACROLEIN AS AN INDUCER OF OXIDATIVE STRESS

The major biochemical pathway for the metabolism of acrolein is the conjugation with GSH. Although acrolein reacts spontaneously with GSH, the formation of Michael adducts between GSH and acrolein is catalyzed by GSTs. The activity of GSTs, was significantly decreased in hippocampus and not in cerebellum from AD brains [36]. These results suggest that the ability of AD brain to detoxify reactive aldehydes is reduced in hippocampus, an affected region in AD, while the brain can detoxify these aldehydes in the cerebellum. Also, GSH reacts with acrolein at the third carbon to produce GS-propionaldehyde [78]. It has been suggested that glutathiolation facilitates aldehyde reduction by aldose reductase, an enzyme implicated in the cellular response against oxidative stress [79]. GS-propionaldehyde can also be subsequently metabolized by ALDH [80]. Any glutathione-acrolein adduct escaping the reductive pathway of metabolism could diffuse into the mitochondria to undergo oxidation by the

ALDH2 isoform. ALDH2 was found to act as a protector against oxidative stress through oxidizing toxic aldehydes. A decrease in ALDH2 activity was proposed to contribute to AD and the genetic deficiency of ALDH2 represents a risk factor for AD. The isoform ALDH2*2 enzyme displays less than 1% of the activity of wild type enzyme [81]. Indeed, one cross-sectional study of an Asian-oriental population demonstrated an increased risk for late onset AD in individuals having an *ALDH2*2* allele and interacting synergistically with the presence of the apolipoprotein E allele 4 [82–84]. A transgenic mice model with low activity of ALDH2 exhibited an age-dependent neurodegeneration accompanying memory loss [85]. These results suggest that, in AD, the ability of the AD brain to detoxify aldehyde derivatives such as GS-propionaldehyde could be reduced. In addition, GS-propionaldehyde may be responsible for some *in vivo* effect of acrolein because GS-propionaldehyde is a more potent stimulator of oxygen radical formation than acrolein [78]. Moreover, it was reported that acrolein is a potent and irreversible inhibitor of ALDH1 and ALDH2 in part by the alkylation of the active site cysteine by the highly electrophilic C3 carbon of acrolein [86].

Due to its high reactivity, acrolein is not only a marker of lipid peroxidation but is also an initiator of oxidative stress by adducting with cellular nucleophilic groups found on proteins, lipids, and nucleic acid [87, 88]. Acrolein can rapidly incorporate into proteins to generate carbonyl derivatives [63,87], a marker of oxidized proteins. For instance, in synaptosomes from forebrains or in SH-SY5Y cell line, acrolein, from 5 μM and in a dose dependent manner, induced an increase in total protein carbonyl level [89,90]. By proteomic analysis, several proteins have been identified that are specifically carbonylated in the presence of acrolein such as β-actin and tropomyosin [90]. The effect of acrolein on the modification of the neurofilament subunits NF-L was also investigated. There was an acrolein concentration-dependent increase in the formation of NF-L aggregation. This aggregation involves the formation of free radicals and was suppressed by a free radical scavenger such as N-acetylcysteine [91]. As neurofilament proteins are major cytoskeletal components of neurons, abnormality of neurofilaments is proposed in brain with AD. This effect of acrolein could be involved in the abnormal distribution of neurofilament-L observed in neurons with AD [92]. These results indicate that acrolein is able to induce the oxidation of some key proteins involved in neuronal functions such as in neurotransmitter release or on neuronal growth.

The toxicity of acrolein involves the modification of the cellular redox potential and reactive oxygen species production. The mechanisms by which acrolein causes oxidative damage and neurotoxicity are not completely defined, but accumulating data indicates that acrolein, as a highly electrophile compound, binds to and depletes GSH levels [93], which is the major target for acrolein. The depletion of GSH induced by acrolein due to alkylation reactions could be responsible of the modification of the redox state. This hypothesis is supported by the protective effect of N-acetylcysteine, a precursor of GSH, and of GSH against oxidative damage induced by acrolein [76,88]. In AD, failure of glutathione synthesis could render cells particularly susceptible to acrolein [10,94]. In cells the ratio of GSH to GSSG is maintained extensively towards the more reduced state, and any alteration in this ratio may induce reversible formation of mixed disulfide bonds between protein sulfhydryl groups and GSH with the S-glutathionylation of a large number of proteins [95]. By reacting with GSH, acrolein induced the alteration of the ratio GSH/GSSG supporting the formation of S-glutathionylation or S-glutathiolation. These compounds are implicated in the buffering of oxidative stress, stabilization of extracellular proteins, protection of proteins against irreversible oxidation of critical cysteine residues, and regulation of enzyme activity [96, 97]. S-glutathionylation is a reversible posttranslational modification from which the release of GSH can be catalyzed enzymatically by glutaredoxin, a thioltransferase. In AD, the expression of glutaredoxin-1, the cytoplasmic form, was found to be elevated in neurons from frontal cortex and hippocampus as to counteract the elevation of S-glutathionylated proteins [98]. In addition, by altering the ratio GSH/GSSG, acrolein will modify the regulation of several pathways involved in key intracellular functions such as protein kinase B, calcineurin, NF-κB, and MAPK. The reaction of acrolein and GSH occurs spontaneously at neutral pH [78], generating glutathionylpropionaldehyde (GS-propionaldehyde). There is evidence that GS-propionaldehyde can elicit superoxide anion formation in the presence of xanthine oxidase.

