2. LITERATURE SURVEY

2.1. Role of herbals and potential phytochemicals including polyphenolics and flavonoids

There is evidence that indigenous antioxidants may be useful in preventing the deleterious consequences of oxidative stress and there is increasing interest in the protective biochemical functions of natural antioxidants contained in spices, herbs, and medicinal plants (Osawa et al., 1994).

Plants like *Bacopa monniera*, *Azadirachta indica*, *Withania somnifera*, as well as *Ocimum sanctum* have been investigated for their effect on cognitive functions of the brain (Singh and Dhawan, 1992). These plants have been grouped under the general class of rejuvenators (Jaiswal et al., 1994; Battacharya et al., 1995; Rodrigues et al., 1999) i.e. drugs that counter the degenerative changes associated with ageing. In recent years, there has been phenomenal rise in the interest of scientific community to explore the pharmacological actions or to confirm the veracity of claims made about herbs in the official books of Ayurveda and Siddha.

The interface of molecular psychiatry and the active principles of some of these plants will be a major field for new developments in neuropharmacology and CNS-active drugs (Vaidya, 1997).

Flavonoids, tannins and other phenolic constituents from plant origin are potential antioxidants (Saskia et al., 1996) and they play an essential role in the prevention of neurodegenerative diseases, including Parkinson’s and Alzheimer’s diseases (Di Matteo and Esposito., 2003).

A direct relationship between antioxidant activity and phenolic content of plant extracts has been reported. In recent years, there has been increasing interest in investigating polyphenols from botanical source for possible neuroprotective effects against neurodegenerative diseases (From the Department of Biochemistry, University of Missouri, Columbia, Missouri, USA).

Youdim and co-workers, in the current series, review the beneficial effects of dietary flavonoids on the neuropathology of Parkinson’s disease. (Youdim et al., 2004). Only recently have studies been performed that focus on the potential for flavonoids *per se* to mediate neuroprotection. It is not clear whether the
neuroprotective effects of flavonoids involve their reducing properties or some other mechanism independent of their antioxidant activities.

Although numerous studies have reported flavonoid-mediated neuroprotection, there is little information about the interaction of flavonoids or their circulating metabolites with the brain endothelial cells forming the blood–brain barrier (BBB), which has complicated identification of flavonoid compounds entering the CNS.

The exact mechanism(s) by which flavonoids modulate P-gp efflux is still unclear. For example, they could bring about changes in the physicochemical properties of the drug recognition pockets in the protein without changing conformation of the transporter. There is also the possibility that flavonoids do not interact with P-gp directly but modulate its function by altering plasma membrane properties or factors contributing to the activity of the transporter. The BBB is formed by the endothelium of brain microvessels, under the inductive influence of associated cells, especially astrocytes (Abbott., 2002).

Protection afforded to endothelial cells against reactive oxygen and nitrogen species and inflammatory insults (Youdim et al., 2002) by dietary forms of flavonoids has also been reported. New research into brain activity shows that flavonoids, the antioxidant nutrients that naturally occur in plants, have a special role in protecting our glial cells. (Al Sears, MD). Flavonoids assist hard-working glial cells (oligodendrocytes) in getting rid of free radicals and other brain-robbers that play the biggest role in memory decline, slowing of body movements and mental fatigue (Ibarretxe et al., 2006).

2.2. About *Ocimum sanctum, L* (Holy Basil)/*Ocimum tenuiflorum, L*.

Among the plants known for medicinal value, the plants of genus *Ocimum* belonging to family Labiatae are very important for their therapeutic potentials. Tulsi is a Sanskrit word, which means “matchless one”. Several medicinal properties have been attributed to the Tulsi plant not only in Ayurveda and Siddha but also in Greek, Roman and Unani systems of medicine (Jeba et al., 2011).
Tulsi is a well-known sacred plant of the Indian subcontinent. Its scientific name is *Ocimum sanctum*/*O.tenuiflorum* (OS). Tulsi is also called by names like Manjari/Krishna tulsi (Sanskrit), Trittavu (Malayalam), Tulshi (Marathi) and Thulsi (Tamil & Telegu). It is called Holy Basil in English.

