THE PROTECTIVE EFFECTS OF HONEY PROPOLIS ON OXIDATIVE STRESS IN KAINIC ACID MEDIATED EXCITOTOXICITY IN RAT BRAIN

BY

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IN THE NAME OF ALLAH,THE MOST BENEFICIENT AND MERCIFUL

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ABTS	2,2'-Azino-di-[3-ethylbenzthiazoline sulphonate]
ADP	adenosine diphosphate
AIF	apoptotic-inducing factor
α-KG	α-ketoglutarate
ALP	alkaline phosphatase
ALT	alanine transaminase
AMPA	α-Amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid
AST	aspartate transaminase
ATP	adenosine triphosphate
BS	brain stem
CA	caffeic acid
CAPE	caffeic acid phenethyl ester
Ca2+-A/K	ca2+ permeable alpha-amino-3-hydroxyl-5-methyl-4-isoxazole propionate (AMPA)/kainate
Ca2+	calcium ion
CAT	catalase
^{0}C	degree celsius
CNS	central nervous system
CB	cerebellum
CGNs	cerebellar granule neurons
CC	cerebral cortex
cGMP	cyclic guanosine monophosphate
DG	dentate gyrus
DMH-	1,2-dimethlyhydrazine

DNA	deoxyribonucleic acid
EAE	experimental autoimmune encephalomyelitis
eNOS	endothelial nitric oxide synthase
$FeCl_3$	ferric III chloride
g	gram
GABA	γ-aminobutyric acid
Glc	glucose
Glu	glutamate
Gln	glutamine
GR	glutathione reductase
GS	glutamine synthetase
GSH	glutathione
GPx	glutathione peroxidase
H	honey
HIF	hypoxia-inducibe factor
HP	honey propolis
HCL	hydrochloric acid
H_2O_2	hydrogen peroxide
IFN-γ	interferon gamma
K+	potassium ion
KA	kainic acid
kg	kilogram
LDH	lactate dehydrogenase
LDL	low density lipoprotein
LPO	lipid peroxide

MDA	malondialdehyde
Mdr- 1 gene	multi drug resistance – 1 gene
ml	millilitre
µl	microliter
µmol	micromol
µM	micromolar
mΔΨ	mitochondrial membrane potential
MPT	mitochondrial permeability transition pores
Na+	sodium ion
NF-κB	nuclearfactor kappa B
NH_3	ammonia
nm	nanometer
NMDA	N-methyl-D-aspartate
NMDARs	N-methyl-D-aspartate receptors
NO	nitric oxide
NOx	nitrate/nitrite
NOS	nitric oxide synthase
NADPH	nicotinamide adenine dinucleotide phosphate
OD	optical density
O_2	oxygen
OS	oxidative stress
(· OH)	hydroxyl
($ONOO^-$)	peroxynitrite
(O2.-)	superoxide radicals
%	percent

P	polyphenol constituents
PARP	poly(ADP-ribose) poly-merase
Prxs	peroxiredoxins
RNS	reactive nitrogen species
rpm	*revolutions per minute*
($ROO^{\cdot}$)	peroxide radicals
ROOH	lipid hydroperoxide
ROS	reactive oxygen species
SD	standard deviation
SE	status epilepticus
SOD	superoxide dismutase
Na+/K+-ATPase	sodium/pottasium-ATPase
TBA	thiobarbituric acid
TBARS	thiobarbituric acid reactive substance
TAC	total antioxidant capacity
TAS	total antioxidant status
TCA	trichloroacetic acid
TLE	temporal lobe epilepsy
TNF/NF-ĸB	tumour necrosis factors/ nuclear factor kappa B
TOS	total oxidant stress
USA	United States of America
USM	Universiti Sains Malaysia
VGLUT	vesicular glutamate transporters
V_{cyc}	glutamate-glutamine neurotransmitter cycling
V_{ana}	anaplerosis

V_{NH3}	ammonia fixation
V_{gln}	glutamine synthesis

ABSTRAK

KESAN PERLINDUNGAN MADU PROPOLIS PADA STRES OKSIDATIF DALAM KEADAAN EXCITOTOXICITY ASID KAINIC DALAM OTAK TIKUS

Pengenalan : Propolis telah dicadangkan untuk menjadi pelindung terhadap gangguan neurodegeneratif. Ia telah terbukti mempunyai aktiviti biologi yang luas, iaitu pada dasarnya dikaitkan dengan kehadiran flavonoid dan caffeic asid ester fenil (CAPE).

Objektif : Untuk memahami kesan saraf dari madu propolis, ujian aktiviti glutamin sintetase (GS), nitric oksida (NO), thiobarbiturik bahan asid reaktif (TBARS) dan jumlah status antioksidan (TAS) telah dikaji di dalam otak tikus mengikut bahagian tertentu iaitu korteks serebrum (CC), serebelum (CB) dan batang otak (BS). Tikus tersebut telah diberi madu propolis dan disuntik asid kainic.

Kaedah : Dua puluh empat tikus Sprague Dawley jantan dengan berat 250-300 gram telah mengambil bahagian dalam kajian ini. Tikus-tikus ini telah dibahagikan kepada empat kumpulan dengan enam tikus dalam setiap kumpulan. Kumpulan 1 ; kawalan, kumpulan 2 ; dirawat asid kainic, kumpulan 3 ; dirawat madu propolis dan kumpulan 4 ; dirawat asid kainic dan madu propolis. Tikus –tikus yang dikorbankan pada masa yang tertentu dan dipancung menggunakan alat pemenggal kepala dan otak dikeluarkan dengan cepat dan kawasan otak yang berbeza iaitu korteks serebrum (CC), serebelum (CB) dan batang otak (BS) diasingkan dan digunakan untuk menyediakan homogenat untuk bagi pemeriksaan parameter biokimia. Data dianalisis menggunakan one-way ANOVA, SPSS versi 20.

