# CHAPTER 2
# LITERATURE REVIEW

## 2.1    Medicinal Mushrooms

Over the last few decades, the herbal medicines and treatment remedies used in traditional medicine have emerged as an important theme in the prevention and treatment of various human diseases and disorders. The development of traditional medicine of various cultures has earned this distinguish branch of medical-related discipline the term "Complementary and Alternative Medicine" (CAM) (World Health Organization, 2000). Furthermore, there has been an increasing popularity in integrative medicine, where conventional Western medical treatments are combined with CAM for which there is evidence of safety and effectiveness (National Center for Complementary and Alternative Medicine, 2008, Updated July 2011). Herbal medicines or dietary supplements, being the most popular and lucrative form of traditional medicine, form the major domain in CAM. This eventuates to the development of "mushroom nutriceuticals" which refers to extracts derived from mycelium or fruiting body of mushrooms having potential therapeutic application (Chang & Buswell, 1996).

Mushroom, as defined by Change & Miles (2004), is "a macrofungus with a distinctive fruiting body which can be either epigeous (above ground) or hypogeous (underground) and large enough to be seen with the naked eye and to be picked by hand". Mushroom has been consumed as food and medicine since the ancient times in many different parts of the world. Early civilizations including Greeks, Egyptians, Romans, Chinese and Mexicans regarded mushrooms as a delicacy and often used them in religious ceremonies. The Romans regarded mushrooms as "Food of the Gods" – serving them only on festive occasions, while the Chinese treasured mushrooms as the "elixir of life" (Chang & Miles, 2004). The profound health promoting benefits in mushroom has long been recognized in the Orient which leads to its incorporation in

Asian traditional medicine practices especially in China, Japan and Korea (Poucheret et al., 2006). In traditional medicine, *Auricularia* spp. are used to treat haemorrhoids, *Tremella fusiformis* for maintaining healthy lung tissue and *H.erinaceus* for suffering of gastric ulcers (Chang & Buswell, 1996). The Chaga mushroom, *Inonotus obliquus*, is widely used in folk medicine in Russia, Poland and most of the Baltic countries in the form of hot water extract to improve overall health and prevent various diseases (Asatiani et al., 2010).

The intervention of modern science in exploring the medicinal properties of mushrooms has provided a scientific basis for empirical observations in traditional medicine. Of the 15,000 of mushroom species currently known, it has been identified that among them 700 species possess certain medicinal attributes. Yet, it is estimated that there are about 1800 species of mushrooms that possess potential medicinal attributes (Chang & Miles, 2004). A growing body of research in mushroom studies is increasingly confirming the medicinal efficacy and bioactive molecules responsible for various therapeutic actions in mushrooms. This leads to the emergence of the term 'medicinal mushrooms'- referring to mushrooms that are considered to have medicinal applications. Medicinal mushrooms, together with other tonic remedies used in various traditional medicines, are usually used as adaptogens and immunostimulants. By definition, an adaptogen is a relatively non-toxic substance that can modify the host's biological response by stimulating the immune system thus resulting in various therapeutic effects. An adaptogen has a nonspecific action on the body, whilst supporting and regulating all the major body systems (Wasser & Weis, 1999).

The pharmacological potential and effectiveness of medicinal mushrooms in treating numerous human disorders has gained great attention in scientific and medical research. This is mainly due to the therapeutic effects of mushrooms demonstrated against incurable diseases including cancer and degenerative diseases, with multiple

complex pharmacological actions acting on different cellular and molecular targets. The bioactive compounds influence cell surface receptors and trigger various downstream signaling events which lead to high pharmacological efficiency and specificity. Furthermore, fungal bioactive metabolites can be conveniently obtained from various origins such as wild or cultivated fruiting bodies as well as mycelia biomass and supernatant of submerged culture using bioreactors (Poucheret et al., 2006). A large number of mushroom-derived compounds including cellular components and secondary metabolites have been demonstrated to possess immunomodulating effects and could be used for the treatment of diseases. The bioactive molecules identified include polysaccharides, glycoproteins (lectins), triterpenoids and fungal immunomodulatory proteins (Chang & Miles, 2004). Polysaccharides are the best known and most potent mushroom-derived substances with antitumor and immunomodulating properties (Wasser, 2002). Several medically important polysaccharides have undergone anti-cancer clinical trials including lentinan (from *Lentinula edodes*), schizophyllan (from *Schizophyllum commune*), Grifon-D (from *Grifola frondosa*), polysaccharide-K (commercially sold as Krestin) and polysaccharopeptide (both from *Trametes versicolor*) (Smith et al., 2002).

### 2.1.1 *Hericium erinaceus* –The monkey head mushroom

The genus *Hericium* was originally described by Christian Hendrik Persoon in 1794. Recent molecular taxonomy studies have placed *Hericium* under the Russulales (formerly under Aphyllophorales), in the family *Hericiaceae* (Kirk et al., 2008). All species in the genus *Hericium* produce basidiomata as a mass of fragile icicle-like spines that are suspended from a branched supporting framework or from a tough unbranched tissue; their basidiomata are considered edible and delicious mushrooms (Ko et al., 2005). In the wild, these beautiful spine fungi are saprotrophs, commonly seen growing on wood of broadleaved trees especially beech (Boddy et al., 2011). The

*Hericium* genus encompasses a number of species which is widespread throughout north temperate areas, including *H.cirrhatum* ('spine face'), *H.coralloides* ('coral spine fungus') and *H.erinaceus* (known as 'monkey head' in China) (Pegler, 2003).

    *Hericium erinaceus* (Bull.: Fr.) Pers. is a highly sought-after mushroom species due to the medicinal properties ascribed to it. The mushroom can be found in the wild throughout the North temperate regions of the world, comprising Europe, East Asia and North America. The fruiting body is 5 to 30 cm in size with a rudimentary stipe; it has an exotic appearance with beautifully cascading long white tendrils of 3 to 6 cm in length, hanging down from its base (Chang & Miles, 2004) (**Figure 2.1**). This profound appearance has earned the mushroom various names across the globe. In the West, it is generally known as 'lion's mane', 'tree hedgehog', and 'bearded tooth'. In North America, fresh fruitbodies are sold as a gourmet food under the name of 'Pom poms' (Pegler, 2003). In Japan, *H.erinaceus* is mainly known as *Yamabushitake* because it resembles the ornamental garment worn by the hermit Buddhist monks who practice asceticism in the mountains (*Yamabushi*, literally means "those who sleep in the mountains", refering to the hermit monks; "*take*" means mushroom, in Japanese). Also, with reference to its shape, it is also named *Jokotake* (drinker fungus)*, Usagitake* (rabbit fungus) and *Harisenbon* (balloon fish). In China, the mushroom goes by the name *shishigashira* , which means "lion's head" and *houtou*, which means "monkey head", as the fruiting bodies are reminiscent of the head of a baby monkey (Mizuno, 1999). In Korea, the mushroom is well known as *norukung-daei* (roe deer's hip) (Ko et al., 2005). *Hericium erinaceus* has been successfully domesticated in Malaysia and cultivated in lowland conditions. In Malaysia, the mushroom is known as 'cendawan bunga kobis' which means 'cauliflower mushroom'(Sabaratnam et al., 2007).

**Figure 2.1**: Fruiting bodies of *H.erinaceus*.

(A) Wild *H.erinaceus* found in Hampshire, United Kingdom [Taken from Pegler (2003)].

(B) Fresh fruiting bodies obtained from a local mushroom farm in Tanjung Sepat, Selangor.

(C) Freeze-dried fruiting bodies used in the preparation of aqueous extract used in this study.

In Chinese cuisine, *H.erinaceus* is regarded as a delicacy and ranked as one of the "four most famous dishes" in China, together with sea cucumber, bear's palm and bird's nest. In the olden Chinese society, this culinary delicacy was only dedicated to the royalties due to its distinctiveness and rarity. In gastronomic terms, the monkey head mushroom is considered a delicious mushroom with a succulent texture, mild and sweet flavour comparable with that of a lobster (Pegler, 2003). Prior to 1960, this edible mushroom was available only in the wild. The Shanghai Agricultural Academy of Science successfully domesticated the mushroom in 1960 which eventually led to large-scale commercial production. *Hericium erinaceus* is mainly produced in China with its annual production estimated to be 9547 million tonnes in 2001 (Chang & Miles, 2004). *Hericium erinaceus* can be grown on a wide range of substrates including sugar cane culms, sawdust, cotton waste, corn cobs and old paddy straws. Each material is usually supplemented with rice or wheat bran, sucrose, gypsum as well as water to provide moisture content of 65% (Pegler, 2003). Mycelial growth was at its best at 25℃ while the formation of fruiting bodies requires the optimum temperature at 20℃ (Chang & Miles, 2004).