Tulsi is a much branched, fragrant and erect herb having hair all over. It attains a height of about 75 to 90 cm when mature. Its leaves are nearly round and up to 5 cm long with the margin being entire or toothed. These are aromatic because of the presence of a kind of scented oil in them. A variety with green leaves is called Shri Tulsi and the one with reddish leaves is called Krishna Tulsi.

The botanical name *Ocimum sanctum* (Latin) of basil depicts that it is sacred plant not only in India but also throughout the world because of the outstanding medicinal and purifying virtues contained in its essential volatile oils in the leaves.

Tulsi, the Queen of herbs is one of the most potent general adaptogens known to modern science, strengthening the body’s natural capacity to adapt to a wide variety of stresses, and restore and maintain healthy homeostatic equilibrium. It thus offers remarkable preventative and curative potential with respect to many stress-related degenerative disorders, such as cancer, heart disease, arthritis, diabetes and neurological dementia.

Substantial evidence has accumulated that, in addition to Tulsi’s many specific therapeutic applications, the herb’s powerful general adaptogenic properties offer significant preventative and curative potential with respect to the stress-related degenerative diseases endemic to industrialized societies. It enhances the efficacy of many other therapeutic treatments as well (Pushpangadan and Sobti, 1977).

'Adaptogens' are still new to western medicine, and like other adaptogens, contains many nutrients and active phytochemicals, which act synergistically to bring about a state of balance in almost all of the body's systems. Research indicates that
Tulsi has a very high safety margin with exceptionally low toxicity, providing general beneficial effects at doses without adverse reactions or other undesirable side effects.

2.2.1. Phytochemical constituents:

The important bioactive constituents of *Ocimum sanctum* are ursolic acid (UA), a triterpenoid and rosmarinic acid a phenylpropanoid. The other constituents of the plant include phytosterols viz. campesterol, stigmasterol, β-sitosterol etc.; flavonoids viz., apigenin-6,8-C-diglucosides, luteolin-5-O-glucoside; phenolics viz., gallic acid, procatechuic acid, vanillic acid, caffeic acid, alkaloids, saponins, phenylpropane glycosides and tannins etc., (Rastogi and Mehrotra, 1998; Wagner et al., 1994; Devi et al., 1998; Balanehru and Nagarajan, 1991)

The leaves & flowers of the *O. sanctum* contain an essential oil possessing eugenol as the major constituent. Other terpenoids present include β-caryophyllene with minor terpenes like bornyl acetate, β-elemene, methyl eugenol, neral, β-pinene etc. Eugenol has demonstrated 97% cyclooxygenases-1 inhibitory activity when assayed at 1000 mM concentration. Civsilineol, civsimavitin, isothymonin, apigenin and rosmarinic acid displayed 37, 50, 37, 65 and 58% cyclooxygenase-1 inhibitory activity, respectively, when assayed at 1000 mM concentrations (Kelm et al., 2000)

2.2.2. Therapeutic uses and experimental pharmacological studies:

The plant has medicinal properties. The leaves are nerve tonic and sharpen memory. They promote the removal of catarrhal matter and phlegm from the bronchial tubes. It has been reported that *Ocimum sanctum* has antistress (Dadkar et al., 1988; Singh et al., 1991a), anti-ulcerogenic, radio protective, anti-inflammatory effects and nootropic potential (Joshi and Parle, 2006).

The ethanol extract of Os leaves was found to prevent the reduction in adrenergic neurotransmitters in brain of rats exposed to swimming stress and gravitational stress (Singh et al., 1991b). Essential oil (E.O) from leaves and seeds of Os showed anti-stressor effects in rats exposed to restrained stress (Sen et al., 1992).