Konklusi : Hasil kajian ini jelas menunjukkan pemulihan aktiviti GS dan kadar NO dalam excitotoxicity asid kainic. TBARS yang merupakan penanda stress oksidatif telah meningkat dengan ketara dalam ketiga-tiga bahagian otak yang diuji dalam kumpulan KA, namun peningkatan kosentrasi TBARS oleh KA telah dihalang dengan suplemen madu propolis. Manakala kepekatan TAS telah menurun dengan ketara dalam kumpulan KA berbanding kumpulan disuplemen propolis dan KA. Ini menunjukkan bahawa penurunan TAS dicegah dengan pemberian propolis. Oleh sebab itu, propolis boleh menjadi ejen yang mungkin berpotensi melindungi gangguan neurodegeneratif dan excitotoxicity asid kainic dalam otak.

ABSTRACT

THE PROTECTIVE EFFECTS OF HONEY PROPOLIS ON OXIDATIVE STRESS IN KAINIC ACID MEDIATED EXCITOTOXICITY IN RAT BRAIN

Introduction : Propolis has been proposed to be protective on neurodegenerative disorders. It has been shown to have broad biological activities, which are principally attributed to the presence of flavonoids and caffeic acid phenyl ester (CAPE).

Objective : To understand the neuroprotective effects of honeybee propolis, glutamine synthetase (GS) activity, nitric oxide (NO), thiobarbituric acid reactive substances (TBARS) and total antioxidant status (TAS) are studies in different brain regions- cerebral cortex (CC), cerebellum (CB) and brain stem (BS) of rats supplemented with propolis and subjected to kainic acid (KA) mediated excitotoxicity.

Materials and method : Twenty four Sprague Dawley male rats weighing 250-300 grams were used as subjects in this study. The animals were divided into four groups with 6 rats in each group. Group 1; control, group 2 ; kainic acid treated, group 3 ; propolis treated and group 4 ; kainic acid and propolis treated . The animals were sacrificed at the specific time and decapitated using the guillotine and brains were quickly removed and the different brain regions, namely cerebral cortex (CC), cerebellum (CB) and brain stem (BS) were separated quickly and were used to prepare the homogenates for the assay of biochemical parameters. Results were analyzed by one-way ANOVA using SPSS software version 20.

Conclusion : The results of this study clearly demonstrated the restoration of GS activity and NO levels in kainic acid mediated excitotoxicity. TBARS which is the marker of oxidative stress was increased significantly in all the three brain regions tested in KA group, but the increase of TBARS concentration by KA was prevented by prior supplementation with propolis and the concentration of TAS was decreased significantly in KA group compared to propolis and KA group indicating the depletion of TAS concentration by KA was prevented by supplementation of propolis. Hence, propolis can be a possible potential candidate of protective agent against excitotoxicity and neurodegenerative disorders.

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Associate Professor Dr Mummedy Swamy : Supervisor

Associate Professor Dr K.N.S Sirajudeen : Co-Supervisor

1. INTRODUCTION

1.1 EPILEPSY

Neurological disorders are diseases of the central and peripheral nervous system. In other words, the brain, spinal cord, cranial nerves, peripheral nerves, nerve roots, autonomic nervous system, neuromuscular junction, and muscles. These disorders include epilepsy, Alzheimer disease and other dementias, cerebrovascular diseases including stroke, migraine and other headache disorders, multiple sclerosis, Parkinson's disease, neuroinfections, brain tumours, traumatic disorders of the nervous system such as brain trauma, and neurological disorders as a result of malnutrition.

Hundreds of millions of people worldwide are affected by neurological disorders. Approximately 6.2 million people die because of stroke each year; over 80% of deaths take place in low- and middle-income countries. More than 50 million people have epilepsy worldwide. It is estimated that there are globally 35.6 million people with dementia with 7.7 million new cases every year - Alzheimer's disease is the most common cause of dementia and may contribute to 60–70% of cases. The prevalence of migraine is more than 10% worldwide (World Health Organization, 2014).

Epilepsy is a chronic disorder of the brain that affects people in every country of the world. It is brain disorder characterized predominantly by recurrent and unpredictable interruptions of normal brain function, called epileptic seizures. Seizures are brief episodes of involuntary shaking which may involve a part of the body (partial) or the entire body (generalized) and

sometimes accompanied by loss of consciousness and control of bowel or bladder function. An epileptic seizure is the result of a sudden excessive discharge of cells in part of the brain.

Seizures can vary from the briefest lapses of attention or muscle jerks, to severe and prolonged convulsions. Seizures can also vary in frequency, from less than one per year to several per day (World Health Organization, October 2012).

Epilepsy is one of the most prevalent neurological disorders that can be effectively prevented and treated at an affordable cost. It is the most common serious brain disorder worldwide with no age, racial, social class, neither national nor geographic boundaries. Currently, there are over 50 million sufferers in the world today, 85 % of whom live in developing countries. An estimated 2.4 million new cases occur each year globally. At least 50% of cases begin at childhood or adolescence. 70% to 80% of people with epilepsy could lead normal lives if properly treated. In developing countries, 60% to 90% of people with epilepsy receive no treatment due to inadequacies in health care resources and delivery, and due to social stigma (World Health Organization, October 2012). In recent systematic reviews, the lifetime prevalence rates for active epilepsy varied from 1.5 to 14 per 1,000 person-years in Asia. Figures for annual incidence in China and India are similar to those in the United states of America (USA) and Europe but lower than those reported from Africa and Latin America (Mac *et al.*, 2007).

Epilepsy is in most cases multifactorial. Aetiology varies among various age groups, patient groups and geographical locations. In general, congenital and perinatal conditions are the most common causes of early childhood

epilepsy onset; while in adults, epilepsy is more likely to be due to external non-genetic causes. The disorder may be caused by brain disease or injury such as traumas, infections such as meningitis or encephalitis, vascular disease, degenerative disease, tumour or abuse of alcohol, some drugs or other toxic substances. In developing countries many cases of epilepsy are related to preventable parasitic diseases, e.g. neurocysticercosis, malaria, schistosomiasis.