In addition to GSH, thioredoxin (Trx) and thioredoxin reductase (TR) contain thiol groups and may react with electrophiles such as acrolein. TR is a selenoprotein that catalyzes the reduction of oxidized Trx. TR enzyme contains a selenocysteine residue near the C-terminal which plays an essential role in the catalytic activity of TR [99,100]. TR is a central antioxidant enzyme, with the potential to detoxify hydrogen per-

oxide and lipid peroxides [101], to induce superoxide dismutase [102], to reduce S-nitrosoglutahione [103], to maintain the cell redox state, and to regulate the activity of several transcription factors and genes expression [104]. In addition to the redox regulation, the Trx/TR system plays an important role in a number of biological functions such as in apoptosis and in immunomodulation. It has been shown that among glutathione peroxidase, glutathione reductase, superoxide dismutase, and TR, only TR was rapidly inactivated by acrolein [105]. The mechanism by which TR is inactivated by acrolein is not known but it has been proposed that acrolein may modify the selenocysteine residue in TR active site [105]. Moreover, this inactivation is dependent on the presence of NADPH suggesting that the modification site(s) may be located in active site(s) of the TR. The reduction of the activity of TR was gradually recovered thereafter without any treatment within 24 h [105]. This result suggests that the inactivation of TR by acrolein triggers a compensatory signal for inducing TR gene expression. Treatment of cells with acrolein, from 5–25 μM, for 30 min induced a depletion of Trx in a dose-dependent manner with more than 90% lost at 25 μM of acrolein [106]. A significant depletion of Trx1 was also observed in AD brain limiting the substrate available for TR and thus reduced its protective abilities [77,107].

ACROLEIN REPRESENTS ONE OF THE MOST TOXIC METABOLITES OF POLYAMINES IN BRAIN

Acrolein is also one of the most toxic by-products of polyamines [108]. Polyamines compounds are a family of relatively low-molecular-weight biologic amines that are protonated under physiological conditions. Endogenous polyamines such as putrescine, spermidine, and spermine are required for cell growth, proliferation and differentiation [109] and in brain, the concentrations of spermidine and spermine can reach micromolar range. Polyamines can be metabolized by polyamine oxidase (PAO) or serum amine oxidase (SAO) pathways. When polyamines are metabolized through the SAO activity, spermine and spermidine produce aldehydes and hydrogen peroxide [109]. On the other hand, PAO can catalyze N-acetylpolyamines that have been acetylated by spermidine/spermine N-acetyltransferase (SSAT). This reaction produces 3-acetoamidopropanal and hydrogen peroxide. 3-acetoamidopropanal is spontaneously decomposed to acrolein and acetamide with acrolein being one of the most toxic metabolites.

Polyamines can modify the interaction between ligand and their respective receptor. For instance, polyamines can potentiate the activation of the N-methyl-D-aspartate (NMDA) receptor [110] and block the AMPA receptor [111]. NMDA receptors play central roles in a number of physiological processes, including long-term potentiation in the hippocampus, synaptogenesis, and synaptic plasticity. Excessive NMDA receptor activation has been implicated in the pathophysiology of several chronic neurodegenerative diseases, such as AD. Moreover, polyamines are also released following NMDA receptor activation [112, 113].

In AD, an abnormal polyamine system activity has been observed. The levels of spermidine were markedly and significantly increased in temporal cortex (70%) whereas putrescine and spermine levels were decreased in temporal cortex (28%) and in occipital cortex (35%) from AD [114,115]. However, the results are still conflicting as in frontal cortex, spermidine and spermine were markedly decreased [116]. The involvement of polyamines in AD is strengthened by the up-regulation of polyamine metabolism by $A\beta_{1-42}$ and its 11-amino acid subset, $A\beta_{25-35}$ [117]. The mechanism for increased endogenous polyamine levels in response to $A\beta$-induced oxidative stress remains unknown but it is suggested that the elevation of polyamines in AD could aggravate neuronal damage. As acrolein is also one of the most toxic by-product of polyamines, acrolein could thus also contribute to neuronal damage induced by polyamines.