Administration of the 70% ethanolic extract of Os had a normalizing action on discrete regions of brain and controlled the alteration in neurotransmitter levels due to noise stress, emphasizing the antistressor potential of this plant (Samson et al., 2006; Ravindran et al., 2005).

The effect of methanolic extract of *O. sanctum*, *L* leaves in cerebral reperfusion injury as well as long-term hypoperfusion was studied by Yanpallewar et
al., 2004 Studies with ursolic acid, a major constituent in *Ocimum sanctum*, has shown that it protects hippocampal neurons from kainic acid induced injury (Shih et al., 2004).

### 2.3. About L-DOPA containing *Mucuna pruriens*, *L.*

*Mucuna pruriens* (Mp), an important medicinal plant with wide medicinal properties, is frequently used in a large number of traditional herbal preparations. L-DOPA, a major bioactive was selected as a chemical marker of *M. pruriens*. *Mucuna pruriens* Linn (Leguminosae), commonly known as “the cowhage” or “velvet” bean; and “atmagupta” in India, is a climbing legume endemic in India and in other parts of the tropics including Central and South America (Vacchani et al., 2011).

The seeds of *Mucuna pruriens* containing Levodopa as an important constituent, have been reported for anti-Parkinson’s activity (Vaidya et al., 1978; Katzenschlager et al., 2004), aphrodisiac activity (Amin et al., 1996), antioxidant activity (Tripathy and Upadhyay, 2002), neuroprotective activity (Manyam et al., 2004), learning and memory enhancing effect (Poornachandra et al., 2005) and anti-inflammatory activity (Hishikar et al., 1981).

Even L-DOPA free fraction of seed showed significant antiparkinsonism activity (Nagashayana et al., 2000). The possible mechanism as reported for the neurorestorative activity on the 6-OHDA lesioned rats was shown due to the increased complex I activity and the presence of other constituents of NADH and co-enzyme Q in the cotyledon powder (Sathiyanarayanan and Arulmozhi, 2007).

### 2.4. Animal models

Animal research offers an ideal solution in testing therapeutic strategies. Model organisms provide an inexpensive and relatively quick means to perform two main functions: target identification and target validation.

The tragedy of a group of drug addicts in California in the early 1980s who consumed a contaminated and illicitly produced batch of the synthetic opiate MPPP brought to light MPTP as a cause of PD symptoms (Langston et al., 1983). Other
predominant toxin-based models employ the insecticide rotenone, the herbicide paraquat and the fungicide maneb (Cicchetti et al., 2009). Models based on toxins are most commonly used in primates. Transgenic rodent models also exist (Harvey et al., 2008).

Rotenone, MPTP, and paraquat are used to produce animal models of PD, the mechanism(s) by which these compounds target and kill dopaminergic neurons has remained elusive. Complex I inhibition is required for rotenone toxicity (Sherer et al., 2003a), and it is well established that MPP+ targets dopamine neurons through selective uptake by the DAT (Javitch et al., 1985; Uhl, 1998).

6-Hydroxydopamine (6-OHDA) is one of the most widely used models of PD; it uses the same catecholamine transport system as dopamine and norepinephrine, and produces specific degeneration of catecholaminergic neurons. To specifically target the nigrostriatal dopaminergic pathway, 6-OHDA must be injected stereotactically into the substantia nigra tract or the striatum of the brain (Przedborski et al., 1995) as it is unable to cross the blood-brain barrier.

In contrast to neurotoxin-based models, which show acute neuronal death, genetically modified rodent models were thought to allow progressive degeneration, in the same way as in human PD, hence it was disappointing to discover that this was not the case. Other toxins that cause PD-like symptoms- Mn, toluene, hexane, Hg, CN, Cu or drug-induced (haloperidol).