There is proposed mechanism of cell damage and seizure generation in acquired epilepsy. Seizure attacks result in synaptic disturbance accompanied by neuronal hypersensitivity and more of excitation than inhibition (Figure 1.1). The seizure attacks may also lead to post traumatic inflammatory or other exogenous brain injuries, as well as disturbances in the capillary–neuron–glial cell system. The involvement of large neuronal populations and the brain structures in epileptic process may accelerate impairments of the functions performed by certain structures, such as cognitive disorders, catatonia, changes in behavior, personality, memory, and other dysfunctions. Thus, the role of medicines is to eradicate epileptiform seizures and decrease their duration and severity, as well as protection of the brain against neurodegenerative processes (Neganova *et al.*, 2011).

Epilepsy can be either idiopathic or symptomatic. It is estimated that up to 50% of epilepsy cases are symptomatic or acquired, therefore associated with a previous neurological insult (DeLorenzo *et al.*, 2005). Acquired form of epilepsy does not have a genetic link and becomes more common as people age. It is more widespread than genetic or idiopathic form. Idiopathic epilepsy is mainly due to genetic causes or developmental central nervous system (CNS)

disorders and malformations, that most often affect mitochondrial or ion channel function.

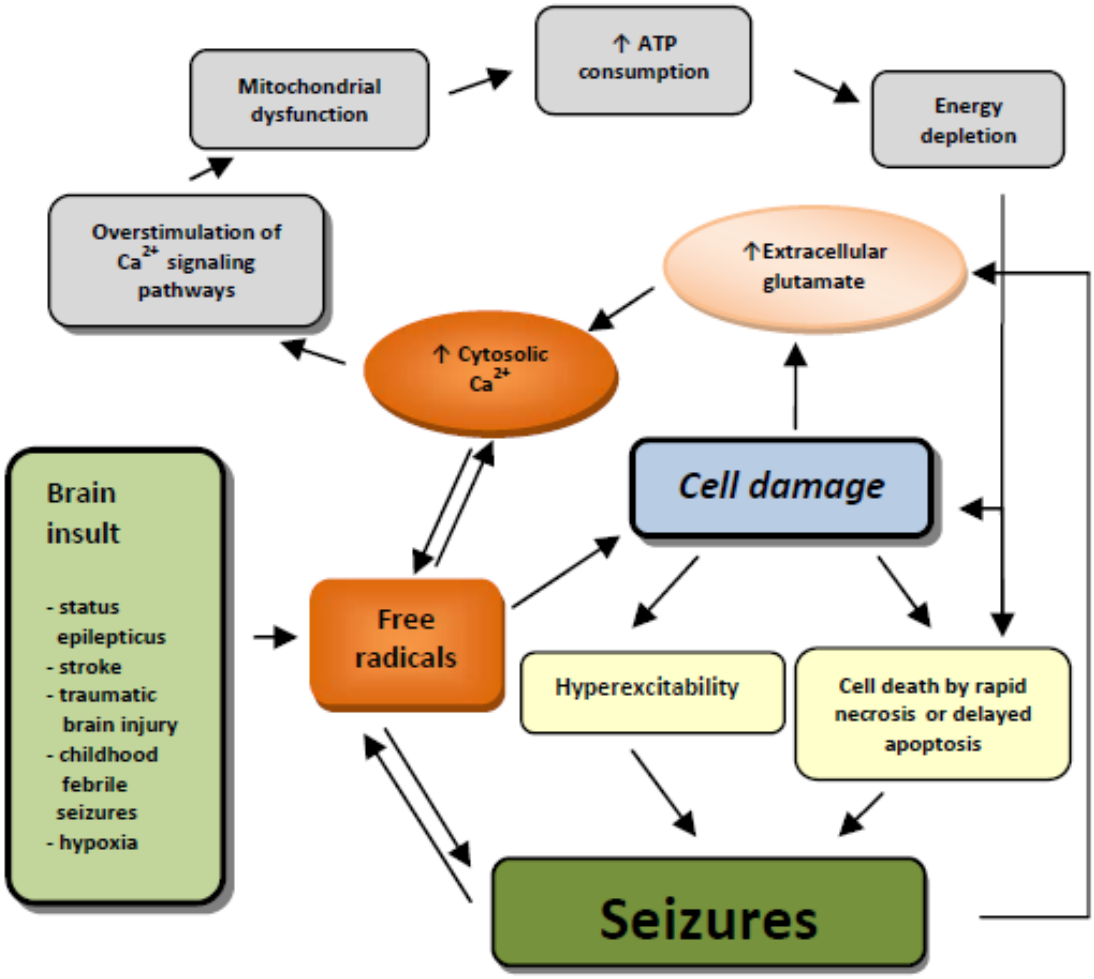

Figure 1.1 A schematic presentation of proposed mechanism of cell damage and seizure generation in acquired epilepsy. Seizures or initial brain insult lead to accumulation of free radicals. Seizures alone, or through cell damage, cause hyperexcitability and increased extracellular glutamate concentration, which results through increased cytosolic calcium ion (Ca2+) concentration and consequently overstimulated Ca2+ signalling pathways in mitochondrial dysfunction, increased adenosine triphosphate (ATP) consumption and energy depletion. This is associated with cell damage and necrotic or delayed apoptotic cell death. Cell death in itself is again considered as a cause of seizures. Beyond that, hyperexcitability may lead to lower seizure threshold, which can result in propagation reaction (Martinc *et al.*, 2012).

1.2 OXIDATIVE STRESS AND SEIZURE

Oxidative Stress (OS) is defined as an imbalance between the production and removal of reactive oxygen species (ROS), such as the free radicals [e.g. hydroxyl (・OH), nitric acid, superoxide (O2.-)] or the non-radicals (e.g. hydrogen peroxide (H_2O_2), lipid peroxide) lead of damage (called oxidative damage) to specific molecules with important injury to cells or tissue and lead to many types of nerve-cell death in the CNS (Nakajima *et al.*, 2009; Seven *et al.*, 2012). The imbalance can result from a lack of antioxidant capacity caused by disturbance in production, distribution, or by an over-abundance of ROS from an environmental or behavioral stressor. In addition, excess ROS further reacts with nitric oxide (NO) to generate reactive nitrogen species (RNS) such as peroxynitrite (Brown and Borutaite, 2001) . While ROS/RNS serve as signalling molecules at physiological levels, an excessive amount of these molecules leads to oxidative modification and, therefore, damage all components of the cell, including proteins, lipids, and deoxyribonucleic acid (DNA) (Wang and Michaelis, 2010).