### 2.1.2 Medicinal and therapeutic properties of *H.erinaceus*

During the past decades, there has been an upsurge interest in the medicinal herbs used in traditional remedies for the prevention and treatment of prevalent diseases. This is evident with the burgeoning of CAM treatments and increasing production of herbal-based nutriceuticals in the healthcare industry. In traditional Chinese medicine, the monkey head mushroom is prescribed for gastrointestinal ailments and discomfort including gastritis, stomach disorders and ulcers. The dried fruiting bodies are used in a Chinese traditional drug preparation known as "Houtou" for treating chronic stomach diseases. In a research study done in a Shanghai hospital, patients who were given the monkey head mushroom in the form of tablet, have been reported to have improvement

in ulcers, inflammations and tumours of the digestive tract (Stamets, 2000). It is worth noting that *H.erinaceus* mushroom grown in the tropical climate of Malaysia was reported to have cytoprotection against ethanol-induced gastric ulcers in rats with less gastric mucosal damage, decreased edema and the absence of submucosal leukocytes infiltration (Abdulla et al., 2008).

Biologically active polysaccharides from medicinal mushrooms are extensively studied for their antitumor and immunomodulatory properties as they pose to be potential candidates for the treatment and prevention of cancer (Smith et al., 2002). Antitumor-active polysaccharide fractions containing heteroglycans and glucan-protein complexes isolated from the fruiting body of *H.erinaceus* inhibited the growth of Sarcoma 180 implanted in mice (Mizuno et al., 1992). A recent study by Kim et al. (2011a) revealed that intraperitoneal injections of *H.erinaceus* extracts reduced the tumor weight by induction of natural killer cells, macrophages activation and angiogenesis inhibition. When used in combination with doxorubicin (a chemotherapeutic drug), *H.erinaceus* polysaccharide enhances the sensitization of doxorubicin-mediated apoptotic signaling resulting in the inhibition of human hepatocellular carcinoma growth (Lee & Hong, 2010). The antitumor of mushroom polysaccharides is exerted via the activation of the immune response of the host organism by stimulating natural killer cells, T-cells, B-cells and macrophage-dependent immune system responses (Wasser, 2002). Polysaccharides from the liquid culture broth and fruiting body of *H.erinaceus* induced functional activation of macrophages by upregulating cytokine expression of tumour necrosis factor-alpha (TNF-$\alpha$) and interleukin-1 beta (IL-1$\beta$) (Lee et al., 2009a; Lee et al., 2009b). Recent reports showed that *H.erinaceus* induces maturation of dendritic cells and induces intercellular adhesion molecule-1 (ICAM-1) expression in human monocytes through ERK and ROS-dependent pathways (Kim et al., 2010; Kim et al., 2011b).

Several studies revealed the hypolipidemic and hypoglycaemic effect in *H.erinaceus* which rendered this mushroom to be a potential therapeutic agent for the treatment and prevention of cholesterol and diabetes. An exo-biopolymer extracted from the submerged mycelial culture of *H.erinaceus* had a significant hypolipidemic effect whereby the levels of plasma total cholesterol and low-density lipoprotein (LDL) cholesterol were substantially reduced; while the plasma high-density lipoprotein (HDL) cholesterol level increased (Yang et al., 2002, 2003). Administration of *H.erinaceus* combined with a high-fat-level diet in mice resulted in a significant decrease in body weight gain and fat weight, in addition to reduction of serum and hepatic triacylglycerol levels (Hiwatashi et al., 2010). The methanol extract of *H.erinaceus* demonstrated hypoglycemic effect on streptozotocin-induced diabetic rats with significant reduction in blood glucose level, serum triglycerides and total cholesterol level (Wang et al., 2005).

The *H.erinaceus* mushroom has been reported to have antioxidant properties with total polyphenols being the major antioxidant components (Mau et al., 2002). The locally grown *H.erinaceus* was reported to have moderately high antioxidant potential. The phenolic content of *H.erinaceus* varied greatly according to the preparation method in which hot water extract was found to have higher phenolic content compared to methanolic extracts (Abdullah et al., 2012). Malinowska et al. (2009) reported that selenium-containing extracellular polysaccharides from *H.erinaceus* submerged culture showed potent antioxidant properties. Interestingly, *H.erinaceus* polysaccharides were reported to enhance skin antioxidant enzymes, MMP-1, TMP-1 activities and collagen protein levels, indicative of anti-aging effects on the skin (Xu et al., 2010).

The earliest isolated phenols from the fruiting body of *H.erinaceus*, known as hericenone A and hericenone B, showed cytotoxicity against HeLa cells, which is a cell line derived from cervical cancer (Kawagishi et al., 1990). Similar cytotoxic activity

was also found in erinapyrone A and erinapyrone B from the culture broth of *H.erinaceus* mycelia (Kawagishi et al., 1992). Ergosterol peroxide, a steroidal derivative with cytotoxic activity, has been isolated from the mycelium of *H.erinaceus* (Krzyczkowski et al., 2009). The lectin of *H.erinaceus* is a heterotetramer which exhibited specificity towards sialic acids especially *N*-glycolylneuraminic acid (Kawagishi et al., 1994a). A novel lectin from *H.erinaceus* was reported to exhibit mitogenic activity towards mouse splenocytes and antiproliferative activity towards hepatoma (HepG2) and breast cancer (MCF7) cells. The lectin also manifested HIV-1 reverse transcriptase inhibitory activity (Li et al., 2010).

The *H.erinaceus* mushroom has been reported to possess antimicrobial activities. Erinapyrone C, a new γ-dihydropyrone, demonstrated moderate inhibitory effect against the growth of Gram-positive bacteria (Arnone et al., 1994). Chlorinated orcinol derivatives, isolated from the mycelia of *H.erinaceus*, exhibited antimicrobial activity towards *Bacillus subtilis*, *Saccharomyces cerevisiae*, *Verticillium dahliae* and *Aspergillus niger*. The production of these compounds may be a self-defensive system of the mushroom (Okamoto et al., 1993). It has been reported that erinacines A, B (Kawagishi, 2005) and K (Kawagishi et al., 2006), isolated from the cultured mycelia of *H.erinaceus*, showed anti-methicillin-resistant *Staphylococcus aureus* (anti-MRSA) activity. MRSA is one of the most prevalent pathogens in nosocomial infections. Kawagishi (2005) reported that MRSA in some patients disappeared when they consumed the mushroom during a clinical test conducted in a hospital in Japan.

## 2.2    Neurotrophic Factors

The formation of axons and dendrites from individual neurons, which are collectively known as neurites, is pertinent to the functionality and development of the human nervous system. The development of neurite engages a series of complex processes including neurite initiation, elongation, branching and synapse formation

which are critical determinants of neuronal connectivity (Audesirk & Audesirk, 1998). The morphological appearance of the neurite heavily relies on the cytoskeletal building blocks including microtubules, neurofilaments and microfilaments. Microtubules provide structural support in the development and maintenance of neurites besides acting as conveyers for transport of membrane-bounded organelles. In neurons, neurofilaments play an important role in regulating cellular and axonal volumes in addition to providing mechanical strength and a stable cytoskeletal framework. The major class of cytoskeletal element – actin microfilaments, maintain the distribution of plasma membrane proteins and mediate interactions between neurons and the environment. Microfilaments form the basis of filopodia and lamellipodia which are essential for cell migrations, growth cone motility and myelination (Pigino et al., 2006).

The extension of axons and dendrites is the hallmark of differentiation in neurons. On the onset of neurite outgrowth, neurons extend lamellipodia (flat, veil-like structures in constant motion) around the cell body, which develop into short neurites, designated as minor processes (**Figure 2.2**). As the neuron continues to mature, one of the minor processes rapidly elongates and forms the axon. The remaining minor processes continue to develop and become dendrites (Dotti et al., 1988). The further development of the neurites relies on the growing tips of the neuron, known as growth cones which are highly motile and dynamic. Growth cones explore their environment through surface protrusions known as filopodia and lamellipodia. Filopodia are spike-like projections that grow and retreat rapidly from the growth cone surface whereas the web-like lamellipodia are found in between filopodia (Pigino et al., 2006) (**Figure 2.2**). The neuronal growth cone plays a critical role in the development of axons especially in neurite elongation and pathfinding (Tessier-Lavigne & Goodman, 1996). Neurite outgrowth is regarded as an *in vitro* marker of differentiation and an equivalent of axonal extension, thus, this model system is commonly used to assess potential