2.5. Screening of AntiParkinson’s agents

**MPTP MODEL**

MPTP (1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine) is a potent neurotoxin which results in selective degeneration of dopaminergic neurons projecting from substantia nigra pars compacta (SNpc) into striatum and mimics PD-like symptoms in experimental models (Smeyne and Jackson-Lewis, 2005).

<table>
<thead>
<tr>
<th>Toxicant</th>
<th>Regional target</th>
<th>Cellular target</th>
<th>Subcellular target</th>
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<tbody>
<tr>
<td>MPTP</td>
<td>Neostriatum</td>
<td>Dopaminergic neurons</td>
<td>Nerve terminals</td>
</tr>
</tbody>
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It is highly lipophilic and enters the dopaminergic neurons via dopamine reuptake and transporter system. First MPTP is converted to 1-methyl-4-phenyl-2,3-
dihydropyridine through MAO-B which is present in the proximate glia. This further
is converted to MPP+ i.e. 1-methyl-4–phenyl-pyridine. Dopaminergic neurons are
selectively more sensitive to MPTP since they have less VMAT2 [neuronal specific
vesicular monoamine transporters]. VMAT2 function by concentrating toxic
substances in the synaptic vesicle and sequester MPTP thereby protecting intracellular
components.

MPP+ along with MPTP causes massive dopamine release, which is
metabolized by oxidative degradative process and forms ROS like H$_2$O$_2$, super oxide
radicals, etc and finally dopaminergic cell loss occurs. Evidence presented from both
in vitro and in vivo studies support that MPTP exposure generates ROS resulting in
oxidative stress (Krishnan et al., 1997).

1–methyl-4–phenylpyridinium ion (MPP(+)), an inhibitor of mitochondrial
complex I, has been widely used as a neurotoxin because it elicits a severe Parkinson's
disease-like syndrome with elevation of intracellular ROS level and apoptotic death
(From the Institute of Biophysics, Chinese Academy of Sciences, Beijing, China).

MPP+ impairs oxidative phosphorylation by inhibiting complex I of the
mitochondrial electron transport chain (Nicklas et al., 1985). This leads to a rapid
decrease in ATP content, particularly in the striatum and ventral midbrain (Chan et
al., 1991; Fabre et al., 1999), the brain regions most sensitive to MPTP toxicity.

Along with respiratory chain inhibition, neuronal cell death by MPP+ is
suggested to be mediated by formation of the mitochondrial permeability transition
that leads to the release of cytochrome c and activation of caspases, and by
disturbance of intracellular Ca$^{2+}$ homeostasis (Cassarino et al, 1999; Lee et al, 2006,
2007).

Down-regulation of glutaredoxin (thioltransferase, a thiol disulfide oxido-
reductase) using antisense oligonucleotides results in the loss of mitochondrial
complex I activity in mouse brain (Kenchappa and Ravindranath, 2003).

Treatment of SH-SY5Y cells with MPP (+) caused the loss of cell viability,
and condensation and fragmentation of nuclei, which was associated with the
elevation of ROS level, the increase in Bax/Bcl-2 ratio, and the activation of caspase-
3. MPP (+) induced mitochondria dysfunction characterized by mitochondrial
membrane potential loss and cytochrome c release.
Challenge with MPTP stimulates certain neuroinflammatory cascade and activates glial cells which upregulates COX-2 expression and enhanced expression of pro-inflammatory mediators causing the neuronal death (Vroon et al., 2007).

In mice, chronic treatment with MPTP causes irreversible dopaminergic cell loss, whereas a single dose of MPTP causes only a transient mitochondrial complex I dysfunction in the striatum and midbrain (Sriram et al., 1997). The work of Sanjay Kasture has reported the efficacy of *Mucuna pruriens* in behavioral rescue in MPTP mice models (Kasture et al., 2009).

2.6. Hypothesis and rationale of the research work undertaken

- Age-dependent neuropathology is a cumulative response to alterations induced by reactive oxygen species. Therefore cognitive aging, according to this hypothesis should be slowed and possibly even reversed, by appropriately increasing levels of antioxidants or decreasing overproduction of free radicals in the body.