ROS are transient, unstable, and largely localized to cellular compartments, thus their direct measurement are difficult. Therefore, the role of free radicals in pathologic conditions has been derived from the measurement of indirect markers of oxidative stress such as lipid and protein oxidation products and the activities of free radical scavenging enzymes [e.g superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GPx), glutathione reductase (GR), and peroxiredoxins (Prxs)] (Patel, 2004).

The pathogenesis of epilepsy is closely related to oxidative stress. It is not only a consequence of excessive neuronal activity but it also may be involved in the triggering and development of seizures (Figure 1.2).

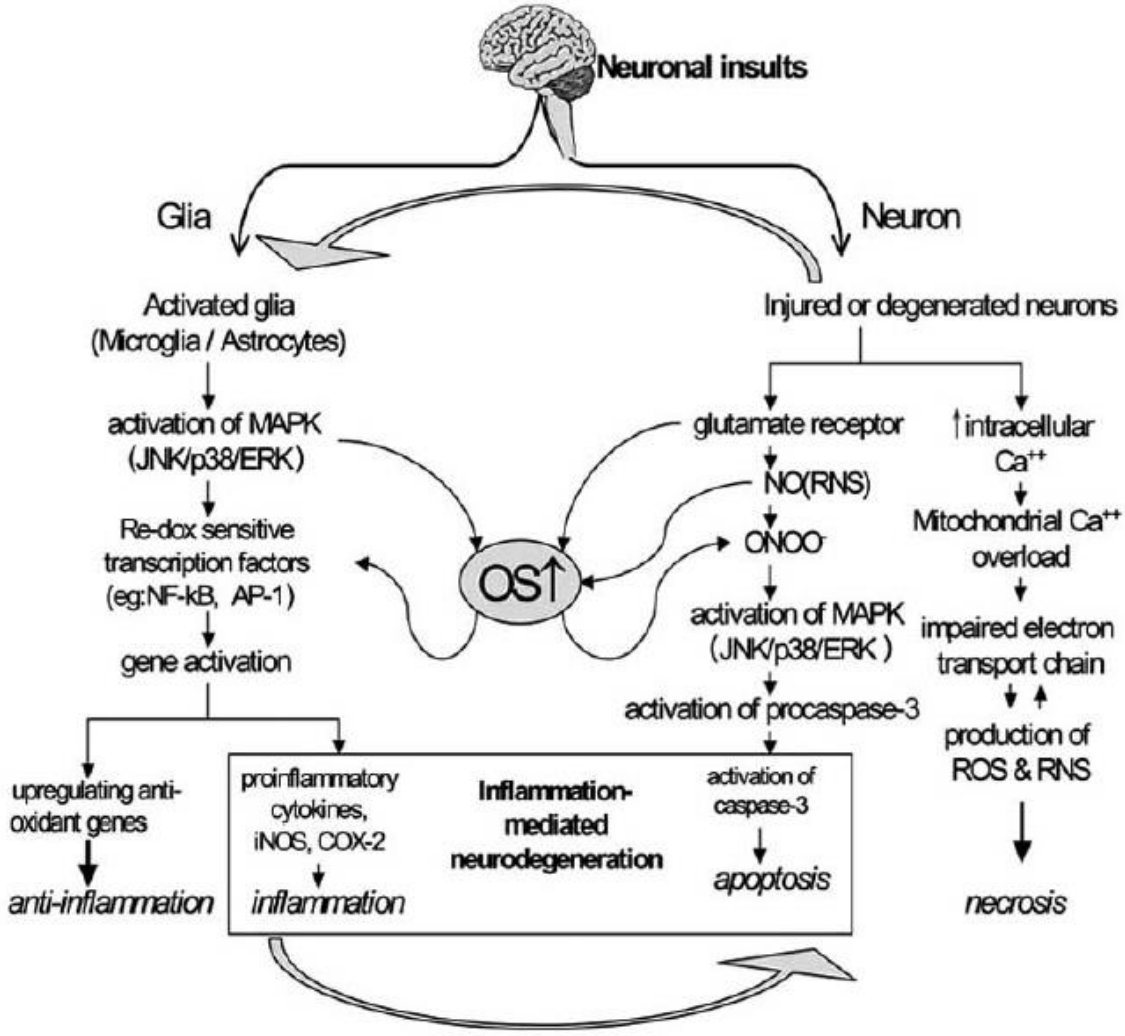

Figure 1.2: Schematic depicting the several possible pathways following neuronal insults leading respectively to inflammation, anti-inflammation, apoptosis and necrosis, and the pivotal role of OS. The glial inflammatory factors and neuronal apoptotic elements can form a vicious cycle leading to progressive degeneration. Source : (Wang *et al.*, 2006)

In the CNS, excessive production of ROS/RNS has been brought up as a mechanism for neurodegeneration associated with various insults to neurons, such as hypoxia and hypoglycemia as well as with the neurodegeneration seen in Alzheimer's and Parkinson's disease. The highly reactive ROS and RNS, when over-produced or under-detoxified due to factors such as aging and disease, are harmful to cells since they chemically modify lipids, proteins, and nucleic acids.

The brain is particularly susceptible to oxidative stress-induced damage due to a large quantity of mitochondria, a high degree of oxidizable lipids and metals, a high oxygen consumption, and less antioxidant capacity compared with other bodily organs. Thus, oxidative stress is responsible to neurological disorders such as epilepsy. The brain also contains high concentrations of polyunsaturated fatty acids that are prone to lipid peroxidation. Apart from that, it is rich in iron, which can catalyze hydroxyl radical formation, and is low in CAT activity (Patel, 2004; Shin *et al.*, 2011). Latest updates showed that the generation of free radicals during epileptic activity has a significant role in the death of neurons (Neganova *et al.*, 2011).