relevance for enhancing repair/regeneration following spinal cord or peripheral nerve injury following trauma, chemically induced toxicity and other neuronal damaging conditions (Calabrese, 2008b).
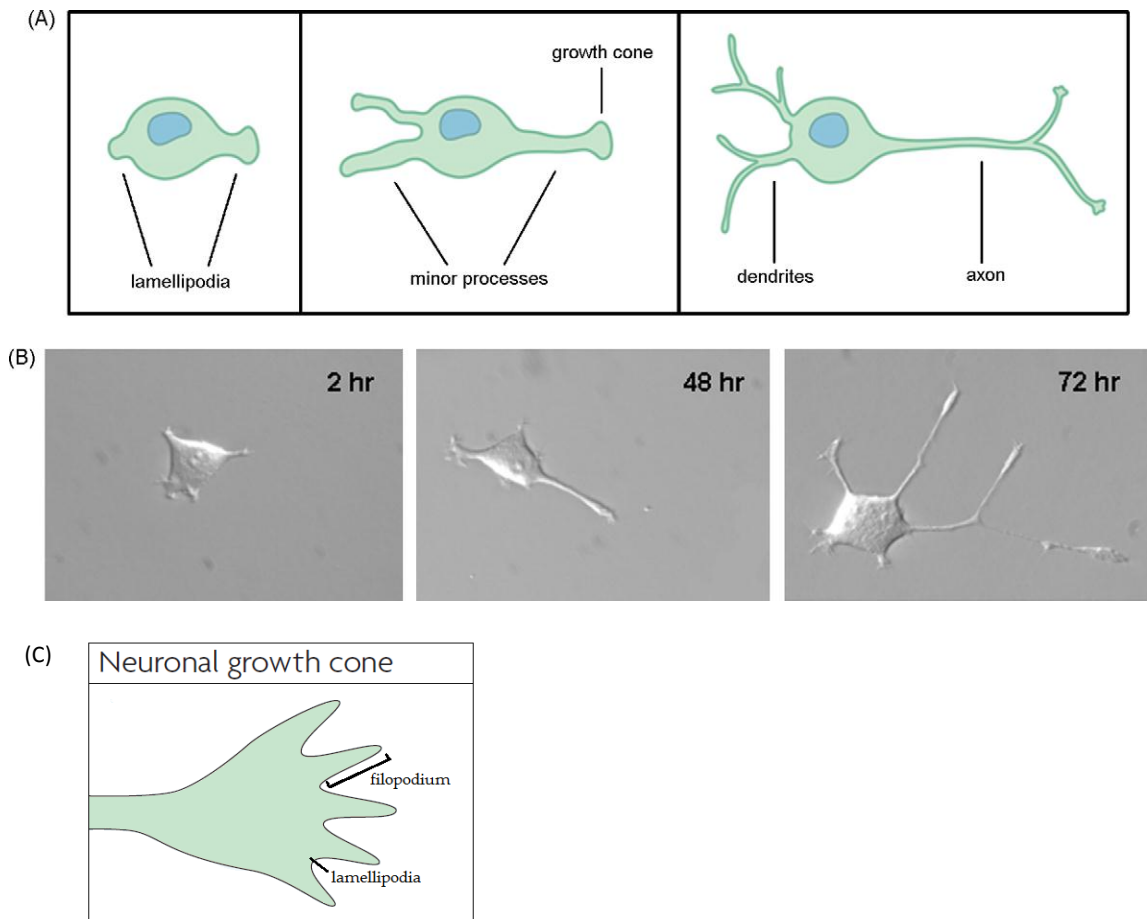


**Figure 2.2**: Early morphological events in neurite outgrowth [Taken from Radio and Mundy (2008)].

(A) Diagrams illustrating a neuron extending lamellipodia, leading to the development of minor processes (tipped with a growth cone) and eventually forming an axon and dendrites.

(B) Contrast photomicrographs of neurite outgrowth in PC12 cells upon treatment of 100 ng/mL NGF after 2, 48 and 72 hr.

(C) Neuronal growth cone comprised of filopodia and lamellipodia.

The extension of neurites is regulated by intrinsic and extrinsic factors which are intertwined. Many intrinsic phenotypes are programmed in at the progenitor stages and the expression of receptors for survival, growth and initial axon guidance is probably inherent to the neuron. The intrinsic growth state of a neuron is a crucial regulator of axon and dendrite elongation can be affected by extrinsic signals (Goldberg, 2004). Axons and dendrites response to a multitude of extracellular cues including trophic factors, extracellular matrix and activity-dependent depolarization which may lead to neurite outgrowth or inhibition (Letourneau et al., 1994; Van Ooyen et al., 1995; Weisenhorn et al., 1999). Among them, trophic factors remain to be the most extensively studied in relation to neurite outgrowth due to their ability to induce the process of proliferation and differentiation.

The word *trophic* literally means *nourish* and neurotrophic factor simply refers to an agent that nourishes or supports the growth of neurons (McMahon & Murinson, 2005). Neurotrophic factors are proteins that regulate the development of nervous system, neural plasticity and maintenance of structural integrity (Hefti, 1997). The first discovered neurotrophic factor, nerve growth factor (NGF), formed the concept basis of 'neurotrophic factor hypothesis' where developing neurons compete for a limited supply of neurotrophic factor produced by the target tissue, resulting in selective survival of neurons in the neuronal population. This results in the death of neurons which failed to obtain trophic support (Yuen et al., 1996). This mechanism largely accounts for the sophisticated control of the number of neurons connecting with their targets in the periphery to the size of the target field which leads to the formation of a stable neuronal population (Landreth, 2006). Neurotrophic factors exert a vast range of physiological and pharmacological functions on neurons including promotion of survival, proliferation and differentiation. These molecules are required in the adult nervous system to maintain the morphology, function and connectivity of mature neurons. The

ability of neurotrophic factors in preventing neuronal death and atrophy render them as potential therapeutic candidates in neural injury and neurodegenerative diseases (Johnson & Tuszynski, 1999).

Over decades of extensive research, several major classes of neurotrophic factors have been identified including neurotrophins, neurokines, fibroblast growth factors, transforming growth factor β superfamily, glial-derived neurotrophic factors and epidermal growth factor superfamily (Landreth, 2006). Examples of neurotrophic members in each category are tabulated in **Table 2.1**. Neurotrophins, the first neurotrophic family, are the most prominent and best studied among neurotrophic factors. Members of this family include NGF, brain-derived neurotrophic factor (BDNF), neurotrophin 3 (NT-3) and neurotrophin 4/5 (NT-4/5). New members of the family include neurotrophin 6 (NT-6) and neurotrophin 7 (NT-7) that have been isolated from fish and are not found to have mammalian homologues (Windebank & McDonald, 2005). Neurotrophin signalling is mediated by two different transmembrane receptors, namely the Trk (tropomyosin related kinase) receptor and the p75 neurotrophin receptor (p75$^{NTR}$). The Trk receptors belong to the tyrosine kinase family of receptors that share an amino acid sequence homology with the tropomyosin receptor kinase (Perez-Polo, 2010). The Trk receptors binds with individual neurotrophin with well-defined specificity where TrkA specifically binds to NGF, TrkB binds to BDNF and NT-4/5, and Trk C preferentially binds to NT-3. Neurotrophins also bind to the p75$^{NTR}$ to form multimeric receptor complexes with Trk and other receptors in mediating apoptosis, neurite outgrowth and myelination (Price et al., 2007).

## 2.2.1 Nerve growth factor (NGF)

Nerve growth factor (NGF) is the prototypical member of the neurotrophin family which forms the basis of our knowledge in the indispensable role of neurotrophic factors in the nervous system. The NGF molecule is a hetero-hexamer made up of a

**Table 2.1**: Major classes of neurotrophic factors. Adapted from Landreth (2006).

| | |
|---|---|
| **Neurotrophins** | Nerve growth factor (NGF) |
| | Brain-derived growth factor (BDNF) |
| | Neurotrophin 3 (NT-3) |
| | Neurotrophin 4/5 (NT-4/5) |
| **Neurokines** | Ciliary neurotrophic factor (CNTF) |
| | Leukemia inhibitory factor (LIF) |
| | Interleukin-6 (IL6) |
| **Fibroblast growth factors** | FGF-1 (acidic fibroblast growth factor) |
| | FGF-2 (basic fibroblast growth factor) |
| **Transforming growth factor β superfamily** | Transforming growth factors β (TGF-β) |
| | Bone morphogenetic factors |
| **Glial-derived neurotrophic factor family** | Glial-derived growth factor (GDNF) |
| | Neurturin |
| | Artemin |
| **Epidermal growth factor superfamily** | Epidermal growth factor (EGF) |
| | Transforming growth factor α |
| | Neuregulins |
| **Other growth factors** | Platelet derived growth factor (PDGF) |
| | Insulin-like growth factor I |

biologically active β-NGF dimer, protein dimer kallikreins - γ-NGF and α-NGF, together with 2 zinc atoms (Perez-Polo, 2010). NGF was discovered by Levi-Montalcini and Hamburger in the 1940s due to its ability to stimulate outgrowth in the sensory ganglia (Levi-Montalcini, 1987). Eventually, the NGF was identified as an essential survival factor for peripheral sensory and sympathetic neurons in the development of nervous system. Continued studies further explored the involvement of NGF in the adult central nervous system (CNS) and protective effects on the cholinergic neurons of basal forebrain which revolutionized the concept of CNS plasticity in neurobiology (Johnson & Tuszynski, 1999).