  It is well known, for example, that central nervous system (CNS) vulnerability to oxidative stress (OS) (Joseph et al., 1998a; Joseph et al., 1998b) and inflammation (Chang et al., 1996) increases during aging, and dietary antioxidant/anti-inflammatory agents may reduce these sensitivity increases.

- Oxidative stress and neuroinflammation resulting from neuroglial activation, at level of neurons, microglial cells and astrocytes, are key factors in the etiopathogenesis of both neurodegenerative and neurological diseases. Therefore, emphasis is attributed to the antioxidant and anti-inflammatory activity exerted by specific molecules present in food plants or in remedies prescribed by herbal medicines.

- A variety of antioxidant compounds derived from natural products (nutraceuticals) –polyphenolics, flavonoids have demonstrated neuroprotective activity in either *in vitro* or *in vivo* models of neuronal cell death or neurodegeneration, respectively such as curcumin from tumeric and resveratrol from grapes. They are classified as antioxidants either by directly scavenging free radicals or they indirectly increase endogenous cellular antioxidant defenses, for example, via activation of the nuclear factor erythroid-derived 2-related factor 2 (Nrf2) transcription factor pathway.
**O. sanctum, L** is amongst the Rasayanas (longevity) referred in western medicine as adaptogenic that nourishes nervous system (Vaidya, 1997) has its therapeutic role as neuro protective or symptomatic treatment. Substantial evidence for *Ocimum sanctum, L*’s antioxidant’s properties provides an approach to serve as nervines (Yanpallewar et al., 2004) in the prevention and treatment of stress-related degenerative diseases (Govindarajan et al., 2000).

Behavioral studies suggest that it facilitates activation of dopaminergic neurons (post-synaptic) (Wagner, 1994) and antioxidant properties as well. The ethanolic leaf extract has been found to increase DA levels in corpus striatum and influences the levels of neurotransmitters in stress (Ravindran et al., 2005; Nair, 2007).

An ethanol extract of the leaves of *Ocimum sanctum* have shown to prolong the time of lost reflex in mice due to pentobarbital, decreased the recovery time and severity of electroshock- and pentylenetetrazole-induced convulsions, and decreased apomorphine-induced fighting time and ambulation in "open field" studies.

Using a behavioural despair model involving forced swimming in rats and mice, the extract lowered immobility in a manner comparable to imipramine. This action was blocked by haloperidol and sulpiride, indicating a possible action involving dopaminergic neurons (Sakina et al., 1990; Husain, et al., 2007).

NR-ANX-C is a polyherbal formulation containing the aqueous extract of *W. somnifera* (17% withanolides, 2.1% w/w), 70% ethanolic extract of *O. sanctum* (17% Ursolic acid, 2.9% w/w) and 70% ethanolic extract of *C. sinensis* (34%, total polyphenols 60.1% w/w).

With the exception of *C. sinensis* in NR-ANX-C, its antioxidant potential and its individual constituents has contributed to the reduction in the oxidative stress and the catalepsy induced by haloperidol administration (Rao et al., 2004; Arjuman et al., 2007). It has been demonstrated that cataleptic effects of haloperidol are apparently mediated by dopamine receptors localized postsynaptically on striatal neurons (Sanberg, 1980).

Eugenol and UA are the chief components present in the volatile oil and extract of *O.sanctum, L*, respectively.
The neuroprotective efficacy of eugenol against N-methyl-D-aspartate (NMDA), oxygen-glucose deprivation and xanthine/xanthine oxidase-induced neurotoxicity in primary murine cortical cultures have been exhibited by modulating both NMDA receptor and superoxide radical (Wie et al., 1997). Eugenol, a spicy nutraceutical obtained from cloves, was shown to prevent 6-OHDA-induced reduction in the dopamine level in the mouse striatum. This reduction was associated with a reduction in 6-OHDA-induced lipid per oxidation and an increase in the GSH level (Kabuto et al., 2007).