Studies showed that there was a relationship between status epilepticus (SE) and oxidative stress. The pathophysiology behind that are increased blood flow, energy and oxygen during seizure and that SE induces the production of redundant ROS (Shin *et al.*, 2008b).

Some researchers have discovered that prolonged seizure activity initiates calcium influx via voltage-gated and N-methyl-D-aspartate-dependent

ion channels and then activates biochemical cascades, which result in neuronal cell death and the production of ROS (Frantseva *et al.*, 2000b).

Up to now, various experimental seizure models have been developed to investigate the role of endogenous antioxidants in response to excitotoxic oxidative stress. Impairment of endogenous antioxidant factors against oxidative stress is involved in seizure generation. Increased oxidative stress also lead to seizure-induced brain injury and afterwards results in epilepsy. Antiepileptic drugs help to improve antioxidant systems. The ability of antioxidants are to weaken seizure generation and the accompanying changes in oxidative burden further support an important role of antioxidants as having antiepileptic potential (Shin *et al.*, 2011).

1.3 KAINIC ACID-MEDIATED EXCITOTOXICITY

Morphologically, CNS neurons differ in size, the number and complexity of dendrites, number of synaptic connections, length of axons and distance across which synaptic connections are established, extent of axonal myelination, and other morphological characteristics. Neurons can also be classified chemically on the basis of the neurotransmitters they use for chemical transmission or neuromodulation, e.g., glutamate, γ-Aminobutyric acid (GABA), acetylcholine, dopamine, adenosine, or peptide transmitters and neuromodulators (Wang and Michaelis, 2010).

Glutamate is the major excitatory neurotransmitter in the mammalian CNS and is normally stored intracellularly. However, for examples cases of CNS trauma, stroke, epilepsy, and in certain neurodegenerative diseases, increased concentrations of extracellular glutamate (caused either by excessive vesicular release or transporter reversal, disruption of cellular uptake, or liberation of glutamate following necrotic cell lysis) can result in the over-activation of ionotropic glutamate receptors and trigger neuronal cell death (termed excitotoxicity).

Glutamate-induced neurotoxicity is a superior example of “excitotoxicity”, also results in either rapid necrosis or delayed apoptosis of the neuron depending on the severity of the insult (Bonfoco *et al.*, 1995).

Kainic acid (KA) (2-carboxy-4-isopropenylpyrrolidin- 3-ylacetic acid) (Figure 1.3), also known as alga-*kaininso,* is isolated from *Digenea,* a red alga found in tropical and subtropical waters (Coyle, 1987). Studies indicated KA as a non-degradable analog of glutamate and it is 30-fold more potent in neurotoxicity than glutamate. This neuroexcitant can bind to the α-Amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA)/KA receptors, which are subtypes of the ionotropic glutamate receptors in the brain (Bortolotto *et al.*, 1999).

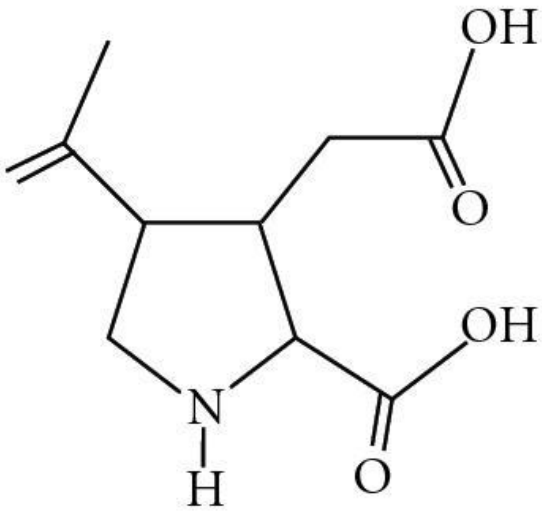

Figure 1.3: The molecular structure of kainic acid (2-carboxy-4-isopropenylpyrrolidin 3-ylacetic acid). The molecular formula of KA are $C_{10}H_{15}NO_4$ (Zheng *et al.*, 2010).

Administration of KA has been shown to increase production of reactive oxygen species, mitochondrial dysfunction, and apoptosis in neurons in many regions of the brain, particularly in the hippocampal subregions of CA 1 region and CA 3 region, and in the hilus of dentate gyrus (DG).

Systemic injection of KA to rats also results in activation of glial cells and inflammatory responses typically found in neurodegenerative disease (Wang *et al.*, 2005). Excitation resulting from stimulation of the ionotropic glutamate receptors is known to cause the increase in intracellular calcium and trigger calcium-dependent pathways that lead to neuronal apoptosis (Figure 1.4).