Neurotrophins are a group of proteins that interact with the Trk family of receptors present in the cytoplasmic domain with specific affinity. It is known that NGF specifically binds to TrkA, BDNF and NT-4/5 interacts with TrkB and NT-3 binds to TrkC. The Trk receptors share a common structural organization of their extracellular domains which consists of an array of three leucine-rich repeats flanked by two cysteine clusters (**Figure 2.3**). This structure is followed by two C2-type immunoglobin-like domains in which the second domain acts as the major ligand-binding interface in TrkA and TrkB (Huang & Reichardt, 2003). The binding of NGF to the transmembrane TrkA receptor leads to dimerization and activation of the intracellular kinase domain of TrkA by phosphorylation of tyrosine residues in its autoregulatory loop. This further induces tyrosine phosphorylation which creates adaptor protein docking sites and activates downstream signal transduction pathways including the Ras/ERK mitogen-activated protein kinase (MAPK) pathway, the phophatidylinositol 3'-kinase (PI3K)/Akt pathway and the phospholipase C (PLC)γ pathway (Price et al., 2007). The signalling of MAPK and PI3K pathways mediate neuronal survival and protection upon unfavourable conditions whereas PLCγ pathway plays a key role in synaptic plasticity (Manadas et al., 2007).
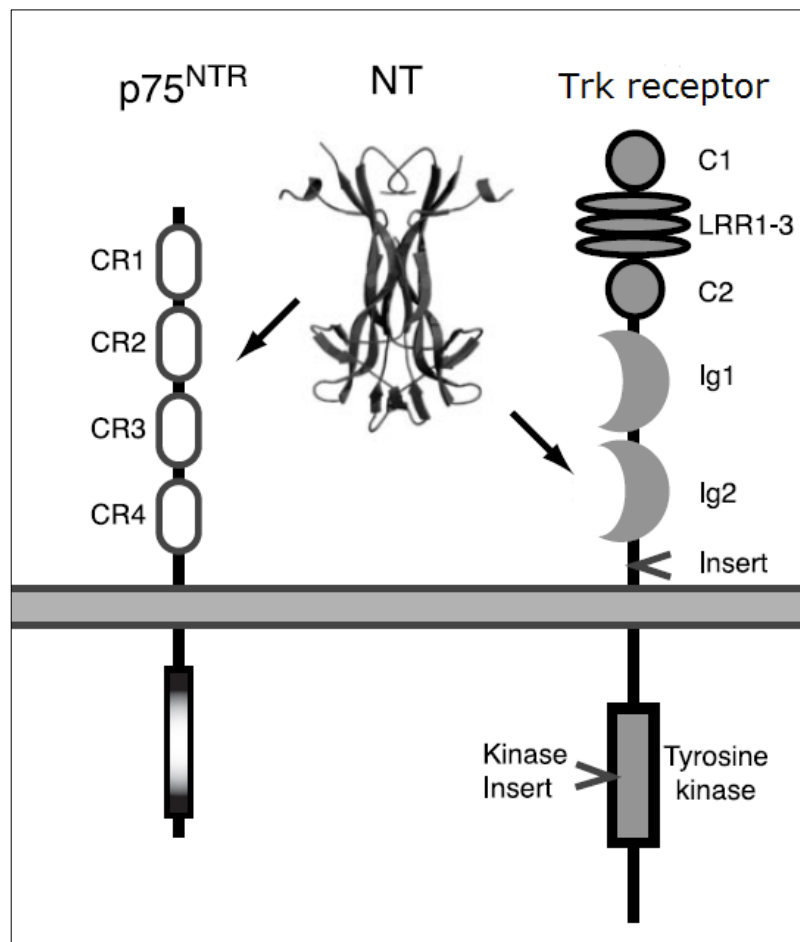
**Figure 2.3**: The structures of Trk receptors and p75 neurotrophin receptor (p75$^{NTR}$). The extracellular domain of p75$^{NTR}$ consists of four cysteine-repeat domains (CR). Both CR2 and CR3 play a role in neurotrophin (NT) binding interactions. The extracellular domain of Trk receptor consists of cysteine clusters (C), leucine-rich repeats (LRR) and immunoglobin-like domains (Ig). The second immunoglobin-like domain (Ig2) serves as the major ligand-binding interface in TrkA and TrkB. Each Trk receptor has a single transmembrane domain and a single cytoplasmic tyrosine kinase domain. [Adapted from Huang & Reichardt (2003)]

Besides Trk receptors, all members of the neurotrophins, including NGF, also bind to p75$^{NTR}$ without selected specificity. The p75$^{NTR}$ is a transmembrane glycosylated protein which belongs to the tumor necrosis factor-alpha (TNF-α) family (Johnson & Tuszynski, 1999). p75$^{NTR}$ regulates the responsiveness of Trk receptors to their neurotrophin ligands thus its presence enhances the specificity of TrkA for NGF. Conversely, the p75$^{NTR}$ regulates apoptotic signals when its own set of intracellular cascade is activated (Johnson & Tuszynski, 1999). The extracellular domain of p75$^{NTR}$ consists of four cysteine-repeat domains in which the second and third cysteine domains

play a role in neurotrophin-binding interaction (Huang & Reichardt, 2003). The structure of Trk receptor and p75$^{NTR}$ are depicted in **Figure 2.3**.

NGF is synthesized and secreted by sympathetic ganglia and sensory target organs. The growth factor is then captured at the axon terminal by receptor-mediated endocytosis and retrogradely transported to the cell body in membrane vesicles via dynein motor proteins along axonal microtubules. In the cell body, the transport vesicles fuse with late endosomes and lastly lysomes, where the NGF is degraded (Windebank & McDonald, 2005). This 'signalling endsome hypothesis' provides a mechanism for long-distance communication between the synapse and the cell body (Howe & Mobley, 2005). The amount of retrogradely transported NGF is proportional to the size of the innervated target area (Lessmann et al., 2003). NGF plays an indispensable role during the developmental stages of peripheral neurons including trigeminal ganglia, dorsal root ganglia and sympathetic ganglia (Huang & Reichardt, 2001). In the PNS, NGF is also produced by non-neuronal target cells such as keratinocytes, melanocytes, vascular and smooth muscle cells, as well as endocrine tissues, in response to stimuli (Sofroniew et al., 2001).

Upon peripheral nerve injury, macrophages release cytokines which induce the synthesis of NGF in Schwann cells and fibroblast around the injured area. The synthesized NGF is postulated to be an essential factor for survival and regeneration of the injured neurons (Huang & Reichardt, 2001). Administration of exogenous neurotrophic factors after nerve injury mimics the effect of target-organ derived trophic factors on neuronal cells. In regeneration of peripheral nerve, NGF and other neurotrophins promoted survival and phenotypic expression of primary sensory neurons in dorsal root ganglia and of motoneurons in spinal cord (Terenghi, 1999).

The expression of NGF in the central nervous system (CNS) neurons is confined to basal forebrain cholinergic neurons, striatal cholinergic neurons and Purkinje cells

(Landreth, 2006). The basal forebrain neurons provide primary cholinergic innervations to the neocortex, hippocampus and olfactory bulb which express high levels of NGF mRNA and protein (Mufson et al., 1999). NGF plays a vital role in hippocampal plasticity and function which are involved in memory and learning (Conner et al., 2009). NGF is a potent and rather selective neurotrophic factor for forebrain cholinergic neurons and prevents cholinergic neuron atrophy caused by aging or experimental injury (Hefti, 1997). NGF is also produced by the major glial cells in the CNS, such as microglia, astrocytes and oligodendrocytes. The growth factors produced by glial cells not only influence the development and survival of themselves but also neuronal development in particularly the maturation of axon (Manadas et al., 2007).

Adequate level of NGF is important in the maintenance of a functional basal forebrain cholinergic system and this dependence of trophic support is augmented is aging brains (Auld et al., 2002). The basal forebrain cholinergic neurons undergo atrophy and cell loss which is correlated with cognitive decline, particularly in patients of aged-related neurodegenerative disorders such as Alzheimer's disease (Schliebs & Arendt, 2011). As NGF is viewed as a potent survival factor in the cholinergic neurons, this growth factor has been conceived as a promising therapeutic agent for the treatment of Alzheimer's disease. Early attempts of NGF treatment in Alzheimer's disease via intracranial infusion were shown to counteract cholinergic deficits (Seiger et al., 1993). Further investigation of NGF treatment has revealed its side effects in treated patients, i.e. back pain and weight loss (Jonhagen, 2000). However, some studies have reported the increased levels of NGF, along with decreased BDNF levels, in surviving neurons of hippocampus and neocortical regions (Siegel & Chauhan, 2000). ProNGF, the immature precursor of NGF, is upregulated in Alzheimer's disease. The accumulation of proNGF in the presence of reduced cortical TrkA and sustained levels of $p75^{NTR}$ shifts the balance between cell survival and death molecules in Alzheimer's disease (Schliebs &

Arendt, 2011). Impairments in the retrograde transport of NGF may explain the increased levels of the neurotrophin in the brains of Alzheimer's disease (Niewiadomska et al., 2011).