Ursolic acid (UA), a triterpenoid compound, has been reported to possess antioxidant and anti-inflammatory properties (Lu et al., 2011). The pretreatments of PC12 cells with UA significantly attenuated subsequent H$_2$O$_2$ or MPP$^+$-induced release of IL-6 and TNF-α ($P < 0.05$). Based on the observed antioxidative and anti-inflammatory activities from UA, this compound was a potent agent against neurodegenerative disorder (Tsai and Yin, 2008).

The protective effect of UA against the lipopolysaccharides (LPS)-induced cognitive deficits in mice have been assessed which suggest that UA may be useful for mitigating inflammation-associated brain disorders by inhibiting pro-inflammatory factors production, at least in part, through blocking the p38/NF-κB signaling pathways. These data suggest that UA could be recommended as an anti-brain inflammation agent (Wang et al., 2011).

2.7. Objectives underlying the research work

- A comparative study of free radical scavenging activities of various extracts by different methods of extraction and solvents of varying polarity.
Spectrophotometric methods for determination of plant polyphenolics and their *in vitro* antioxidant activity assessment and comparison with a standard antioxidant

Any correlation between chelatory activity and the content of phytochemicals in the extracts

In addition, available literature data indicates that there is a great deal of diversity in the composition of essential oil of *O. sanctum* cultivated in different localities (Dharmagadda et al., 2005). The present study was therefore aimed at determination of yield of volatile oil and its components, evaluation of the total phenolic content and antioxidant activity of the essential oil hydrodistilled from fresh leaves of *O. sanctum, L* that comes from the area of Vasai area in Thane district, Maharashtra to study the influence of geographical variability

*Therefore, a more extensive investigation was undertaken to understand effect of* *Ocimum sanctum, L* *on various biomarkers for its probable neuroprotective properties in MPTP-inflicted animals at three levels-

**At in vivo level:**
- A. Essential oil and active antioxidant –rich extracts of hydromethanolic (HM) and EtOAc fraction at three graded doses
- B. Synergism of bromocriptine + hydromethanolic extract of *O. sanctum*
- C. L-DOPA rich and free fraction of *Kapi kacchu*

**At in vitro levels (sagittal slices of brain simulated in artificial cerebrospinal fluid [ACSF]):**
- Effects of ursolic acid and eugenol on few selected biomarkers

*This prompted us to investigate its:-*

- Unravel the probable cellular mechanisms and gain insight into probable neuroprotective properties through its effects on various biomarkers in the brain homogenate/blood serum/mitochondria on MPTP-inflicted mice.
- The probable neuroprotective activity against a neurotoxin-mediated neurodegeneration by understanding its mechanistic approach at biochemical,
neuro chemical and molecular level induced by modified MPP model (MPTP-probenecid).

✓ Whether therapeutic treatment with natural iron chelators or antioxidant enzyme boosters is a real possibility or a good approach in treatment of polygenic/multifactorial disorders including PD.

✓ Extrapolate the *in vitro* antioxidant results with the *in vivo* data where effects on surrogate biomarkers are studied that can enable the assessment of antioxidant efficacy.

### 2.8. Significance of the research project

The work primarily provides deeper insights into the antioxidant approach as therapeutic intervention for neurodegenerative disorder of PD by understanding probable mechanism of neuroprotection at the biochemical, cellular and molecular levels.

The Medhya rasayanas including *O. sanctum* that have exhibited reduction in both oxidative stress and haloperidol-induced catalepsy provides an approach to its therapeutic role as neuroprotective probably through dopaminergic or non-dopaminergic influences. So also, the phytochemicals including ursolic acid and eugenol have shown to protect neuronal cells against chemical-induced apoptosis *in vitro*.

With respect to clinical treatment, it would be interesting to determine its effects on various parameters associated with age-related neurodegeneration.