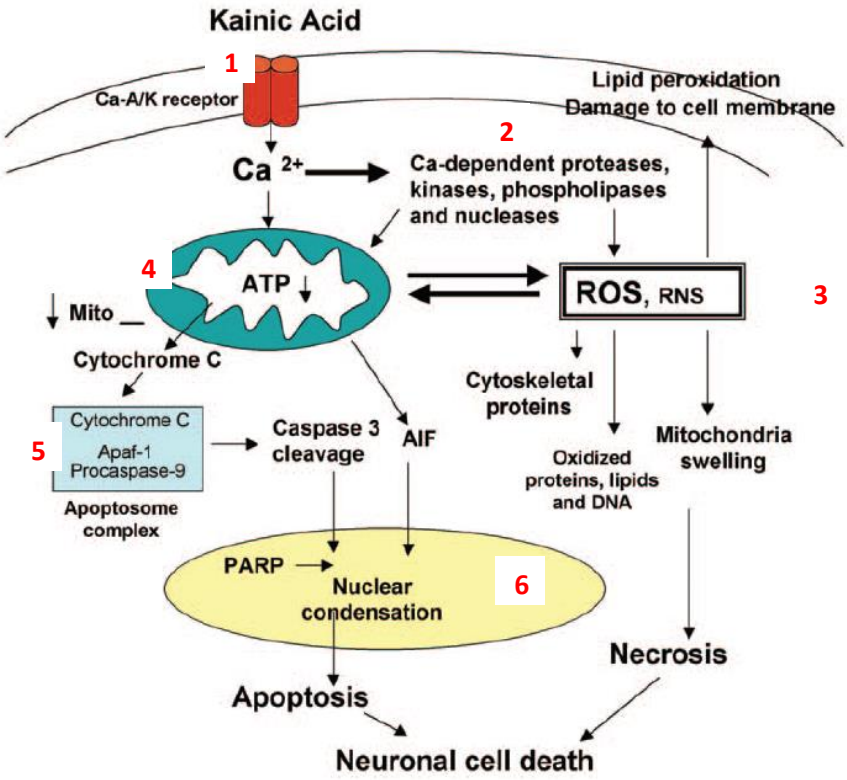

Figure 1.4: Scheme depicting the neuronal cell death pathway induced by KA. (1) KA stimulates the Ca2+ permeable alpha-amino-3-hydroxyl-5-methyl-4-isoxazole propionate (AMPA)/kainate (Ca2+-A/K) receptors, leading to rapid Ca2+ entry; (2) activation of Ca2+-dependent enzymes and generation of ROS; (3) excessive Ca2+ and ROS lead to collapse of mitochondrial membrane potential ($m\Delta\Psi$) and opening of mitochondrial permeability transition pores (MPT); (4) release of mitochondrial factors (e.g., cytochrome-*c* and apoptotic-inducing factor (AIF); (5) cytochrome-*c* binding to Apaf-1 and caspase-9 to form apoptosome complex and activation of caspase-3 pathway; (6) nuclear condensation and DNA fragmentation. Alternatively, intense Ca2+ overload could directly cause mitochondrial swelling and damage, decrease in ATP, and increase in ROS, which oxidize protein, lipid, and DNA, causing acute neuronal necrosis. PARP ; poly(ADP-ribose) poly-merase. Source : (Wang *et al.*, 2005)

Activation of glutamate receptors and consequent calcium-dependent depolarization of mitochondrial membrane potential lead to incomplete oxygen (O_2) consumption, reduced production of ATP, overproduction of ROS, NO, and peroxynitrite, and consequent damage of cell structures including lipids, proteins, and DNA. Thus, impaired mitochondrial respiratory chain function and the ensuing lipid peroxidation associated with seizure activity may precede neuronal damage and death in vulnerable brain regions (Frantseva *et al.*, 2000b).

KA exposure can significantly increase the production of malondialdehyde (MDA) and 4-hydroxy-alkenals, suggesting an increase in lipid peroxidation (Shin *et al.*, 2008a). Apart from increase in lipid peroxidation, systemic administration of KA also caused a decrease in reduced form of glutathione (GSH) levels in the hippocampus (Shin *et al.*, 2008b). Injection of KA also causes the increase in nitric oxide synthase (NOS) in neurons (Yasuda *et al.*, 2001). NO or its derivatives could cause accumulation of RNS, which, then, alters mitochondrial function and induces apoptosis or necrosis (Brown and Borutaite, 2001). Studies done by Swamy *et al* demonstrated that increased production of NO, increased activity of NOS, decrease activity of glutamine synthetase (GS) and increased oxidative stress in KA mediated excitotoicity (Swamy *et al.*, 2009; Swamy *et al.*, 2011a).

1.4 HONEY PROPOLIS

Propolis is a natural product that derived from plant resins collected by honeybees and also known as bee glue. It is an adhesive, dark yellow to brown colored balsam and has a pleasant aromatic odor. Bee gather propolis from the buds, leaves and similar parts of trees different plants, in the temperate climate zone (Seven *et al.*, 2012).

Propolis has a long history of being used for folk medicine and foods for centuries. Currently it become more popular as a health drink and is used extensively in food and beverages in various parts of the world including Japan, the USA and Europe, where it is claimed to improve health and prevent diseases such as inflammation, heart disease, diabetes and even cancer (Banskota *et al.*, 2002).

The chemical composition of propolis is quite complex. It varies with collection time (season), geographic origin, local flora, multiple of trees and plant species used for collection. The compounds of honey are such as polyphenols, phenolic aldehydes, sequiterpene quinines, coumarins, amino acids, steroids and inorganic compounds. The common components of poplar propolis are the phenolics: flavonoid aglycones, (flavones and flavanones), phenolic acids and their esters (Farooqui and Farooqui, 2012).

Different compound of propolis have a broad spectrum biological and pharmacological activities such as antibacterial, antiviral, antifungal, antioxidant, antiaging, antiulcer, antitumor, antiallergic, antiinflammatory, antiosterporotic, antitrombogenic, antiatherosclerosis, cardioprotective, immunomodulating and hepatoprotective (Table 1).

Component, propolis type	Biological Activity
Polyphenols and flavonoids Mostly poplar, but present in most propolis types	Antibacterial, antiviral, antifungal, antioxidant, antiaging, antiulcer, antitumor, antiallergic, antiinflammatory, antiosterporotic, antitrombogenic, antiatherosclerosis, cardioprotective, immunomodulating, hepatoprotective, sicatrising
Caffeic acid phenethyl ester (CAPE) and other caffeates Mostly poplar	Antioxidant, anti-inflammatory, antitumor, antibacterial, antiviral, fungicide, immunomodulatory, cardioprotective, hepatoprotective, antiosteoporosis
Caffeic acid (CA) Poplar, Baccharis	Antiviral, Antioxidant, antiulcer, antitumor
Polyprenylated benzophenones Cuba, Venezuela and Brazil	Antioxidant, antiinflammatory, antitumor
Artepillin C Baccharis	Antioxidant, antiinflammatory, antitumor, apoptosis inducing
Prenylated flavanones (propolins) Taiwan	Antioxidant, anticancer, apoptosis inducing,
Terpenes Greece, Crete, Croatia, Brazil	antibacterial, antifungal
Essential oils Brazil, Poland	antibacterial
Furfuran lignans Canary islands	antibacterial

Table 1.1 : Biological effects of propolis components. Source (Bogdanov, 2011).