Current research developments are focused on CERE-110, an adeno-associated virus-based gene delivery vector that encodes for human NGF, which enables an effective and safe delivery method of NGF to the targeted brain region. CERE-110 has shown to be neuroprotective and neurorestorative to basal forebrain cholinergic neurons in the rat fimbria-fornix lesion and aged rat models, also its bioactive effects were demonstrated on young rat cholinergic neurons (Bishop et al., 2008). The phase 1 clinical trial of CERE-110 in early and mild stages of Alzheimer's disease patients demonstrated that it was safe and well tolerated, together with improvements of cognitive decline. The phase 2 multicenter, sham surgery-controlled trial of CERE-110 gene transfer is currently on-going (Lim et al., 2010).

### 2.2.2   Herbs and mushrooms with neurotrophic activtiy

Extensive research over the decades has revealed the therapeutic potential of NGF in brain pathologies. Nonetheless, its large molecular size and inability to cross the blood-brain barrier have been identified as the major limitations in NGF therapies (Aloe & Micera, 1999). The blood-brain barrier regulates the transport of endogenous and exogenous compounds by controlling their selective and specific uptake in the brain. Selective uptake of compounds allows the influx of small, lipid-soluble substances and certain water-soluble peptides (Gomes et al., 2009). Thus, the discovery of low molecular weight NGF-stimulating compounds which are able to cross the blood-brain barrier has attracted much attention. Alongside, secondary metabolites extracted from plants, herbs and fungi possessing neurotrophic properties similarly worth further exploration.

Several herbs and mushrooms have been reported to have neurotrophic activity due to the presence of NGF-stimulating compounds. *Liriope platyphlla*, a common medicinal herb in Korea, induced NGF secretion in primary astrocytes and in C6 rat astrocyte cell line via a PKC-dependent pathway. The *Liriope platyphlla*-conditioned media of both astrocytes stimulated neurite outgrowth in PC12 cells (Hur et al., 2004). Cistanches Herba, a holoparasitic plant of *Cistanche deserticola* Y.C. Ma, is classified as a tonifying agent in oriental traditional medicine. The Cistanches Herba extract increased NGF induction in C6 cells and lead to neurite extension in PC12 cells. NGF was upregulated in the cortex and hippocampus of extract-treated mouse which improved learning and memory function (Choi et al., 2011). *Mycoleptodonoides aitchisonii*, an edible mushroom found in Japan and the Kashmir region of India, has been reported to have enhancing effects on the synthesis of NGF and neurotransmitter metabolism in the brain of treated rats (Okuyama et al., 2004). Two eudesmane-type sesquiterpenes, dictyophorines A and B, isolated from the mushroom *Dictyophora indusiata*, were reported to promote NGF-synthesis in astroglial cells (Kawagishi et al., 1997). Cyathane diterpenes from the mushroom *Sarcodon scabrosus* - scabronines A, B, C, E and G, were reported to increase NGF mRNA expression in human astrocytoma 1321N1 and the methylester form of scabronine G enhanced the secretion of neurotrophic factors via activation of protein kinase C (PKC-ζ) (Kita et al., 1998; Obara et al., 2001; Obara et al., 1999). Cyrneine A, a cyathane diterpenoid isolated from *Sarcodon cyrneus*, induced neurite outgrowth in PC12 cells via Rac-1 dependent mechanism and stimulated NGF gene expression in 1321N1 cells (Obara et al., 2007).

Plants of the genus *Polygala* have been commonly used as herbs in traditional Chinese medicine. The roots from *Polygala caudata* have been used as a sedative agent under the name of "wubangzi" in Chinese medicine. Euxanthone isolated from *P.caudata* induced differentiation in neuroblastoma cells with the expression of neurite

specific marker MAP-2 protein (Mak et al., 2000). The compound similarly exerted marked stimulatory action on neurite outgrowth in chick embryo dorsal root ganglia upon activation of the MAP kinase pathway (Naidu et al., 2007). A small molecular compound, polygalasaponin G (PS-G), extracted from *P.japonica* promotes neurite outgrowth of neurons cultured on myelin substrates and inhibits the activation of RhoA. Inactivation of RhoA may antagonize inhibitory components in the CNS and promote axonal regeneration (Xu et al., 2009). Eleven alkyl 2,3-dihydroxybenzoates, gentisides A − K, isolated from the traditional Chinese medicine *Gentiana rigenscens* Franch showed significant neuritogenic activity in PC12 (Gao et al., 2010a; Gao et al., 2010b). The gentiside derivative − tetradecyl 2,3-dihydroxybenzoate was a promising lead compound that induces neurite outgrowth via activation of the ERK signalling pathway (Luo et al., 2011). Honokiol is a main biphenyl neolignan isolated from *Magnoliae* cortex which has been used as traditional crude medicine in China and Japan for the treatment of anxiety-related disorders and digestive complains.

Six neuritogenic cerebrosides, termitomycesphins A − F, were isolated from the edible Chinese mushroom *Termitomyces albuminosus* (Qi et al., 2001). Both termitomycesphins A and C with C16 α-hydroxy fatty acid showed more potent neuritogenic activity than termitomycesphins B and D with C18 α-hydroxy fatty acid (Qi et al., 2000). Three diterpenes, tricholomalides A − C, isolated from the fruiting body of *Tricholoma* sp. induced neurite outgrowth in rat pheochromocytoma PC12 cells (Tsukamoto et al., 2003). The discovery of these diterpenes has led to the synthesis of neurotrophically active tricholomalides A and B (Wang et al., 2009). The hot water extract of *Tremella fuciformis*, a common food and traditional drug used clinically in China as a tonic, induced neurite outgrowth in PC12h cells and reduced the degree of β-amyloid toxicity (Park et al., 2007). Oral daily treatment of *T.fuciformis* in rats

significantly reversed the scopolamine-induced deficit in learning and memory functions through enhancing the central cholinergic activity (Kim et al., 2007).

### 2.2.3 Neurotrophic activity of *H.erinaceus*

Over the past two decades, *H.erinaceus* has attracted considerable attention in neurobiology research owing to its neurotrophic activity attributed to the presence of potent NGF stimulators discovered by Professor Hirokazu Kawagishi of Shizuoka University (Japan). Hericenones C, D and E from the fresh fruiting bodies of *H.erinaceus* were the earliest reported NGF-stimulating compounds present in mushrooms (Kawagishi et al., 1991). This roused great interest in the scientific community and further studies were engaged to study the mushroom. Hericenones F, G and H with similar NGF-stimulating activity were later discovered and the difference of activity among the hericenones was found to be dependent on the nature of the fatty acids (Kawagishi et al., 1993). Hericenones I, J and 3-hydroxyhericenone F were the latest members of the hericenone group. Among them, 3-hydroxyhericenone F was reported to show protective activity against endoplasmic reticulum stress-dependent death but not in hericenone I and J (Ueda et al., 2008). It is not known whether hericenones I and J possess NGF-inducing activity as other members of the hericenone family. Hericenes A, B and C (Arnone et al., 1994) together with erinacerins A and B (Yaoita et al., 2005), were isolated from the fruiting bodies of *H.erinaceus* with neurotrophic activities yet to be explored.

Erinacines are a group of diterpene-xyloside derivatives with a cyathane skeleton. These potent NGF-inducing compounds are found in the mycelium of *H.erinaceus*. Erinacines A, B and C were the first discovered members of the group (Kawagishi et al., 1994b). Erinacine A was found to increase NGF levels in the brain regions of locus coeruleus and hippocampus but not cerebral cortex and olfactory bulb. Catecholamine levels were similarly augmented in the locus coeruleus area of treated

rats (Shimbo et al., 2005). Erinacines D (Kawagishi et al., 1996b), E, F and G (Kawagishi et al., 1996a) were reported to possess similar NGF-inducing activity on rat astroglial cells. Erinacine E is also reported to be a κ opioid receptor agonist (Saito et al., 1998). Erinacines H and I were subsequently isolated with only erinacine H reported to have NGF–stimulating activity (Lee et al., 2000). Erinacines J and K were isolated and erinacine K showed anti-MRSA activity (Kawagishi et al., 2006). Alongside, erinacine A and B were also isolated as anti-MRSA compounds and it was reported that MRSA in some patients disappeared when they were given the mushroom during a clinical test in Japan (Kawagishi, 2005). Erinacine P is regarded as the parental metabolite of the erinacine family as it can be successfully converted chemically into erinacine A and B (Kenmoku et al., 2000). Erinacine Q is regarded as the precursor of erinacine P and can be converted into erinacine C, the most potent simulator of the erinacines (Kenmoku et al., 2002). Erinacine R (Ma et al., 2006) and erinacol (Kenmoku et al., 2004) were isolated from mycelia of *H.erinaceus* but their activity has not been studied. Both hericenones and erinacines are low molecular weight compounds that easily cross the blood-brain barrier. However, the mechanism of these compounds in stimulating NGF biosynthesis in the brain remains elusive.