ACROLEIN AND NEUROTRANSMISSION

The neurotoxic mechanism of acrolein involves a disruption of membrane polarization, which would allow calcium influx through voltage-dependent channels. In acrolein-treated neurons, a 2.5 fold increase in intracellular calcium concentrations was observed at a concentration of acrolein as low as 500 nM which could lead to neuron death [76]. This neurotoxic process is similar to that described for HNE [55]. Moreover, the toxicity of acrolein on neurons may be mediated through the inhibition of the glucose transporter and glutamate uptake, since acrolein at 5 nM and 100 nM, led to a 43% and 63% decrease, respectively [118]. The decrease in glucose uptake is higher with acrolein than with HNE [77]. Glutamate is the major excitatory neurotransmitter in

mammalian brain and, in excess, can disrupt calcium homeostasis leading to neuronal death [119]. Acrolein from 750 nM, led to a significant decrease in glutamate uptake [77]. Again, the effect of acrolein on glutamate uptake is stronger than the effect observed with HNE. With 10 μM concentration of acrolein, the decrease in glutamate uptake reached 86% while it represents only 37% with HNE at the same concentration. The effect of acrolein on glutamate uptake depends on the cell type as on astrocytes culture the effect is significant only with concentrations higher than 10 μM [77,120]. These effects could contribute to the impairment of glutamate uptake observed in AD [121–123] or during aging [124].

The binding of acrolein with sulfhydryl groups could modify some components involved in synaptic activity. The cysteine sulfhydryl group can exist in multiple oxidation states; it can be oxidized to sulfenic acid (RSOH), sulfinic acid (RSO_2H), sulfonic acid (RSO_3H), or in combination with another thiol to form disulfide bond (RSSR). This versatility demonstrates the sulfur's widespread importance in biological processes. The redox state of these sulfhydryl groups on cysteine residues could influence many critical steps in neurotransmission such as channel-mediated voltage- and ligand-gated ion flux, receptor binding, membrane fusion, presynaptic neurotransmitter uptake and synaptic vesicles refilling [125].

CONCLUSION

A large database suggests that these by-products of lipid peroxidation could mediate, at least in part, many chronic diseases involving oxidative stress. A large body of evidence indicated the role of HNE, IsoPs, and acrolein in preclinical AD, in MCI, in early AD and in AD. Despite the pathogenic relevance of these molecules, the precise mechanisms of their neurotoxicity remain to be established. These mechanisms are multistep processes initiated, for instance, by the covalent interaction with a nucleophile compound. In AD, these by-products of lipid peroxidation are present in neurofibrillary tangles and dystrophic neuritis surrounding senile plaques and are elevated in amygdala, hippocampus, and parahippocampus. Moreover, lipid peroxidation in hippocampus may precede protein oxidation [25] and may represent the earliest detectable oxidative damage in the progression of AD. These early changes in lipid peroxidation in hippocampus correlate with the histopathological changes in hippocampus.

Being strong electrophiles, HNE and acrolein display the highest reactivity with nucleophiles such as the sulfhydryl group of cysteine, the imidazole group of histidine and the amino group lysine. More research is needed to establish to toxicological relevance of lysine, histidine, and cysteine adducts in HNE and acrolein toxicities. However, the relatively high thiolate reactivity of acrolein and HNE and the well-known critical roles of this sulfhydryl in cellular functions suggest that the initial aspects of toxicity are mediated by cysteine adduction. The slower rate of lysine or histidine adduction might be relevant to toxicities in the presence of high concentrations or subchronic exposure durations. These by-products of lipid peroxidation have the potential to inhibit several enzymes, and could rapidly deplete GSH levels and modify the cellular redox potential. Acrolein is an inducer of oxidative stress not only by GSH depletion but also by inactivating TR enzyme and decreasing Trx levels. Proteomic analysis indicated that HNE and acrolein could alter some key proteins in neurons. In addition to reactions with proteins, acrolein and HNE can also react with a variety of nucleophilic sites in DNA. Results from numerous studies indicated that the endogenous production of HNE, IsoPs, and acrolein in oxidatively stressed neurons was linked to nerve terminal dysfunctions. This neuropathogenic mechanism is particularly involved in neocortex, hippocampus, and other relevant brain regions of AD [126]. The environmental exposure to these electrophilic compounds could accelerate the onset and development of AD. Although evidence suggests that the AD process involves several factors (aging, environmental, genetic, and non genetic), all of them converge to initiate neuronal oxidative stress and the subsequent production of by-products of lipid peroxidation leading to the pathophysiological cascade. Thus, lipid peroxidation has to be considered within the framework of the pathophysiological mechanisms as a secondary process that might lead to further neuronal damage by different mechanisms. In this context, the reduction of lipid peroxidation or the regulation of different pathways activated by by-products of lipid peroxidation might be relevant in the prevention or in decelerating disease progression. Given the potential role of these by-products in AD, the development of tools directed to limit their production could be an appealing therapeutic strategy. One strategy that has been tested is the treatment with nucleophilic scavengers such as N-acetyl-cysteine [127]. However, the results were inconsistent likely due to the pKa of the corresponding compound (pKa = 9.6), and therefore

the electrophile scavenging activity was not appropriate at physiological pH. Other nucleophiles with lower pKa (6.5) have failed to display neuroprotection [125]. Altogether, these results strengthen the question of the source of the oxidative stress as a central explanation of this pathology and pharmacological targets to prevent or to delay the progression of AD.

DISCLOSURE STATEMENT

Authors' disclosures available online (http://www.j-alz.com/disclosures/view.php?id=422).

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