Most recent studies have shown that natural preventive compounds have gained popularity day by day as some of the widely used synthetic pharmaceuticals and therapeutics might have some undesirable effects. Many of these compounds, such as plant phenolics, often exhibit antioxidant activities; therefore the addition of these compounds into food products may be helpful to the health of consumers and also to the stabilization of food products.

Apart from that, propolis does not show toxicity if given orally. Studies done, after administration to rat or to humans, propolis does not appear to have side effects (Sforcin, 2007). On the other hand, intraperitoneal administration of ethanolic propolis extracts has slight effects on animals under narcotic-induced hypothermia. Propolis oral administration does not show any significant alteration in some important enzyme levels in rats (Sforcin *et al.*, 1995).

In another study, determination the possible toxicity and side effects of propolis done in *Rainbow trout*, where they were fed on diets containing 0, 0.5, 1.5, 4.5 and 9 gram (g) propolis/ kilogram (kg) diet for 8 weeks. Their results showed that all dosages induced no significant alterations in growth parameters and the levels of total protein, albumin, globulin, low-density lipoprotein cholesterol, highdensity lipoprotein cholesterol, triglycerides and activities of alanine transaminase (ALT), aspartate transaminase (AST), alkaline phosphatase (ALP) and lactate dehydrogenase (LDH), when compared to the control group. On the basis of their findings, propolis is a non-toxic substance for *Rainbow trout* and its long-term administration might not have any side effects (Beyraghdar Kashkooli *et al.*, 2011).

Preliminary scientific studies show some types of propolis have *in vitro* antibacterial and antifungal activity with active constituents including flavonoids

like galangin and hydroxycinnamic acids like caffeic acid (Cafarchia *et al.*, 1999; Cushnie and Lamb, 2005; Orsi *et al.*, 2005). In the absence of any *in vivo* or clinical studies however, it is not clear if this antimicrobial activity has any therapeutic relevance.

Preliminary *in vivo* studies with rats suggest propolis may be effective in treating the inflammatory component of skin burns (Hoşnuter *et al.*, 2004; Ocakci *et al.*, 2006). Also, a clinical trial has shown Brazilian propolis skin cream to be superior to silver sulfadiazine for the treatment of partial thickness burn wounds (Gregory *et al.*, 2002).

Propolis has been reported to exhibit both immunosuppressive and immunostimulant effects (Brätter *et al.*, 1999). Propolis has been the subject of recent dentistry research, and there is some *in vivo* and clinical evidence that propolis might protect against dental caries and other forms of oral disease, due to its antimicrobial properties (Botushanov *et al.*, 2000; Koo *et al.*, 2002; Duarte *et al.*, 2006). Propolis is also being investigated for its efficacy in the treatment of canker sore (Samet *et al.*, 2007) and in reducing the inflammation associated with canal debridement and endodontic procedures.

Apart from that, in *in vitro* tests, propolis induces cell cycle arrest, apoptosis and reduces expression of growth and transcription factors, including NF-κB. Notably, caffeic acid phenethyl ester down-regulates the *mdr-1* gene, considered responsible for the resistance of cancer cells to chemotherapeutic agent (Wu *et al.*, 2011). In *in vivo* studies with mice, propolis inhibits 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone-induced tumorigenesis (Sugimoto *et al.*, 2003).

It is considered as a useful product and already used in alternative medicine. Recently, there has been a growing interest in its utilization by the food processing, cosmetic, and pharmaceutical industries. Propolis is important not only for their nutritional properties but also for their functional and biological properties. Due to the large number of beneficial effects that honey propolis presented on the body, these products could be considered as potential ingredients for different foods. In any case, some precautions must be taken for their use in foods to avoid some problems in persons who suffer from allergy by bee-related allergens.

1.4.1 Honey propolis as an anti-oxidant

One of the most important properties of honey propolis is their antioxidant capacity, which involved in the prevention of certain illnesses, including cardiovascular diseases, cancer, and diabetes (Viuda-Martos *et al.*, 2008). Propolis extracts exhibited the highest antioxidant activity compared to pollen and royal jelly (Nakajima *et al.*, 2009).

Free radicals and other oxidative agents are of great importance in the action mechanism of many toxins. The antioxidant acts defensive factor against free radicals in the body. These free radicals provoke oxidative damage in biomolecules, such as nucleic acids, proteins, lipids, and carbohydrates, which may alter the cell and induce its death (Diplock *et al.*, 1998).

Chemical structures of major flavonoid constituents in propolis are chrysin, galangin, CAPE, kaempferol, acacentin, pinocembrin, pinobaskin, apigenin, luteolin, and quercetin. Propolis also contains other phenolics such as vanillin, *p*-coumaric acid, quinic acid, cinnamic alcohol, cinnamic acid derivatives, caffeic acid, ferulic acid, and isoferulic acid. Flavonoids and phenolic which are main chemical compositions of honey propolis act as an anti-oxidant and anti-inflammatory agent (Farooqui and Farooqui, 2012).

Several studies have been done in Eastern Europe and South America showed that flavonoids concentrated in propolis are powerful antioxidants which are capable to scavenge free radicals (Banskota *et al.*, 2001).

The presence of phenolic compounds and flavonoids is basically cause the antioxidant activity. Although the exact mechanism is unknown, some mechanism that are considered such as free radical sequestration, metallic ion

chelation, hydrogen donation or their acting as substrate for radicals such as superoxide and hydroxyl (Van Acker *et al.*, 1996; Al-Mamary *et al.*, 2002).

On the other hand, Chyrisin is one of the propolis compounds which has hepatoprotective and antioxidant activities in rats (Sathiavelu *et al.*, 2009). Benzoic acid derivate exhibits antioxidant effects using inhibition assays of luminol luminescence, 2,2-diphenyl-1-picrylhydrazyl, and lipoperoxidation. Particularly caffeic acid, caffeoylquinic acid and cinnamic acid are effective O2.- scavenging activity (Nakajima *et al.*, 2007).