The neurotrophic action of *H.erinaceus* extract was observed in rat brain slices *in vitro* in which the extract suppressed the excitation of hippocampal neurons caused by L-glutamic acid. The inhibition of spike activity was due to the hyperpolarization of neuronal membrane during the application of extract. The *H.erinaceus* extract also improved the myelination process in the mature myelinating fibers without evoking a toxic effect on nerve cell growth (Moldavan et al., 2007). *Hericium erinaceus* extract upregulated NGF mRNA expression in 1321N1 human astrocytoma cells; thereby the secretion of NGF protein from 1321N1 cells in the culture medium enhanced the neurite outgrowth of PC12 cells. The *H.erinaceus* extract promoted NGF gene expression via

JNK signaling yet the hericenones C, D and E were not found to be responsible for the activity indicating the presence of unknown neuroactive compounds in the mushroom (Mori et al., 2008). A high molecular weight exo-biopolymer purified from the liquid culture broth of *H.erinaceus* mycelium enhanced the growth and neurite extension of PC12 cells with efficacy higher than growth factors such as NGF and BDNF (Park et al., 2002). The intake of *H.erinaceus* cookies for 4 weeks has managed to reduce depression and anxiety in thirty female subjects in a clinical study and the effects were attributed to the NGF-enhancing action of *H.erinaceus* (Nagano et al., 2010).

Although *H.erinaceus* is a temperate mushroom, its cultivation in tropical Malaysia has not affected its neurotrophic activity. Aqueous extracts of *H.erinaceus* stimulated neurite outgrowth in neural hybrid clone NG108-15 with maximal outgrowth recorded in mycelia extracts and the least outgrowth in oven-dried fruit body extract (Wong et al., 2007). The administration of *H.erinaceus* extract enhanced functional recovery of injured rat peroneal nerve in the early stages of regeneration (Wong et al., 2009a). Aqueous extract of *H.erinaceus* promoted regeneration of axons and reinnervation of motor endplates in an axonotmetic model during peripheral nerve regeneration (Wong et al., 2011).

### 2.2.4 Neurotrophic factors in combination

In view of the importance of neurotrophic factors in the development of nervous system and neurodegenerative diseases, recent studies have delved into exploring the pharmacological properties of two or more neurotrophic factors, when used in concert, to promote neuronal survival and regeneration. Although different growth factors employ distinct signalling pathways in neuronal functions and developments, the cellular responses were seen to overlap. Thus, the interactions between neurotrophic factors may have an additive or synergistic effect in neuronal functions.

Different combinations of GDNF, BDNF and CNTF were shown to have either additive or synergistic effects on the activity of choline acetyltransferase and motoneuron number in 8-day-old mesencephalic culture. GDNF and CNTF promoted neurite outgrowth and increased the size of cell body whereas BDNF enhanced cholinergic properties of the subpopulations of motoneurons (Zurn et al., 1996). The combination of the three neurotrophic factors CNTF, BDNF and NT-3 or four neurotrophic factors CNTF, BDNF, NT-3 and NT-4 increased the choline acetyltransferase levels by four fold in cultured human spinal cord cholinergic neurons (Kato & Lindsay, 1994). The combined application of NGF (50 ng/mL), GDNF (10 ng/mL) and CNTF (10 ng/mL) at their respective optimum concentration enhanced the neurite outgrowth and longest neurite length in rat dorsal root ganglion explants (Deister & Schmidt, 2006). The combined concentration gradients of NGF and NT-3 showed significant synergism in axonal guidance of embryonic lumbar dorsal root ganglion cells where the guidance distance was increased with lowered concentration gradients required for individual neurotrophin (Cao & Shoichet, 2003). Rat embryonic nigral explants cultures exposed to GDNF and NT-4/5 showed a steady increase in culture volume, protein content and dopamine levels. The combined treatment also showed pronounced reduction of cellular degeneration and a marked increased in counts of nigral neurons containing calbindin-D28k, a calcium-binding protein (Meyer et al., 2001). The combination of GDNF and NGF exerted a synergistic effect on the axonal elongation, axonal branching and growth kinetics in dorsal root ganglion explants from 9 days old chicken embryos (Madduri et al., 2009).

Neurotrophic factors are able to protect neurons under acute injury conditions by providing trophic support as their therapeutic strategy. A suitable combination of neurotrophic factors may enhance the neuroprotective efficacy of neurotrophins on neuronal injury and improve sensory motor functions. The combined treatment of

BDNF and GDNF was seen to attenuate motor dysfunction and the pathophysiology of spinal cord injury even when applied over the traumatized cord at later stages which suggest the combined factors work in synergy to potentiate their beneficial effects leading to enhanced neuroprotection (Sharma, 2007). The combined administration of basic fibroblast growth factor (bFGF), NT-3 and BDNF synergistically enhanced neuronal survival, disinhibited axon growth and promoted axon regeneration without the intervention of scar tissue at the lesion site (Logan et al., 2006). The application of NGF stimulated a neuritogenesis response in auditory neurons cultured from adult rat acoustic ganglia when used in combination with substrate bound bFGF. Conversely, transforming growth factor β (TGFβ$_1$) enhanced the survival effect of bFGF on adult auditory neurons but does not by itself promote their survival in dissociated acoustic ganglion cultures (Lefebvre et al., 1991). The application of BDNF, GDNF and dibutyryl cyclic adenosine monophosphate (db-cAMP) produces convergent signals to activate protein kinase A (PKA) and mitogen-activated protein kinase (MAPK) pathways involved in the survival of postnatal mesencephalic dopamine neurons *in vitro* (Lara et al., 2003).

### 2.2.5 Enhancement of neuritogenic activity of neurotrophic factors by plants and herbs

In the search of bioactive phytocompounds possessing neurotrophic activity, it was found that several phytochemicals from plants and herbs were reported to enhance the neuritogenic activity of neurotrophic factors. As NGF is the best studied neurotrophic factor, it is widely used as the positive standard in the screening of compounds for neurotrophic activity.

Several secondary metabolites isolated from medicinal plants used in folk medicine demonstrated NGF-potentiating effect on neurite outgrowth activity *in vitro*. The neuritogenic activity of these compounds was greatly dependent on the presence of

NGF. Clerodane diterpenoids and flavonoids isolated from medicinal plants *Baccharis gaudichaudiana* (Guo et al., 2007), *Ptychopetalum olacoides* (Tang et al., 2009) markedly increased the percentage of neurite-bearing cells induced by NGF. Similar neurite inducing activity was reported in iridoid glycosides of *Picrorhiza scrophulariiflora* (Li et al., 2000), prenylated xanthones from *Garcinia xanthochymus* (Chanmahasathien et al., 2003), protoberberine alkaloids from Coptidis Rhizoma (Shigeta et al., 2002), and visbane-type diterpenoids from *Viburnum sieboldii* (Kubo et al., 2010). Several neuritogenic compounds were reported to be isolated from marine brown alga including pheophytin (Ina et al., 2007), sargachromenol (Tsang et al., 2005) and sargaquinoic acid (Tsang & Kamei, 2004).

*Centella asiatica*, commonly known as Indian pennywort, is highly regarded in Ayurvedic medicine as a rejuvenating herb (rasayana) reputed to increase intelligence and memory. The ethanolic extract of *C.asiatica* elicited a marked increase in neurite outgrowth in human SH-SY5Y cells in the presence of NGF; however, the water extract was reported to be inactive (Soumyanath et al., 2005). The water extract of *Astragalus membranaceus*, a commonly used herbal medicine in Asia, markedly enhanced NGF-mediated neurite outgrowth and the expression of growth-associated protein 43 (GAP-43), a marker for growth cones, in PC12 cells. Alongside, the administration of *A.membranaceus* extract promoted the maturation of regenerating nerves with larger mean values of myelinated axon number, endoneurial area and total nerve area in rats with sciatic nerve defects (Lu et al., 2010). (+)-Eudesmin isolated from the stem bark of *Magnolia kobus* DC. var. *borealis* Sarg. was found to induced neurite outgrowth by itself and enhance NGF-mediated neurite outgrowth in PC12 cells. The neurite outgrowth activity of (+)-eudesmin in PC12 cells was expressed via stimulation of upstream MAPK, protein kinase C (PKC) and protein kinase A (PKA) pathways (Yang et al., 2006).

## 2.3    Neuroprotection

The term "neurodegeneration" refers to progressive loss of structure or function of neurons, culminating in the death of neurons. Neurodegeneration induced by damaging insults is a major risk factor for the onset of neurodegenerative disorders which is prevalent among the elderly population (Repici et al., 2007). Neurodegeneration develops as an outcome of a lifetime of free radical cellular insults, triggered by excitatory amino acids. This alters the functioning of neurons and neuroglial cells resulting in impaired energy production, loss of membrane fluidity, receptor decay, protein oxidation and DNA injury, both mitochondrial and nuclear (Blaylock, 1999). The discovery of multiple injury cascades in neurodegeneration including free radical generation, excitotoxicity, oxidative stress, apoptotic cell death and inflammation renders these cellular events to be prime targets in the development of novel therapeutic interventions (Danton & Dietrich, 2005). Treatments interfering with a specific event in the death-signalling pathway have been reported to elicit neuroprotection against neuronal death (Repici et al., 2007). A broad spectrum of agents display neuroprotective effects including neurotrophic factors, anti-excitotoxins, antioxidants, bioenzymatic supplements, antiapoptotics, immunosuppresants and steroids (Calabrese, 2008a).

### 2.3.1   Oxidative stress

Reactive oxygen species (ROS) are defined as molecular entities that react with cellular components resulting in detrimental effects on their functions. ROS include free radicals which contain highly reactive unpaired electrons such as superoxide ($O_2^{-\bullet}$), nitric oxide ($NO^{\bullet}$) and hydroxyl radical ($OH^{\bullet}$), and other molecular species such as hydrogen peroxide ($H_2O_2$) and peroxynitrite ($ONOO^-$) (Andersen, 2004). Under physiological conditions, superoxide radicals are broken down to harmless products such as $O_2$ and $H_2O$ via the action of antioxidant enzymes. The superoxide radical is

readily converted to $H_2O_2$ by dismutation via superoxide dismutase (SOD) or by interaction with transition metals. $H_2O_2$ is then reduced to $H_2O$ by glutathione peroxidase (GPx) or converted to $O_2$ and $H_2O$ by catalase. The toxicity of $H_2O_2$ observed in cortical neuron culture is attributed to the generation of reactive hydroxyl radical ($OH^\bullet$) by the interaction of $H_2O_2$ with transitional metals (such as copper and iron) via Fenton reaction (Gorman et al., 1996).

In the human body, free radicals are produced as by-products of cellular metabolism during the electron transfer process within mitochondria. Under normal physiological conditions, the tissues are protected with a three-tier system of antioxidant defenses which includes antioxidant vitamins, antioxidant enzymes and thiol compounds. Antioxidant vitamins such as vitamins E and D function within the lipid portions of the cells whereas vitamin C and minerals operate in the aqueous portion of the cells, mainly in the cytosol. Antioxidant enzymes including superoxide dismutase (SOD), glutathione peroxidase (GPx), glutathione reductase and catalase metabolize different types of free radicals and their precursors. Thiol compounds including albumin, glutathione and alpha lipoic acid are found intracellularly and extracellularly within the cells (Blaylock, 1999). The fine balance between oxidant and antioxidant reactions is vital for regular neuronal function. Any imbalance in this equilibrium favouring free radical formation is defined as a state of oxidative stress (Gorman et al., 1996).

When the generation of free radicals and oxidants exceeds the rate at which the endogenous antioxidant defenses can scavenge, macromolecules such as proteins, lipids and nucleic acids become targets for oxidative modification which lead to deterioration of cellular structural architecture and neuronal death (Ischiropoulos & Beckman, 2003). The peroxidation of polyunsaturated fatty acids in cell membranes causes deterioration of membrane functions, reduction in the electrochemical potential and increased permeability of the membrane (Gallego et al., 2011). Several cellular proteins

functioning as enzymes, transport molecules, second messengers and intracellular support structures were subjected to reduced functional capacity in the event of oxidation (Blaylock, 1999). The formation of carbonyl derivatives includes direct oxidation of amino acid side chains and oxidation-induced peptide cleavage (Finkel & Holbrook, 2000). Protein carbonyl formation is a major component and contributor to reduced function in neurodegenerative diseases such as Alzheimer's disease, Parkinson's disease and Amyotrophic lateral sclerosis (Blaylock, 1999). Ageing cells and organisms accumulate substantial levels of oxidant-damaged nuclear DNA as well as mitochondrial DNA. Increasing damage to mitochondrial DNA leads to compromised mitochondrial function and integrity which results in higher level of ROS release, forming a vicious cycle of DNA damage (Finkel & Holbrook, 2000).

The brain, one of the most metabolically active organs of the body, utilizes approximately 20% of the total oxygen uptake at resting state. The requirement for uninterrupted oxygen-rich blood supply produces a high amount of reactive free radicals; yet, the brain is relatively deficient in enzymes that metabolize oxygen-based reactants to innocuous species. Furthermore, the central nervous system is highly enriched with polyunsaturated fatty acids vulnerable to oxidation by toxic oxygen derivatives. The blood-brain barrier which protects the brain from toxins, reduces the uptake of antioxidants such as vitamin E into the brain (Shukla et al., 2011). It was observed that oxidative stress increases with age in the brain and the ability of cells in response to oxidative damage seems to decline with age and contribute to protein buildup. Increased oxidative alterations to proteins such as β-amyloid in Alzheimer's disease might result in increased protein misfolding and impaired degradation, causing the toxic accumulation of soluble protofibrils or insoluble aggregates within the diseased brain that contribute to neurodegeneration. (Andersen, 2004). Excessive generation of ROS leading to dysregulation of intracellular calcium signalling has been observed in

neurodegenerative diseases in which aberrant calcium levels stimulate multiple pathways that induce the apoptotic cascade (Barnham et al., 2004). The damage of oxidative stress observed in Alzheimer's disease includes advanced glycation end products (AGEs), nitration and lipid peroxidation adduction products as well as carbonyl-modified neurofilament protein and free carbonyls (Rottkamp et al., 2000).

### 2.3.2 Apoptosis

Originally, the term "apoptosis" is used in Greek referring to "dropping off" or "falling off" of petals and leaves from flowers and trees. The term was then proposed by Kerr et al. in 1972 to describe a controlled cell deletion mechanism which plays a complementary but opposite role to mitosis in the regulation of animal cell populations. The process is regarded as an active and inherently programmed phenomenon, affected by a variety of environmental stimuli (Kerr et al., 1972). Over the years, apoptosis has been recognized and accepted as an important mode of "programmed" cell death which involves the genetically determined elimination of cells. Besides functioning as a homeostatic mechanism for the maintenance of cell populations in tissues during development and aging, apoptosis also acts as a defense mechanism in immune reactions or in damaged cells due to pathological diseases or noxious agents (Elmore, 2007).

Developmental death in the form of apoptosis plays a vital role in normal cellular development as is proliferation and differentiation. In the embryonic and fetal development of mammals, apoptosis is involved in the deletion of redundant epithelium at the plane of fusion of the palatine processes, removal of interdigital webs as well as differentiation of the gut mucosa and retina. In adult tissues, continuous apoptotic deletion of cells is a consistent feature of proliferating mammalian cell populations which quantitatively balances the mitosis process. Extensive apoptosis observed in many tumors is regarded as an intrinsic autoregulatory mechanism operating at early

stages of events which leads to malignant transformation. Apoptosis induced by noxious agents such as radiation, mild hyperthermia, cancer-chemotherapeutic substances and chemical carcinogens serves as a homeostatic function in selectively deleting cells with acquired abnormalities of DNA. Apoptosis is also activated by cell-mediated immune reactions where cellular immune reactions selectively eliminate infected cells thereby eradicate the infection and transformed cells (Walker et al., 1988).

Structural changes in apoptosis are distinct from other forms of cell death and involve two discrete stages which comprise the formation of apoptotic bodies and the phagocytosis or degradation of these apoptotic bodies (Kerr et al., 1972). During the early stages of apoptosis, the occurrence of cell shrinkage and pyknosis appears as a prominent feature. The cells decrease in size; cytoplasm becomes dense and the organelles are more tightly packed. Pyknosis, the condensation of chromatin in the nucleus, is the most characteristic feature of apoptosis. This is followed by extensive membrane blebbing and the cell fragments separate into apoptotic bodies via the "budding" process. Apoptotic bodies consist of cytoplasm with tightly packed organelles either with or without a nuclear fragment. These contents are enclosed within an intact plasma membrane with the organelle integrity maintained. Finally, the apoptotic bodies are phagocytosed by macrophages and degraded within phagolysosomes (Elmore, 2007). The morphological changes seen in apoptosis are associated with the reorganization of cytoskeletal structures regulated by caspases. Caspases are catabolic enzymes that digest proteins including structural proteins which forms the cytoskeleton (Hacker, 2000).