In vitro, propolis inhibits peroxidation of low-density lipoprotein (LDL) and nitration of proteins. Moreover, in bovine aortic endothelial cells, propolis was reported to increase endothelial NOS (eNOS) expression and inhibit nicotinamide adenine dinucleotide phosphate oxidase (Silva *et al.*, 2011). *In vivo,* propolis can increase antioxidant capacity in animals and humans (Jasprica *et al.*, 2007; Zhao *et al.*, 2009), leading to decrease lipid peroxidation, which is strongly associated with the risk of cardiovascular disease.

Turkish propolis inhibited hydrogen peroxide induced damage to DNA in cultured fibroblasts. The antioxidant activity of phenolic components of the Turkish propolis may reduce damage to DNA induced by H_2O_2, which may be related to its chemopreventive activity (Aliyazicioglu *et al.*, 2011).

Red propolis from Cuba has shown protective effects in models of alcohol induced liver damage, most likely due to its antioxidant properties (Remirez *et al.*, 1997). Propolis inhibited macrophage apoptosis via effects on GSH and the tumor necrosis factors/nuclear factor kappa B (TNF/NF-κB) pathway (Claus *et al.*, 2000).

Furthermore, Brazilian propolis from *Baccharis dracunculifolia* modulated 1,2-dimethlyhydrazine (DMH-) induced DNA damage in colon cells (de Lima *et al.*, 2005). There is a study showed that daily intake of powdered propolis for 15 days decreased the plasma malondialdehyde concentration in men (Jasprica *et al.*, 2007). The antioxidant effect of Brazilian red propolis has been attributed to chalcones and isoflavonoids (including 7-Omethylvestitol, medicarpin, and 3,4,2',3'-tetrahydrochalcone) that act as electron donors (Righi *et al.*, 2011). Moreover, total flavonoid content in Brazilian red propolis is correlated with antioxidant activity, suggesting that all the phenolic and flavonoid compounds present contribute to this activity (da Silva Frozza *et al.*, 2013).

Chinese red propolis had a higher antioxidant activity than propolis from other origins, which was attributed predominantly to CAPE (Izuta *et al.*, 2009). Chilean propolis also has antioxidant properties, which are correlated with its chemical composition (Russo *et al.*, 2004).

Additionally, the antioxidant and free radical- scavenging properties of propolis may be due to its phenylpropanoid content (Korkina, 2007). Thus, the available data indicates that propolis of different origins and distinct compositions consistently exhibit antioxidant actions. In addition to this antioxidant effect, bioactive compounds in propolis influence a large number of biochemical signaling pathways, and also physiological and pathological processes.

1.4.2 Honey propolis protective effects on the brain

Brain oxidative injury, resulting from excessive generation of free radicals, likely contribute to the initiation and progression of epilepsy after brain injury. Therefore, antioxidant therapies aimed attenuation of oxidative stress has received considerable attention in the treatment of epilepsy.

The effect of propolis on brain cells had been unknown in professional literature. Tests conducted by the Ruđer Bošković Institute have shown that the propolis could protect the brain from damage and atrophy of nerve cells (Yiş *et al.*, 2013), particularly in cases of nervous system diseases (Alzheimer's disease, Parkinson's disease, Huntington's disease, diabetic neuropathy, neuritis, brain atherosclerosis, epilepsy, depression, schizophrenia, ischaemic-reperfusion brain injury, aging, etc.), because it prevents the brain oxidative stress (Celik and Erdogan, 2008), increases antioxidative defence of the brain tissue (Ramos and Miranda, 2007), neutralizes free radicals in the brain (Ichikawa *et al.*, 2002).

Studies demonstrated that propolis showed neuroprotective effects against neurotoxicity in cell cultures and ischemic neuronal damage in mouse forebrain homogenates by scavenging free radicals (Shimazawa *et al.*, 2005).

Oxygen-derived free radicals have been involved in the pathogenesis of cerebral ischemia–reperfusion injury. CAPE, which is an active component of propolis extract, demonstrates antioxidant properties. Treatment with CAPE significantly inhibited ROS production induced by experimental autoimmune

Encephalomyelitis (EAE), and improved clinical symptoms in rats. These results suggest that CAPE may exert its anti-inflammatory effect by inhibiting ROS production at the transcriptional level through the suppression of nuclear factor kappa B activation, and by directly inhibiting the catalytic activity of inducible nitric oxide synthase (Ilhan *et al.*, 2004).

Caffeic acid (CA) is another component of propolis extract can prevents the aluminum-induced damage of the cerebrum that is associated with neuronal death in the hippocampus as well as learning and memory deficits. *In vitro* treatment with caffeic acid at several different concentrations has been reported to increase the acetylcholinesterase activity in the cerebral cortex, cerebellum and hypothalamus. A similar case is also observed in the cerebellum, hippocampus, hypothalamus, and pons when caffeic acid is administered *in vivo*. All of these findings strongly support the proposition that caffeic acid improves memory by interfering with cholinergic signaling, in addition to its neuroprotective effects (Yang *et al.*, 2008).

Another studies also mentioned that propolis through its antioxidant actions in rat and rabbit brains protects from brain injury after focal permanent cerebral ischemia (Tsai *et al.*, 2006; Altuğ *et al.*, 2008).

The neuroprotective effects of propolis may also be related to its constituents, such as 3,4-di-*O*-caffeoylqunic acid, 3,5-di-*O*-caffeoylqunicacid and / or *p*-coumaric acid (Inokuchi *et al.*, 2006). p-Coumaric acid is the most abundant of the three hydroxy derivatives of cinnamic acid. The study demonstrated the oxidative stress reduction capacity and antigenotoxic capacity of p-coumaric acid. In doxorubicin-induced cardiotoxicity, p-coumaric acid was able to increase the levels of GSH, SOD and CAT activities with a following