Apoptosis is a regulated cellular mechanism which involves an energy-dependent cascade of molecular activity. There are two main apoptotic pathways including the "extrinsic" or death receptor pathway and the "intrinsic" or mitochondrial pathway. The "extrinsic" signalling pathway involves transmembrane receptor-mediated

interactions via death receptors that are members of the tumor necrosis factor (TNF) receptor gene superfamily. The death domain in these receptors are responsible for transmitting the death signal from the cell surface to the intracellular signalling pathways (Elmore, 2007). Currently known death receptors include tumor necrosis factor (TNF) receptor-1, CD95 (Fas/APO-1), TNF-receptor-related apoptosis-mediated protein (TRAMP) and TNF-related apoptosis-inducing ligand (TRAIL) receptor-1 and 2 (Schulze-Osthoff et al., 1998). The activation of caspase-8 is identified as the triggering of the execution phase of death receptor-mediated apoptosis (Elmore, 2007). The "intrinsic" signalling pathway involves non-receptor-mediated stimuli that produce intracellular signals that are initiated in the mitochondria and acted directly on targets within the cell. This pathway can be triggered by unfavourable conditions such as the absence of growth factors, hormones and cytokines, or stimuli such as radiation, toxin, hypoxia, hyperthermia, viral infections and free radicals (Elmore, 2007). During apoptosis, the mitochondrial protein cytochrome c is released to the cytosol and forms a complex by binding to apoptosis protease activating factor (Apaf-1) which activates caspase-9 and initiates the proteolytic cascade that culminates in apoptosis. Agents that cause endoplasmic reticulum stress such as tunicamycin and thapsigargin similarly induce apoptosis via the activation of caspase-12 (Van Cruchten & Van Den Broeck, 2002).

### 2.3.3   Neuroprotection by neurotrophic factors

Neurotrophic factors are required for the maintenance of neuronal function and phenotype in the adult CNS. It was observed that neurons which fail to obtain adequate amount of required neurotrophic factor die by a process of programmed cell death. The neurotrophic factor family has been proposed to play a role in the neuroprotection of neuronal populations by suppressing the expression of 'suicide genes' involved in the induction of apoptosis. The loss of transcriptional suppression of the 'suicide

programme' due to a reduction of neurotrophic factor expression results in neuronal atrophy seen in normal aging or neuronal loss in neurodegenerative disorders (Connor & Dragunow, 1998)

Neurotrophic factors have been vastly studied for their neuroprotective effects. In the presence of basic fibroblast growth factor (bFGF), significant reduction in neuronal death caused by high concentrations of glutamate was observed in cultured hippocampal neurons via the suppression of NMDA-receptor protein expression. Ciliary neurotrophic factor (CNTF), a neurotrophic factor highly expressed in primary cultures of astrocytes, is an injury-related protein and survival-promoting agent after injury. CNTF is shown to attenuate axotomy-induced neuronal degeneration and promotes the survival of neonatal rat corticospinal neurons and cerebellar Purkinje cells *in vitro*. The increased expression of transforming growth factor β1 (TGF-β1) after trauma, excitotoxicity and ischemia indicates its importance in neuronal injury and neuronal plasticity. This neurotrophic factor functions as an endogenous neuroprotectant by reducing the area of ischemia after permanent middle cerebral artery occlusion *in vivo* and reduces the injury of CA1 hippocampal neurons in transient forebrain ischemia rat models (Semkova & Krieglstein, 1999).

The death of septal cholinergic neurons can be largely prevented in both rodents and primates by NGF infusions at the time of axotomy. This neuroprotective effect appears to be mediated via TrkA signalling. NGF is also able to protect a broad spectrum of neurons from ischemia, glutamate receptor-mediated excitotoxicity and metabolic insults such as glucose deprivation and oxidative stress. The neuroprotective effect of NGF in non-NGF receptor-expressing neurons is demonstrated by stabilizing intracellular $Ca^{2+}$ levels and preventing the surge in cytoplasmic $Ca^{2+}$ associated with cell death in excessive glutamate signalling and oxidative stress. After a peripheral nerve injury, reactive Schwann cells produce growth factors, cytokines and growth-

associated proteins which play key roles in axon regeneration and nerve repair (Sofroniew et al., 2001). NGF has demonstrated beneficial effects in aged rats with memory deficits and is currently identified as a potential therapeutic agent for the treatment of Alzheimer's disease. Administration of NGF in early stages of the disease could prevent cholinergic neuronal atrophy observed in the basal forebrain of Alzheimer's disease patients (Connor & Dragunow, 1998). The delivery of NGF across the blood-brain barrier poses a significant problem limiting its therapeutic potential. Consequently, secondary metabolites from plants and herbs with NGF-inducing properties are highly sought after as a promising therapeutic candidate in neurodegenerative disorders.

### 2.3.4 Plants, herbs and mushrooms with neuroprotective activity

Oxidative stress in the nervous system is a marker of neurodegeneration in postmortem tissues. Neurons are highly susceptible to oxidative stress which can induce both neuronal necrosis and apoptosis (Ischiropoulos & Beckman, 2003). Several herbal plants used in traditional medicine prescribed for the prevention and treatment of neurological disorders showed promising therapeutic effects in various clinical studies.

Ginseng, the root of the *Panax* species, is a well-known herbal medicine used in China, Korea and Japan. It is regarded as a tonic and adaptogen to increase the body's ability in resisting diseases, restore homeostasis and reduce detrimental effects of the aging process. Ginsenosides or ginseng saponins, the principal bioactive compounds in ginseng, demonstrated neurotrophic effects whereby these compounds elevated NGF mRNA expression in the rat brain. The neuroprotective effect of ginsenosides was attributed to their ability to attenuate oxidative damage and counteract excitotoxicity in neurons (Rausch et al., 2006). An aqueous extract of *Panax ginseng* root extract demonstrated protective effect against hydrogen peroxide ($H_2O_2$)-induced oxidative damage in astrocytic primary cultures (Naval et al., 2007).

*Withania somnifera*, popularly known as Ashwagandha or Winter Cherry, is considered as the Indian ginseng in Ayurveda medicine. It is known to promote physical and mental health, rejuvenate the body in debilitated conditions as well as increase longevity. This herb is particularly studied for its pharmacological use in CNS-related disorders including cerebral ischemia, epilepsy, Alzheimer's disease and Parkinson's disease (Kulkarni & Dhir, 2008). Bioactive compounds from Ashwagandha including withanolide A, withanoside IV and withanoside VI showed neuritic regeneration and synaptic reconstruction in amyloid-β(25-35)-induced damaged cortical neurons (Tohda et al., 2005).

*Lycium barbarum*, also known as *Fructus Lycii* or wolfberry, has long been used in traditional Chinese medicine for its benefits in anti-aging, vision, kidney and liver. The aqueous extract of *L.barbarum* has shown neuroprotective effects against amyloid-β peptide-induced toxicity in primary rat cortical neurons by inhibiting the JNK signaling pathway (Yu et al., 2005). The wolfberry extract also exhibited cytoprotective effects against reducing stress on the endoplasmic reticulum (Yu et al., 2006). In a 30-day double-blind placebo-controlled clinical study, wolfberry juice containing bioactive *L.barbarum* polysaccharides improved the antioxidant markers in the serum of healthy adult subjects aged 55 to 72 years (Amagase et al., 2009).

Dilinoleoyl-phosphatidylethanolamine (DLPE) is a phospholipid bearing two unsaturated fatty acids (linoleic acids) isolated from the fruiting body of *H.erinaceus* mushroom. This phospholipid was effective at reducing endoplasmic reticulum (ER) stress-dependent death in the mouse neuroblastoma Neuro2a cells via the activation of protein kinase C (PKC) pathway (Nagai et al., 2006). An ER-stress suppressive compound known as 3-hydroxyhericenone F was isolated from fresh fruiting bodies of *H.erinaceus* (Ueda et al., 2008). Further, four compounds isolated from the scrap cultivation beds of *H.erinaceus* mushroom exhibited protective activity against ER-

stress dependent cell death. Endoplasmic reticulum (ER) stress induces the apoptotic pathway in cells with signalling between ER and mitochondria. The triggering of apoptosis in neural cells due to ER-stress is one of the major causes of degenerative disorders (Ueda et al., 2009).

Enzymatic extracts prepared from *H.erinaceus* exhibited significant free radical scavenging activity and neuroprotective effects against $H_2O_2$-induced oxidative stress in PC12 cells (Lee et al., 2010). Treatment with *H.erinaceus* for a period of 14 days prior to middle cerebral artery occlusion showed neuroprotective effect in mice against cerebral ischemia. Also, the treatment significantly increased NGF levels in both the cortex and striatum of mice without affecting cerebral blood flow in the cortex (Hazekawa et al., 2010). The administration of *H.erinaceus* prevented impairments of spatial short-term and visual recognition memory induced by amyloid-β(25-35) peptide in mice, indicating that the mushroom may be a promising treatment in the prevention of cognitive dysfunction (Mori et al., 2011). A double-blind placebo-controlled clinical trial was conducted in Japan to examine the efficacy of oral administration of *H.erinaceus* in improving cognitive functions of elderly subjects (aged 50 to 80 years) diagnosed with mild cognitive impairment. The subjects were given four 250 mg tablets containing 96% *H.erinaceus* dry powder 3 times a day for a consecutive 16 weeks. The administration of *H.erinaceus* tablets significantly improved mild cognitive impairments in the subjects and their cognitive functions continuously improved at the duration of intake (Mori et al., 2009).